What Neuromuscular Systems Tell Us about Hormones and Behavior

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I. WHY STUDY SIMPLE SYSTEMS IF YOU WISH TO UNDERSTAND COMPLICATED PROBLEMS?

A decision every scientist must grapple with is whether to approach directly a problem that is very important and therefore likely to be so complicated as to be nearly insoluble or to approach a problem that is more distantly related to the main topic of interest but almost certainly soluble. Should we attack the dragon’s head or tail? Should we study a very complicated system and risk having a career of frustration in which every finding must be couched with careful reservations because of the countless uncontrolled variables involved? Or should we study a simple system with relatively few variables, which permits us to draw firm conclusions and generate additional hypotheses, all of which are published in the more archival journals to be read by only our closest competitors? Most scientific careers waver back and forth between these tactics, risking the dragon’s fiery breath on occasion. In this essay, we celebrate the tactic of taking good solid hacks at the dragon’s tail while the head of the beast rages on. The larger question covered by this volume is whether and how gonadal steroids direct sexual differentiation of the most complicated machine in the universe, the vertebrate brain. The simpler, more soluble systems we discuss here are neuromuscular systems—spinal and brain-stem motoneurons and their target muscles. Our thesis is that an understanding of the mechanisms by which androgens masculinize these simple neuromuscular systems will illuminate and constrain the possible mechanisms by which steroids masculinize even the most complicated brain systems.

The behavioral functions of a neuromuscular system are usually obvious. If we are interested in how hormones alter behavior, we must always keep in mind the function of whatever neural system catches our eye. Because the job of the central nervous system (CNS) is to monitor the environment and control behavior, even an extensive understanding of a neural system without understanding its behavioral role is a hollow accomplishment. Unfortunately, the CNS neurons with a patent function are a remarkably rare minority—sensory neurons and motoneurons. Even for neurons a few synapses past a sensory receptor cell or a few synapses prior to a motoneuron, it can become difficult to understand exactly what that neuron is doing. But it is much more feasible to ask what sorts of behavior an animal is engaged in when a particular muscle contracts. Sometimes anatomy alone provides an excellent hint of a muscle’s function. Muscles attached to the
base of the penis are quite likely to play a role in male reproduction, and muscles surrounding the larynx are quite likely to play a role in vocal communication. By extension, the function of the motoneurons innervating those muscles can also be inferred. But even discerning the function of a muscle can provide surprises. Discovering precisely what behaviors such muscles mediate also requires clever experimentation and careful observation.

Furthermore, it is easier to keep track of various cell populations in a simple system. One of the problems of studying brain systems is that so many different types of cells are encountered that it is difficult to account for them all. For example, for this reason it has proven much more difficult to study the role of steroids in naturally occurring cell death (apoptosis) in the hypothalamus than in the spinal cord or brain stem. If we see fewer neurons in a region at a later age than an earlier age, it could be due to apoptosis of some cells, but it is difficult to rule out the possibility that neurons might have migrated out of a particular nucleus or that neurons that were previously prominent changed their morphology enough to evade detection. Such transformations are commonplace for most brain neurons. But the fate of motoneurons appears to be less fickle, perhaps because they are firmly rooted by their axons extending into the periphery to innervate target muscles. Motoneuronal cell bodies do migrate after their axons innervate the periphery, but we can readily track those migrations by retrogradely filling the somata with tracer provided to the muscles. Also, because motoneurons are so large, they leave plenty of debris when they die, which is why neural apoptosis is most easily documented in these cells (Chu et al., 1978). Finally, although the number and character of muscle fibers changes over development, it is relatively easy to determine how many of these target cells there are at each stage.

The ease of counting muscle fibers brings up another advantage of motoneurons—their target muscles, by virtue of their residence in the periphery, are readily accessible for observation and manipulation. Neurons deeper in the CNS than motoneurons usually test the experimenters’ patience by sending axonal projections in several different directions. This means that, even when you have mapped the efferents of a brain nucleus, it is difficult to manipulate them all at once.

Furthermore, any manipulation of target nuclei in the brain inevitably leads to collateral damage to the other surrounding neurons, complicating the interpretations of the results. But motoneurons obligingly send their axons to a single target and that target can be lesioned, provided with drug treatment, driven electrophysiologically, and so on, without exerting much influence on other neural regions. There’s an excellent reason why the early understanding of synaptic function was generated from neuromuscular junctions. In those cases, the postsynaptic cells are large and easily accessible for observation and manipulation.

Finally, an important characteristic of neuromuscular systems is that they encompass the minimum number of units (two) to permit cell–cell interaction. The most prominent central theme of developmental neurobiology has been that in all but the very simplest of organisms cell–cell interactions dictate the fate of developing neurons. If we want to understand how a neuron works, we can only learn so much by studying it in isolation. A full appreciation for what neurons are capable of and what factors affect them requires the study of either the cells that innervate the neuron or the cells innervated by the neuron or, ideally, both. The motoneuron–muscle fiber dyad has proven a rich source of neural principles as scientists study the myriad of signals passing back and forth between them. The present excitement over neurotrophic factors is largely due to studies of autonomic and somatic motoneurons and their targets. Sexually dimorphic neuromuscular systems also reveal a rich set of interactions because steroids acting on the muscle can sometimes affect the fate of the motoneuron and vice versa.

II. SEXUAL DIFFERENTIATION OF THE SPINAL NUCLEUS OF THE BULBOCAVERNOUS

The spinal nucleus of the bulbocavernosus (SNB) resides in the dorsomedial ventral horn in the lower lumbar spinal cord in rats (Breedlove and Arnold, 1980). SNB motoneurons innervate the striated perineal muscles bulbocavernosus (BC), levator ani (LA), and external anal sphincter (EAS; McKenna and Nadelaft, 1986). The BC and LA muscles attach exclusively to the base of the penis. Contractions of the BC accompany
cup-like erections of the glans penis (Sachs, 1982) and occur during intromission. Removal of the BC abolishes cup-like erections and impairs the male rat’s ability to apply or remove coitalatory plugs, seriously impairing reproduction. Thus, the BC’s role in male reproduction seems clear. Surprisingly, we do not know the function of the LA. Like the BC, the LA is active during erection and ejaculation, but the removal of the LA does not lead to any discernible deficit in male reproduction, so either the muscle’s contribution is subtle or we have not yet found the proper measure to reveal its role. In adulthood, female rats have only a small vestige of an LA (Joubert et al., 1994) and no detectable BC (Forger et al., 1993). Accordingly, adult male rats have approximately three times as many SNB motoneurons as do females, and the SNB cells of males are approximately twice as large as those of females (Fig. 1). Both the motoneurons and their target muscles possess androgen receptors (Breedlove and Arnold, 1980; Jung and Baulieu, 1972), and the BC and LA muscles possess estrogen receptors (Dubé et al., 1976) as well. We review next the studies showing that androgens, acting during development and adulthood, orchestrate the extreme sexual dimorphism of the SNB system.

A. Role of Apoptosis in Determining Motoneuronal Number

A day or two before birth, the rat SNB system shows no signs of sexual dimorphism. The BC and LA musculature of late fetal males has the same configuration as that of adult males and can also be found in late fetal females (Ghatak et al., 1970). Likewise, female rats have as many SNB cells as do males on embryonic day 20 (E20; the day of sperm-positive vaginal smears is designated E0; Nordeen et al., 1985); at least some of those cells in females have extended axons to the BC and LA by E20 (Sengelaub and Arnold, 1986), and have formed a functional synapse by E22 (Rand and Breedlove, 1987). But during the first few postnatal days, the motoneurons and muscles die. Treating females with androgen either just before or just after birth spares the muscles and motoneurons (Breedlove and Arnold, 1983b). Conversely, blocking androgen action in males by pharmacological treatment before birth and castration at birth causes the system to die, resulting in males with SNB systems indistinguishable from those of normal females (Breedlove and Arnold, 1983a).

Whereas masculinization of the rodent brain is often accomplished by the aromatized metabolites of testosterone (i.e., estrogens), the SNB system of rats is masculinized through the action of androgen receptors (ARs), not estrogen receptors. Evidence supporting this claim includes the effectiveness of prenatal androgen antagonists in demasculinizing males, the effectiveness of neonatal nonaromatizable androgens in masculinizing females, and the feminine SNB system in XY rats with a defective gene for the androgen receptor (Breedlove and Arnold, 1981). So androgen receptors are clearly necessary for sparing the SNB system. These results do not mean that estrogen receptors play no role in SNB development because neonatal estrogen treatment can masculinize the size (Breedlove, 1997), but not the survival (Breedlove et al., 1982), of SNB motoneurons.

B. Precedence of the Target Musculature for Spinal Nucleus of the Bulbocavernosa System Fate

Where does androgen act to spare the developing SNB system from apoptosis? There are many candidates

![Figure 1](image-url)  The spinal nucleus of the bulbocavernosus (SNB) is found in the lower lumbar spinal cord of rats. Males (right) have approximately three times as many motoneurons as do females (left) in this region (indicated by arrows), and the few such motoneurons found in females are approximately one-half the size of those in males. Scale bar, 400 μm.
for the site of action—both the target muscles (Jung and Baulieu, 1972) and the motoneurons (Breedlove and Arnold, 1980) possess androgen receptors, as do many cells in the spinal cord and brain that might provide afferents to SNB motoneurons. But there is considerable evidence that androgen spares the SNB system by acting directly on the target muscles. For example, both steroid autoradiography (Jordan et al., 1991) and immunocytochemistry (Jordan et al., 1997) indicate that SNB motoneurons do not express androgen receptors until after the first week of life, well after the fate of the system has been determined. The BC and LA target muscles, on the other hand, contain ARs on the day of birth (Fishman et al., 1990). Androgen can spare the muscles from death even if the spinal cord has been removed (Fishman and Breedlove, 1985). In addition to this circumstantial evidence, in animals that are mosaic for a functional androgen receptor those SNB motoneurons with a dysfunctional AR are just as likely to be spared by perinatal androgen treatment as motoneurons with a wild-type AR (Freeman et al., 1996). So androgen cannot be acting on the motoneurons themselves to spare them. Conversely, androgen manipulations at the muscle can affect the survival of innervating SNB cells (Fishman and Breedlove, 1992). No one knows which cell population in muscle (e.g., muscle fibers, Schwann cells, or fibroblasts) mediates the androgen-induced survival of the tissue.

Nancy Forger and colleagues have made the case that a neurotrophic factor mediates the survival of the SNB system. Treatment of the BC and LA muscles in newborn female rats with ciliary neurotrophic factor (CNTF) delays the involution of both the muscles and their motoneurons (Forger et al., 1993), an effect that must be independent of androgen because it can also be seen in rats with dysfunctional androgen receptors (Forger et al., 1995). CNTF itself probably does not normally rescue the SNB system in males because mice with their CNTF gene disrupted show the typical sex differences in the SNB system. However, the target muscles possess the CNTF-α receptor (CNTFRα), which may normally mediate SNB survival because male mice lacking the CNTFRα gene have a feminine SNB at birth (Forger et al., 1997). Presumably the CNTF treatments affect SNB survival by stimulating this receptor, a job normally accomplished by some other, so far unidentified neurotrophic factor. The search is on for this CNTF-like neurotrophic factor, its source in developing males, and the locus of its androgen modulation.

### C. Prepubertal Androgen Levels Guide Neuromuscular Synapse Elimination and Dendritic Development

Androgenic modulation of the SNB system does not end with the neonatal period. Male rats secrete low but detectable levels of androgen between birth and puberty (Cipriano et al., 1981), and these low levels affect the SNB. Two examples are the role of prepubertal androgens in SNB dendritic growth and neuromuscular synapse elimination.

SNB dendrites first expand and then retract during the 2 months following birth (Goldstein et al., 1990). The expansion or growth of SNB dendrites during the first prepubertal month after birth is exquisitely sensitive to endogenous androgens. During this period, the SNB dendrites undergo substantial and exuberant growth, increasing their overall length approximately fivefold, so that by the end of the first month SNB dendrites have achieved a length that is nearly twice that in adulthood. During the following pubertal month, dendrites regress to their adult length in the face of climbing androgen levels.

Endogenous androgens during the prepubertal period are crucial to SNB dendritic growth, a rather unexpected finding given their markedly low level during this period. In short, SNB dendrites simply fail to grow without gonadal androgens prepubertally (Goldstein et al., 1990). On the other hand, androgen treatment of castrated prepubertal males reinstates and fully supports SNB dendritic growth. Another fascinating twist is that the growth of SNB dendrites clearly depends on the combined action of androgens and estrogens, whereas by in large the development and maintenance of the SNB system depends only on androgens. Estradiol or dihydrotestosterone (DHT) each partially supports dendritic growth during the first postnatal month, whereas the combination of estradiol and DHT fully supports the prepubertal growth of SNB dendrites (Burke et al., 1997). Moreover, fadrozole, which blocks the conversion of testosterone to estradiol by inhibiting aromatase, partially blocks SNB dendritic growth in gonadally intact males (Burke et al., 1999), further
substantiating the hypothesis that endogenous estrogens normally have a role in the development of SNB dendrites during this period. The requirement for two different sex hormones in SNB dendritic growth may mean different sites of action, involving possibly different cellular mechanisms because, for example, SNB motoneurons have ARs but not estrogen receptors (Breedlove and Arnold, 1983c; Simnerly et al., 1990). Although androgen titers increase significantly during puberty, SNB dendrites retract without aid or interference from endogenous androgens (Goldstein et al., 1990). Treatment with exogenous androgens during puberty can, however, temporarily prevent SNB dendritic retraction (Goldstein et al., 1990).

At the same time that SNB motoneurons elaborate dendrites prepubertally, they retract synapses from their target muscles through a process called synapse elimination (Jordan et al., 1988). During neuromuscular synapse elimination, motoneurons retract a large portion of their axonal arbor so that multiply innervated muscle fibers become singly innervated (Brown et al., 1976). In rats, neuromuscular synapse elimination generally occurs during the first 2 postnatal weeks, and culminates in establishing a uniform pattern of single innervation typical of adult mammalian muscles (Van Essen, 1982). An exception to this rule is the androgen-sensitive LA, which is innervated by SNB motoneurons. The time course of synapse elimination in the LA is unusual compared to other rat muscles because it is delayed, it involves two phases separated by a quiescent period, and it is incomplete.

The major period of synapse loss in the LA occurs between postnatal days 14 and 28 (P14 and P28; delayed by 2 weeks compared to other rat muscles); 60% of the fibers lose their multiple inputs (Jordan et al., 1988). However, even at 28 days, 30% of the fibers remain multiply innervated. During the following month (i.e., during puberty), the level of multiple innervation remains stable, but as puberty ends and androgen titers stabilize, synapse elimination begins again in early adulthood. During the third postnatal month, an additional 15% of LA fibers lose their multiple inputs (Jordan et al., 1989b). By the end of the third postnatal month, the adult pattern of innervation for the LA is finally achieved, characterized by a small proportion (15%) of LA fibers multiply innervated (Jordan et al., 1989b, 1990; Lubischer et al., 1991) compared to no detectable multiple innervation in other adult muscles (Jansen and Fladby, 1990).

Although the final outcome of synapse elimination is not dependent on endogenous androgens after P7, the time course of synapse elimination certainly is (Jordan et al., 1989a,b, 1992). Synapse elimination begins and ends sooner if gonadal androgens are removed. At P14, LA muscles deprived of endogenous androgens for 1 week have significantly less multiple innervation than normal (73% vs the normal 90% multiple innervation). In addition, synapse elimination continues in the LA of castrated males during the second postnatal month when normally synapse elimination pauses as pubertal androgens climb. Consequently, the adult pattern of 15% multiple innervation is achieved 1 full month sooner in males deprived of their own androgens during the prepubertal and pubertal stages of development.

Androgen treatment of juvenile castrated males has a much more pronounced effect on synapse elimination. Androgen treatment, adjusted to approximate adult levels and given during synapse elimination, causes LA muscle fibers to remain multiply innervated (Fig. 2) (Jordan et al., 1989a,b). By P28, nearly 70% of the fibers remain multiply innervated, as opposed to the normal 30% multiple innervation at this stage (Fig. 3). The effect of juvenile androgen treatment on synapse elimination in the LA is also permanent because the same high level of multiple innervation is present more than 1 year later (Lubischer et al., 1991). Androgen treatment during synapse elimination is apparently able to permanently stabilize synapses that would otherwise be eliminated. The ability to demonstrate in this relatively simple system a permanent effect of androgen on synapse elimination raises the possibility that sex differences in the adult number of brain synapses may be engendered through a similar hormonal regulation of synapse (or axon collateral) elimination.

Knowing where androgen acts to stabilize synapses ultimately helps to identify the mechanisms that underlie synapse elimination. Although the question of where androgen acts to regulate synapse elimination in the SNB system has not been answered, the results from several experiments have helped to delineate some hypotheses. Because the effect of androgen treatment on synapse elimination was initially demonstrated using testosterone, one question was whether androgen acted as an estrogen or an androgen or both to influence
the fate of developing synapses. Evidence shows that androgens, acting as testosterone or DHT, but not as estrogens, regulate synapse elimination (Jordan et al., 1995), implicating ARs and not estrogen receptors as important mediators in this effect. Examining the ontogeny of androgen receptors in the SNB system has revealed that both SNB motoneurons and the LA muscle have ARs during synapse elimination (Jordan et al., 1991, 1997). Therefore, androgen could act at either or both sites to stabilize synapses. The effect of androgen on synapse elimination may also be mediated via neurotrophic factors because several (e.g., CNTF; brain-derived neurotrophic factor, BDNF; LIF; GDNF; BFGF) have been shown to delay or halt neuromuscular synapse elimination akin to the effect of androgen on this process in the LA (English and Schwartz, 1995; Kwon et al., 1995; Kwon and Gurney, 1996; Jordan, 1996a,b; Nguyen et al., 1998). Perhaps androgen stabilizes synapses by increasing the production or availability of neurotrophic factors from Schwann cells because Schwann cells are potent sources of many neurotrophic factors (Frostick et al., 1998) and Schwann cells at the neuromuscular junction in particular are responsive to androgen during synapse elimination (C. L. Jordan, unpublished observation).

D. Androgen Sensitivity of the Spinal Nucleus of the Bulbocavernosus System Continues Lifelong

The adult SNB system continues to respond to androgen. For example, the behaviors mediated by the SNB motoneurons and their target muscles include reflexive flips and erections of the penis (Sachs, 1982; Hart and Melese-d'Hospital, 1983) that are crucial to male reproductive success; these reflexes wane after adult castration and are restored by androgen treatment (Hart, 1967). The sheer number of characteristics that are maintained by circulating androgens in adulthood is impressive—muscle fiber size; muscle twitch properties; neuromuscular junction size; motoneuronal dendrites, somata, and nuclei sizes; and number and size of synapses on the motoneuronal somata all change with castration. Furthermore, all these measures are restored to normal by androgen treatment after castration. Probably this continued androgen sensitivity of the SNB
FIGURE 3  The effect of endogenous and exogenous androgens on the normal time course of synapse elimination in the LA muscle. Mean (± standard error of the mean) percentage of muscle fibers contacted by more than one axon based on anatomical counts from normal LA muscles or LA muscles from animals that were castrated 1 week after birth and given daily injections of either oil or testosterone propionate (TP) from P7 until sacrifice, either 1, 2, or 3 weeks later, or until P35, for adult muscles at 2 and 3 months after birth. Each data point is the mean of 4–6 different animals. Synapse elimination in the androgen-sensitive LA is distinct from synapse elimination in other rat muscles—it has a delayed onset (beginning at around P14); it has two phases, rather than one, separated by a quiescent period during the second postnatal month; and it is regulated by androgen. Synapse elimination begins and ends sooner in muscle deprived of endogenous androgens (oil-treated muscles), whereas exogenous androgen permanently halts this process, causing multiple innervation to persist more than 1 year after the end of juvenile androgen treatment (Lubischer et al., 1991). Data from Jordan et al. (1989a,b).

system in laboratory rats and mice represents a remnant of seasonal breeding in the wild ancestors of each because the SNB waxes and wanes naturally in the two seasonally breeding rodent species studied, Feromyscus leucopus (Forger and Breedlove, 1987) and Phodopus sungorus (Fig. 4) (Hegstrom and Breedlove, 1999).

Following the castration of adult male rats, the BC and LA muscles shrink due to a decrease in the size of individual fibers rather than a change in fiber number (Venable, 1966). Treatment with various androgens can prevent or reverse such changes, an effect that appears to be mediated by the muscles themselves because local androgen treatment is effective (Rand and Breedlove, 1991). Interestingly, the denervation of the BC and LA renders them unable to respond to androgen (Rand and Breedlove, 1991; Buresova et al., 1972), indicating that innervation has a permissive effect, perhaps on the expression of ARs by the muscle. The BC and LA muscles are remarkably uniform in their fast-twitch properties, and androgen partially enforces this state because adult castration causes the fibers to slow their twitch capacity, an effect reversed by androgen treatment (Souccar et al., 1982a,b). Presumably these effects of androgen on the muscle, increasing size, capacity, and fast-twitch properties, contribute to their effective function during copulation.

SNB motoneurons also respond to adult androgen because castration causes the somata, nuclei, (Breedlove and Arnold, 1981) and dendritic trees of the motoneurons to diminish (Kurz et al., 1986; Forger and Breedlove, 1987), an effect accompanied by the loss of afferents to the motoneurons (Leedy et al., 1987; Matsumoto et al., 1987). The change in SNB somata has been demonstrated to be a cell-autonomous response because SNB motoneurons using a defective copy of the AR gene fail to show a somatic response to adult androgen, even though their target muscles possess wild-type ARs (Fig. 5) (Watson et al., 2001). It is interesting to note that the effect of androgen on SNB somata, making them larger, is just what we expect if the hormone is maintaining the system's fast-twitch properties because fast-twitch motoneurons tend to be larger than slow-twitch motoneurons.

Although androgen acts directly on the SNB motoneurons to regulate soma size, the normal interaction between the motoneurons and their targets is important for androgen responsiveness of both cell populations. For example, surgically severing the motoneuronal axons prevents the target muscles from showing an anabolic response to androgen (Rand and Breedlove, 1991). Conversely, when SNB motoneurons are axotomized, their somata shrink until they have reestablished contact with the target muscles (al-Shammas and Arnold, 1995; Lubischer and Arnold, 1995a). The dependence of motoneurons on contact with target muscles is regulated during development (Lubischer and Arnold, 1995c). Axotomy during the early postnatal period kills most SNB motoneurons. The SNB motoneurons that survive axotomy on P14 fail to develop normal responsiveness to androgen in adulthood—that
is, androgen manipulations did not affect SNB somal size. Later axotomy (P21) does not abolish androgen responsiveness, suggesting that some critical interaction with the muscle target at approximately P14 is essential for androgen sensitivity of SNB neurons. Axotomy at P14 decreases expression of AR in the SNB (Lubischer and Arnold, 1995b), but only transiently, so the loss of AR cannot account for the permanent loss of sensitivity.

Al-Shamma and Arnold (1995), after showing that adult SNB somata recovered their full size and expression of ARs only after reinnervation of their targets, found that one trophic factor, BDNF, could, when supplied to the proximal ends of the cut nerve, prevent the loss of AR expression by SNB motoneurons (al-Shamma and Arnold, 1997). The effect is specific because a host of other neurotrophic factors had no effect. Such BDNF treatment also increases the ability of SNB motoneurons to accumulate androgens (Yang and Arnold, 2000b). This result indicates that the target muscles provide a permissive signal to the motoneurons, allowing them to respond directly to androgen and that BDNF or some BDNF-like factor may constitute that signal. The BDNF treatment could also prevent SNB motoneurons from shrinking in response to castration (Yang and Arnold, 2000b). Interestingly, these authors found that testosterone treatment could somewhat increase the soma size of axotomized SNB motoneurons, which is also consistent with a cell-autonomous response.

ARs are found in almost all motoneuronal populations that have been examined, and there is evidence
that androgen treatment can accelerate the regeneration of the crushed axons of facial motoneurons in adult hamsters (Jones, 1994). Axonal crush also decreases the expression of mRNA for the AR in these motoneurons (Larkowski et al., 2000) and prevents the motoneurons from increasing AR mRNA in response to androgen (Dengler et al., 1997). Because testosterone treatment increases the expression of cytoskeletal proteins in axotomized facial motoneurons (Jones et al., 1999), androgen may act directly on these motoneurons to augment their regeneration.

SNB motoneuronal dendrites wane with castration and wax again with subsequent androgen treatment. At least some of the androgenic influence on dendritic extent is mediated by the target muscles because local androgen application to the muscles can influence the motoneuronal dendrites (Fig. 6) (Rand and Breedlove, 1995). Interestingly, it appeared that only the contralateral and dorsal dendritic projections were altered by androgen action on the target muscles. The ipsilateral dendrites, known to respond as vigorously as the other fields in response to systemic androgen treatment, failed to respond to androgen acting on the muscle. So the site of androgen action for expanding the ipsilateral dendrites remains unknown. Nor do we know anything about the signal transferred from the muscle to the motoneurons to effect the changes in dendrites. The same application of androgen to muscle has no effect on motoneuronal somata, confirming the conclusion from mosaic studies that the somatic response to androgen is cell-autonomous.

The neuromuscular junctions (NMJs) between SNB motoneurons and their target muscles also respond to changes in adult androgen exposure. The castration of adult mice led to a dramatic decrease in the size of NMJs on the BC 1 month later, a change that could be completely reversed by 1 month of androgen treatment. But, interestingly, the change in size was fairly simple—as no discernible alteration in the overall shape or configuration of the NMJs were seen (Balice-Gordon et al.,

![FIGURE 6](image_url) Effect of androgen application on BC muscles on the dendrites of SNB motoneurons. (A) SNB motoneurons innervating a testosterone-treated target muscle (filled bars) have more extensive dendrites than do SNB motoneurons innervating an antiandrogen-treated muscle (open bars). (B) This difference in overall dendritic extent is due to a significant effect on two dendritic fields in the spinal cord—the dorsal and contralateral projections of SNB dendrites. Androgen acting on the target muscle does not seem to affect the ipsilateral projections of SNB dendrites. Because systemic androgen influences all fields equally, the results depicted suggest that androgen acts elsewhere to affect ipsilaterally projecting dendrites (Rand and Breedlove, 1995).
This suggests that the NMJs changed in passive response to the change in their target muscle fibers rather than as a more active process of sprouting or retraction of the terminal field.

The androgenic modulation of the SNB system in adult rats may seem paradoxical because these animals never normally experience a major drop in circulating androgen after puberty, but it is easier to understand when we recall that most vertebrate species in temperate zones breed seasonally. In the males of such seasonal breeders, presumably including the wild stock that gave rise to laboratory rats and mice, androgen levels fluctuate dramatically between the breeding and nonbreeding seasons. Whereas laboratory rats and mice have been artificially selected for year-round breeding for several hundreds of generations, laboratory hamsters still display robust seasonality. Most individual hamsters when kept in a laboratory with controlled climate and ad libitum food and water nevertheless display a host of physiological and behavioral responses to winter-like short day lengths (SD) or summer-like long day lengths (LD). Many of the same SNB system parameters affected by adult androgen manipulations in rats are also affected by such photoperiod manipulations in gonadally intact Siberian hamsters (Hegstrom and Bredlove, 1999).

Aging provides an interesting glimpse into the role of androgen for the synaptic plasticity of SNB motoneurons. Matsumoto and colleagues (1996) have found that the castration of adult male rats leads to a decrease in AR immunoreactivity (AR-ir) in SNB motoneurons and that testosterone treatment can prevent this loss. Aged male rats display both a slight decrease in circulating androgen levels and a decrease in AR expression by SNB motoneurons (Matsumoto and Prins, 1998), indicating that the decline in androgen with aging may reduce the plasticity of the motoneurons. But when the aged motoneurons are provided with supplemental androgen, they can still respond to the hormone with increased synaptic contacts (Matsumoto, 2001), which suggests that adjusting circulating androgen levels in aged individuals might have beneficial effects on the nervous system and behavior.

One important overall lesson to take from steroidal effects on the SNB system is that androgens seem to influence this system at almost every stage of development from the late prenatal stage until old age (Fig. 7). Another obvious feature of the system is that androgen does not act on a single population of cells to regulate function, but rather seems to act on some cellular sites to induce some changes while acting on another cell group to induce other changes (Table 1). Furthermore, the cell group that shows a response to androgen manipulation may not itself be responding to the hormone, but may be responding to androgen action on some other part of the system, as when androgen acts on the target muscles to alter some dendrites of the innervating SNB motoneurons (Rand and Bredlove, 1995). Perhaps these two characteristics, lifelong steroid responsiveness and a plurality of sites of steroid action, will prove common in hormone-sensitive neural systems.

### III. Sexual Differentiation of a Vocal Neuromuscular System in *Xenopus*

The vocal repertoire of *Xenopus laevis*, the South African clawed frog, includes specific male and female songs (see Kelley, Chap. 27 in this volume). Males have six distinct song types, whereas females have two song
TABLE 1
Sites of Androgen Action on the Spinal Nucleus of the Bulbocavernosus System

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<th>Time</th>
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<td>Development</td>
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<td>Dendritic arbor: length of ipsilateral projections</td>
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<td>Motoneuron soma size</td>
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<td>Number of synaptic contacts on SNB cell bodies and proximal dendrites</td>
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<td>Number of gap junctions on SNB cell bodies</td>
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*CGRP: Calcitonin gene-related peptide; NMJ: neuromuscular junctions; SNB: spinal nucleus of the bulbocavernosus.

...types. For both sexes, the fundamental vocal unit is a click, a brief and noisy burst of sound produced by a contraction of intrinsic laryngeal muscles. Both the rate of click production and the temporal patterns of click trains are distinct for males and females. The sexual differentiation of laryngeal muscle and motoneurons underlies sexually differentiated vocal functions. These functional properties are the result of a developmental program that is guided into sex-specific pathways via the secretion of gonadal steroids, androgens, and estrogens. The cellular programs that underlie sexual differentiation have been outlined (Kelley, 1996). The molecular events that participate in these programs are less well understood and are the subject of intensive study.

A. Sexual Differentiation of Muscle Fiber Number

The adult larynx, or vocal organ, is a sexually dimorphic structure—larger in the male than in the female (Fig. 8). One reason for this dimorphism is that the male larynx contains more muscle and cartilage cells than the female larynx. The adult male larynx contains approximately 10 times the female number of muscle fibers. This sex difference arises during postmetamorphic development, after the tadpole transforms to a small frog (juvenile or postmetamorphic stage). At the end of metamorphosis (PM0), the number of muscle fibers in males and females is the same. Over the next 6 months, the muscle fiber number in males increases at a rapid rate, approximately 150 fibers/day. Muscle fiber addition also occurs in females during this period but the rate is much slower, approximately 15 fibers/day (Marin et al., 1990). The larger number of muscle fibers in males is available for recruitment by the CNS and for facilitation at the neuromuscular synapse (see Kelley and Tobias, 1999). Recruitment and facilitation, in turn, are responsible for the progressive increase in amplitude of the clicks in the male’s advertisement call, a feature that females find particularly attractive.

The male pattern of muscle fiber addition depends on his gonads. The castration of a juvenile male halts but does not reverse the process of fiber addition (Marin et al., 1990). Ovariectomy of a juvenile female, on the other hand, does not affect her lower rate of fiber addition (Fig. 9). The careful examination...
**FIGURE 8** Male and female larynges in *Xenopus laevis*. Top: Photomicrographs from a ventral view. The arytenoids disks (AD) are the sound-producing elements; these pull apart and produce a click when the laryngeal muscles (LM) contract. Middle: Drawing of a ventral view of male and female larynges illustrating the anterior–posterior position of the laryngeal section (line) shown in the lower panel. Bottom: Cross section through larynges. The area from which muscle fibers were counted is shown as a small box above the thyrohyal cartilages (in black). Middle and bottom panels from Marin et al. (1990).
of muscle formation in males reveals that the process has two components—a slow, nongonadal dependent rate that resembles the slow muscle fiber addition of females and a faster rate that requires intact testes. When testes from juvenile males are transplanted into juvenile females, the latter achieve a male-like number of laryngeal fibers. The sexually differentiated program of muscle fiber addition is thus driven by the formation of testes in males and not in females. The default program—that executed in the absence of the gonads—is female.

The masculine program does not begin until metamorphosis is complete and the addition of muscle fibers in males comes to an end approximately 6 months later (PM2). Why is muscle fiber addition confined to this particular developmental period? The addition of muscle fibers is due to the proliferation of muscle stem cells (myoblasts), their differentiation into mononucleated fusion-competent myocytes, and the subsequent fusion of myocytes into multinucleated myotubes (which eventually become the myofibers or muscle fibers of the adult). These cell types are present in *X. laevis* larynx, and the rate of cell proliferation is greater in male than in female juveniles for the first 6 months PM (Sassoon and Kelley, 1986). Thus, the sexually differentiated program for myogenesis in the larynx is largely confined to the first 6 months PM. How do the testes act to increase myogenesis? The next step was the identification of hormonal agents that could mimic the effects of testicular transplants; the obvious candidates were the androgenic steroids, testosterone and its 5α-reduced metabolite DHT. Both androgens stimulate myogenesis in juvenile frogs (Sassoon et al., 1986). Treatment with the antiandrogen flutamide beginning at PM0 blocks muscle fiber addition in intact males. The treatment of juvenile females with DHT increases muscle fiber numbers (Marin et al., 1990).

These observations strongly suggest that the secretion of androgenic steroids is necessary for the masculinization of muscle fiber number. However, masculinization achieved with androgen treatment, although robust, was never as complete as that achieved

**FIGURE 9** Sexually differentiated muscle fiber addition depends on androgen secretion. Top: By postmetamorphic stage (PM1) (3 months after metamorphosis is complete), males have more muscle fibers than females and by PM2 (6 months after metamorphosis), an essentially adult complement of fibers has been attained. Middle: Castration at PM0, but not at PM2, blocks muscle fiber addition in males. Flutamide (FL) does not block an ongoing muscle fiber addition program present in both sexes (see top panel). Bottom: Ovariectomized juvenile (PM0, just after metamorphosis is complete) females that received a testis

**FIGURE 9** (Continued) implant displayed male-like amounts of muscle fiber addition at PM2. Ovariectomy did not interfere with the endogenous muscle-fiber-addition program in females. From Marin et al. (1990).
via a testis transplant (Marin et al., 1990), suggesting that some other factor participates in generating the male-specific program of muscle fiber addition. Androgen, although necessary, may not be sufficient for the realization of the masculine developmental program.

With these data in hand, one obvious candidate for the developmental time frame of masculinization is androgen secretion; perhaps androgen secretion begins at metamorphosis in males and tapers off at 6 months PM. However, measurable androgen can be detected in tadpoles as early as stage 47 (a few days after fertilization); the gonads have not yet formed and androgens are produced by the interrenal glands (Kang et al., 1995). Sex differences in circulating androgen are not present until PM4, 9 months after the completion of metamorphosis and approximately 3 months after male muscle fiber addition is complete. Sex differences in laryngeal muscle fiber number cannot therefore be due simply to sex differences in circulating steroids, nor can the opening and closing of the developmental period for fiber addition be due to androgen-secretion patterns.

Androgen promotes myogenesis and muscle fiber addition slowly (within hours to days) and its effects are presumed due to the transcriptional activation of downstream target genes by hormone binding to the AR. The *Xenopus* AR is, as is the case for other vertebrates, a nuclear protein made up of DNA, ligand, and hormone-binding domains. If it were expressed in the larynx only during particular developmental periods, its regulation could explain the time frame for muscle fiber addition.

The developing larynx is one of the most androgen-sensitive tissues in the vertebrate kingdom. During early juvenile development, the levels of androgen-binding activity range between 250 and 500 fmol/mg protein; adult levels are 10–30 fmol/mg (Segel et al., 1987). Juvenile males maintain high levels of androgen binding until 6 months PM, whereas the levels in females decline; at 3 and 6 months PM binding levels are significantly greater in males than in females (Kelley et al., 1989). Thus, males show a greater sensitivity to androgen between 0 and 6 months PM when androgen-driven myogenesis is occurring. The drop in binding coincides with the end of muscle fiber addition. These correlations suggest that decreased sensitivity to androgen could close the developmental period for androgen action.

A closer examination of receptor expression in developing laryngeal cells suggests an alternate explanation. When the *Xenopus* AR gene was cloned, two mRNA species, α and β, were observed (Fischer et al., 1993). The mRNAs for the ARs are long, 9.6 and 8.0 kb, respectively, and the transcripts differ in the 5' transactivation domain of the shorter (~2 kb) coding sequence (see Fig. 10). It is not clear whether these mRNAs are alternatively spliced products of a single gene or products of separate genes. There are at least two AR genes in the *X. laevis* genome but, because the species is a pseudotraploid, most genes are present as two copies and some are inactivated. The α and β isoforms of the *Xenopus* AR are coexpressed in laryngeal cells. The β isoform is very highly expressed in stem cells, myoblasts, and chondroblasts; the levels of expression decrease as differentiation proceeds.

Thus the decrease in AR expression that occurs in males from PM0 to 6 months PM could be due to downregulation of the β isoform that accompanies the cellular differentiation program (Fischer et al., 1995). It is not clear whether decreases in AR expression limit the period of muscle fiber addition in males or whether AR decreases are simply the consequence of myoblasts becoming depleted by fusion and differentiation.

Females maintain the capacity for muscle fiber addition into adult life. This capacity is only revealed following testicular transplants; androgen implants do not evoke muscle fiber addition in adult females (Watson et al., 1993). This observation suggests that females retain stem-cell populations that can respond to testicular signals into adult life and, again, that although androgens may be necessary for myogenesis other testicular or testicular-evoked factors may also be required to reactivate stem-cell proliferation.

When is the AR first expressed in developing laryngeal tissues? Using the *in situ* hybridization (for AR) and rhodamine-dextran (for fate mapping), AR mRNA expression first appears in laryngeal precursor cells a few days after fertilization as they migrate ventrally and caudally to the midline of the pharynx and form the laryngeal anlage. Thus, the gene for the AR is expressed extremely early and at high levels in laryngeal precursors. The regulation of gene expression cannot be the limiting factor in opening the androgen-sensitive period of muscle fiber addition. Androgen secretion also cannot be the critical factor because it also begins in early tadpole life. What opens the developmental period of muscle fiber addition?
Androgen-regulated muscle fiber addition begins after metamorphosis (Marin et al., 1990). One possibility is that hormonal changes associated with the transition from tadpole to froglet are also required for androgen-evoked cell proliferation (androgen competence). A good candidate is thyroxine, a thyroid hormone absolutely required for progression through metamorphosis in all amphibians. Androgen-evoked cell proliferation and growth in the developing larynx can be dramatic. The androgen-enlarged larynx produces extensive mortality because of the compression of the heart and lungs (Robertson and Kelley, 1996). This effect cannot be observed until metamorphic climax (late stages of tadpole development, 56 to 66), but is completely blocked if tadpoles are reared in the antithyroxine agent propythiouracil (PTU). This observation raises the possibility that androgen competence requires exposure to thyroid hormone. Further experiments then demonstrated that precocious administration of thyroxine induces precocious androgen competence both in the tadpole and in cultured tadpole larynges (Cohen and Kelley, 1996). We can conclude that the sensitive period for androgen competence is opened by another hormone, thyroxine. Only a brief exposure to thyroxine is required. The cellular and molecular events are responsible for this effect of thyroxine are not known.

The generation of sexually differentiated muscle fiber numbers in the larynx is the result of a developmental program that relies on androgen secretion from the testes and is initiated by thyroid hormone secretion. The program includes the stimulation of cell proliferation in cell types, myoblasts and chondroblasts, that express very high levels of AR. The close of muscle fiber addition in males is probably due to the depletion of the myoblast population as these cells differentiate. Females maintain responsive myoblasts that can be induced to enter myogenesis given creation of the proper endocrine milieu by a testis-transplant. Females can achieve masculine numbers of muscle fibers, but are not hypermasculinized, again suggesting a finite pool of the cellular components required for laryngeal muscle.

**B. Sexual Differentiation of Motoneuron Number**

The number of laryngeal motoneurons is also a sexually differentiated property of the X. laevis vocal system. Males achieve masculine muscle fiber numbers in the
larynx via androgen-stimulated cell proliferation. Attaining masculine numbers of laryngeal motoneurons, on the other hand, involves the androgen-mediated rescue from ontogenetic cell death (Kay et al., 1999). Laryngeal muscle fibers are innervated exclusively by the axons of neurons located in cranial nerve nucleus IX–X of the caudal medulla (Simpson et al., 1986). The number of laryngeal motoneurons in adult males is approximately twice that in females (Kelley and Dennison, 1990). Sexual dimorphism in cell number is believed to contribute to vocal behavior via the recruitment of the different motor units that subserve fast and slow trills.

Achieving a sexually differentiated number of laryngeal motoneurons is a developmental process. At the beginning of metamorphic climax, tadpole stage 54, the number of laryngeal motoneurons is high in both sexes and is the same (Kelley and Dennison, 1990). Both sexes then proceed to lose motoneurons via cell death, but the rate of loss is greater in males than in females. Treatment with the antiandrogen hydroxyflutamide increases motoneuron loss in males and reduces the number to female levels (Kay et al., 1999).

Sex differences in laryngeal motoneuron numbers thus appear to result from androgen-regulated differences in ontogenetic cell death. Laryngeal motoneurons express AR during the ontogenetic cell death period, in both sexes, and, as previously described, there are no sex differences in androgen levels during this time. Because androgen ablation in males reduces laryngeal motoneuron numbers to female values, androgen is necessary for cell rescue. As is the case for muscle fiber addition, however, androgen secretion may not be sufficient.

Ontogenetic cell death of laryngeal motor neurons is accompanied by a parallel decrease in axon number in the laryngeal nerve (Kelley and Dennison, 1990). If juvenile or adult females are given testicular transplants, the number of axons in the nerve decreases (Watson and Kelley, 1992). No androgen-evoked proliferation of motoneuron precursors can be observed and the increase takes place long after the normal period of cell death is over. One possibility is that androgen stimulation can evoke axon outgrowth from preexisting medullary neurons whose axons would otherwise remain in the CNS. The process could involve a shift in cell fate (from interneuron to motoneuron) or the differentiation of an immature population of motoneurons capable of projecting to the periphery under permissive conditions. When females are given testis transplants, even as adults, they can produce the male-specific advertisement call (Watson et al., 1993). This ability is very strongly correlated with the production of a masculine number of cells. Simply changing cell type (discussed later) does not result in masculinized vocal behaviors.

What are the mechanisms for androgen-induced rescue from cell death? Although the tadpole, the stage at which the process normally occurs, is accessible, it is small and a relatively difficult subject for experimental manipulation. An experimental model for ontogenetic cell death was thus developed, the axotomized juvenile larynx (Perez and Kelley, 1996). In this preparation, the laryngeal nerve is severed on one side as it enters the larynx during early juvenile stages (1–2 months PM or stage PM2). This procedure results in a gradual loss of laryngeal motoneurons; by 4 months after axotomy, the number is reduced to approximately 50% of their initial value (Perez and Kelley, 1996). In males, castration exacerbates motoneuron loss and androgen replacement protects motoneurons. The effects of axotomy are believed to be due to removing the retrograde influence of the target, the laryngeal muscle, rather than direct injury to the motoneuron itself. Thus the experimental model has several advantages—it preserves the androgen dependence of ontogenetic cell death, death is gradual readily permitting intervention, and the unoperated side is not affected by axotomy, proving a useful internal control.

How do laryngeal motor neurons respond to axotomy? One response is an increase in the expression of the AR gene in laryngeal motoneurons (Perez et al., 1996). This observation suggests that the challenged neurons increases its sensitivity to circulating androgen. Because exogenous and endogenous androgen are protective, the response could contribute to androgen-mediated motoneuron rescue. A direct test of this idea requires the ability to block the AR up-regulation or AR expression entirely. Because there are no known naturally occurring AR mutants in X. laevis, the development of transgenic preparations that interfere with AR expression is a logical next step for these studies.

If androgen is protective, what are its downstream targets? One possibility is the calcium-binding protein, calbindin (Perez and Kelley, 1997). This protein is expressed at very high levels in laryngeal motoneurons and their proximal dendrites, in their axons, in the synaptic terminals (Fig. 11), and in the Schwann cells
that ensheath the axons. Calbindin expression is not usually found in motoneurons and could be associated with maintaining calcium homeostasis in laryngeal motoneurons during the repetitive firing required for vocal production. When motoneurons are axotomized, calbindin expression drops; androgen treatment, however, maintains calbindin levels, suggesting that this downstream hormone target could contribute to its protective effects. In the rodent SNB system (see previous discussion), neurotrophins have been implicated in androgen-mediated rescue from ontogenetic cell death (Forger et al., 1997); this possibility has not been explored in *X. laevis*.

### C. Sexual Differentiation of Muscle Fiber Type

The sex-specific songs of *X. laevis* differ in rate of click production—males can produce very rapid trills, whereas the trills of females are slower (see Kelley Chap. 27 in this volume). The sex difference in vocal behaviors is matched by the properties of laryngeal muscle fibers, illustrated by the *vox in vitro* preparation, in which the larynx is isolated and muscle activity, contraction, and actual clicks evoked by the stimulation of the laryngeal nerve. Muscle contraction pulls on tendons attached to the arytenoid disks, the actual sound-producing elements in the larynx (see Fig. 8). A complete cycle of contraction and relaxation produces a single click (because *Xenopus* calls underwater, air flow is not a required component of vocal production).

At rates of nerve stimulation above 20 Hz, female laryngeal muscle tetanizes and cannot relax and contract rapidly enough to produce a clicks (Tobias and Kelley, 1987). Male muscle readily follows nerve stimulation up to 70 Hz and the muscle can contract and relax completely (producing 100% transient tension on the sound-producing arytenoid disks) at rates as high as 100 Hz.

Sex differences in contractility can be traced to sex differences in muscle fiber type—male muscle is entirely fast-twitch, whereas female muscle is mostly slow-twitch (Sassoon et al., 1987). This characteristic, in turn, is accompanied by sex differences in the expression of a particular myosin heavy-chain gene (MHC) whose expression is confined to the larynx (Catz et al., 1992). Laryngeal MHC was isolated from a laryngeal cDNA library using a tadpole MHC as a probe; it resembles chicken embryonic MHC most closely in sequence and is expressed in all muscle fibers in male larynx and some muscle fibers in female larynx. The laryngeal myosin (LM) protein is a strong candidate for the regulation of sex differences in contractility of laryngeal muscle fibers, and its expression appears to contribute to the rapid rates of click production that characterize male songs.

Sex differences in muscle fiber type are the result of divergent developmental trajectories in males and females. During early juvenile stages, fiber-type distribution is the same in both sexes and is mostly slow or female-like. After the period of sexually differentiated muscle fiber addition is over at 6 months PM, males gradually start to transform their muscle fibers from slow to fast twitch. The process is not complete until the male is 12 months PM, and when it is complete he starts to sing. Females, on the other hand, maintain...
the slow-twitch-fiber composition and the numbers of fast-twitch fibers actually decrease somewhat during this period. The expression of the LM gene parallels muscle fiber type composition. In males, the percentage of LM-expressing fibers increases during PM development, whereas in females it decreases somewhat. The functional consequences of these sex-typical developmental programs are apparent in recordings from the vox in vitro preparation. As PM development proceeds, the male larynx acquires increased numbers of fast-twitch muscle fibers, LM expression increased, and the percentage of transient tension increased, reaching adult values at approximately 12 months PM.

As was the case for muscle fiber number, muscle fiber-type switching is androgen-driven in males. Castration blocks but does not reverse the transition from mostly slow- to all fast-twitch fibers. Muscle fiber type can be switched to entirely fast twitch by exogenous androgen, precociously in juveniles and inappropriately in adult females. The expression of the LM gene parallels fiber-type changes and is up-regulated by androgen treatment. The androgen treatment of females at any developmental stage, including adulthood, transforms muscle contractility into the masculine pattern (Kelley, 1996). The androgen-treated adult females do not produce male songs, even if treated for 2 years. The masculinization of muscle fiber type is thus not sufficient for the production of male behaviors. The cellular and molecular events that underlie fiber transformation are not well understood. The simplest possibility is that the expression of the LM gene is regulated directly by androgen. For example, it might contain an androgen response element in its promoter that, when bound to androgen, stimulates LM expression. Two observations suggest that this is an unlikely scenario—the initiation of LM expression requires androgen, and castration at the end of metamorphosis blocks LM expression (Caiz et al., 1995). Once initiated, however, LM expression can be maintained in castrated males. Thus, continued exposure to androgen is not required for LM transcription, as the simple model suggests. Further, LM expression is not up-regulated by androgen in the presence of blockers of protein synthesis. This result suggests, at the very least, the requirement for a rapidly turning-over protein cofactor or that LM expression may lie well downstream in a chain of androgen-initiated events in laryngeal muscle. Such a chain must include a self-reinforcing component to explain the maintenance of LM expression in castrated males.

Even adult females can be induced to switch their muscle fibers from slow to fast. In adults this is a slow process requiring several months of androgen exposure, whereas in juveniles switching is more rapid and complete within 3 weeks. The adult larynx maintains a population of myoblasts (satellite cells) and it is possible that these participate in muscle fiber switching (Fig. 12). Some laryngeal myoblasts express the LM gene and can be induced to proliferate by androgen stimulation. The expansion of this cell population followed by fusion with existing slow-twitch muscle fibers could account for the slow transformation of fiber type in adult females (see Fig. 12, left). Myoblasts are more numerous in juveniles, perhaps accounting for the more rapid switching of fiber type following androgen exposure at earlier stages of development.

Androgen treatment is a much more powerful modulator of muscle fiber type than of muscle fiber

![FIGURE 12](image-url) Two possible models for the conversion of slow-twitch to fast-twitch muscle fibers in developing male laryngeal muscle. The developing laryngeal muscle fiber expresses myosin heavy-chain genes (MHC) controlled by activity of its myonuclei. It is surrounded by myoblasts expressing the LM (laryngeal myosin) gene. Under the influence of androgen and prolactin, gene expression changes with the muscle fibers so that all are LM positive. One possible mechanism (lower left) is the fusion of LM-expressing myoblasts with the muscle fiber and the extinction of the old pattern of MHC expression by transsuppression between nuclei. The other possible mechanism (lower right) is a change in gene expression in existing myonuclei. The two mechanisms are not necessarily mutually exclusive.
number (or laryngeal axon number, at least in adults). One possibility is that these two processes, myoblast fusion with one another to produce new fibers (Fig. 12, right) and myoblast fusion with existing fibers to change fiber type (Fig. 12, left), differ in their requirements for innervation by laryngeal motoneurons. Nerve-muscle interactions are discussed in further detail in a subsequent section (see also Fig. 7).

Despite the presence of circulating androgen and the expression of AR from tadpole stages, androgen-regulated muscle fiber type does not begin until late in juvenile development. What is responsible for regulating this developmental progression? One of the consequences of thyroid hormone secretion during metamorphosis is the synthesis of prolactin from the pituitary. The expression of the LM gene is blocked by hypophysectomy and induced precociously by exposure to prolactin and androgen (Edwards et al., 1999). It thus seems likely that the developmental period of muscle fiber switching in males is a consequence of prolactin secretion (Fig. 13). As is the case for thyroid hormone, a brief exposure to prolactin suffices to induce androgen-sensitivity of LM expression. The molecular basis for this effect is unknown. At a functional level, however, hypophysectomy at early juvenile stages blocks the ability of androgen to induce increases in transient tension in the developing larynx and thus blocks the ability of males to produce the rapid trills of their songs.

D. Sexual Differentiation of the Laryngeal Synapse

Laryngeal muscle contracts in response to the release of acetylcholine at the laryngeal NM. This synapse has the peculiar property of being generally weak in adult males and strong in adult females (Tobias and Kelley, 1987). Intracellular recordings in physiological saline reveal that action potentials in laryngeal motoneuron axons usually provoke only subthreshold excitatory postsynaptic potential in laryngeal muscle fibers (Tobias and Kelley, 1988). Repetitive activity and facilitation are required to enhance synaptic transmission and bring the muscle fiber to threshold for action-potentiation production. In females, on the other hand, the laryngeal synapse is strong or high safety factor. An action potential in the axon of a laryngeal motoneuron reliably produces a muscle action potential in the laryngeal muscle fiber. Together with the progressive recruitment of motor units, facilitation produces the pronounced amplitude modulation of the male's advertisement call.
Synaptic strength becomes sexually differentiated very late in development (Tobias et al., 1998). At the end of metamorphosis, laryngeal synapses are weak in both sexes. Males maintain low-safety-factor synapses into adult life. Females, on the other hand, convert from weak to strong synapses at some point between 26 and 28 months PM. Thus the default program for synaptic strength, exhibited in the absence of the gonads, is male-like or weak. The conversion requires an intact ovary and can be induced in juveniles of either sex by the administration of estrogen (Tobias et al., 1998). Estrogen secretion in females thus appears necessary for the development of their typically high-safety-factor laryngeal synapses. Unlike the effects of androgen on muscle fiber and motor neuron number and on muscle fiber type, the effects of estradiol are transient rather than permanent (Wu et al., 2001). Ovariectomy in adult females reduced synaptic strength to male levels. The effects of estradiol, like those of androgen, are slow. At least 3 weeks of treatment are required to convert weak to strong synapses in juveniles, and synapses do not weaken in adult females until 6 weeks or more after ovariectomy. The strong synapses of females thus develop from an initially weak state under the influence of estrogen secretion; estrogen secretion is also required for their maintenance in the adult.

Both pre- and postsynaptic elements could contribute to sex differences in synaptic efficacy at the laryngeal synapse. To examine this question directly, a quantal analysis of synaptic transmission was carried out and the characteristics of spontaneous quantal release (miniature endplate potentials, mepps) were analyzed at adult synapses (Tobias and Kelley, 1988). As expected from the intracellular recordings in physiological saline, quantal content values (obtained in reduced calcium saline to block action potentials in females) were considerably lower for male synapses than for female synapses. On the other hand, quantal size (as estimated from mepp amplitudes) did not differ between the sexes (Fig. 14). If the postsynaptic receptor were, for example, less sensitive or efficacious at male than at female synapses, a difference in mepp amplitudes, frequency, or rise times should have been present. Because no difference was apparent (nor were systematic differences in resting membrane potential observed), it is most likely that sex differences in synaptic efficacy are pre-rather than postsynaptic in origin—the probability of transmitter release from the presynaptic terminal is less at male than at female laryngeal synapses. Some support for this hypothesis comes from the rapid increase in transmitter release observed after raising extracellular calcium, an effect generally attributed to the presynaptic element.

The intracellular components responsible for sex differences in transmitter release are not known. The frog neuromuscular terminal is an extended structure; the presynaptic side is punctuated by parallel arrays of active zones identified by double rows of intramembranous particles believed to be the sites of synaptic vesicle fusion (Tobias et al., 1995). The intramembranous particles include the calcium channels through which millimolar increases in calcium are effected upon depolarization and the potassium channels essential for repolarization. Either might differ in males and females. Other candidates include synaptic vesicle-associated proteins that regulate vesicle fusion. A particularly attractive candidate is synaptotagmin because it is believed to be a calcium sensor and exists in a wide variety of isoforms.

The mechanism whereby estrogen regulates the laryngeal synapse is mysterious. Clearly the locus of this sexually differentiated feature is presynaptic; however, the estrogen receptor is not expressed in laryngeal motoneurons (Morrell et al., 1975). Laryngeal muscle does express estrogen receptors and it is possible that the strengthening of the synapse requires a retrograde signal from the muscle to the motoneuron. Alternatively, upstream elements in the vocal circuit (see Kelley, Chapter 27) could regulate the laryngeal motoneuron.

What are the behavioral consequences to the female of having weak or strong laryngeal synapses? Because ovariectomized females without produce the female release call, ticking, weak synapses are clearly not required for this slow trill. One initially appealing hypothesis is that females need strong synapses to produce their rapping call, an acoustic aphrodisiac given by ovipositing females in response to a singing male. However, gonadotropin, which induces oviposition, actually transiently weakens the laryngeal synapse (Wu et al., 2001). It is possible that the strong synapses of females are used to increase the safety factor of transmission under physiological conditions associated with
oviposition that would otherwise silence their vocal behavior.

E. Nerve–Muscle Interactions in Neurouromuscular Development

A key feature of neuromuscular development is the interdependence of the synaptic partners, the motoneuron and the muscle fiber that it innervates. When both the motoneuron and the developing muscle fiber are very sensitive to androgen, as is the case for the vocal neuromuscular synapse in X. laevis, untangling cell–cell interactions from hormone effects is a considerable challenge. One approach is to disconnect the two cell types by cutting the laryngeal nerve and compare responses of the innervated and denervated sides of
the larynx and the medulla with and without androgen treatment (Fig. 15). A series of studies using this approach reveals that some androgen-driven processes are not affected by loss of the synaptic connection, whereas others are affected. In the latter, androgen often appears to substitute for the loss of the partner.

In juveniles, treatment with DHT induces muscle fiber addition in females; significantly fewer fibers are added on the denervated than on the innervated sides (Fig. 15). Intact juvenile males do not respond to DHT by muscle fiber addition, probably because myogenesis is already androgen-saturated. Nonetheless, denervation reduces ongoing muscle fiber addition in untreated males, an effect that is mitigated by androgen treatment. In vivo, denervation does not block myoblast proliferation but rather appears to affect the ability of new myoblasts to fuse and form muscle fibers. In the cultured tadpole larynx, however, androgen only evoked proliferation in chondroblasts, even after exposure to thyroxine (Cohen and Kelley, 1996). This reduced preparation thus lacks factors that permit myoblast proliferation. One such factor could be the presence of the nerve. This cannot, however, be the only factor given the proliferation seen in vivo.

Androgens are trophic agents for laryngeal motor neurons both during tadpole development and in the axotomized juvenile preparation. The latter allows us to examine the role of nerve–muscle interactions in the response to androgen. Motorneurons respond to denervation by increasing the expression of the AR gene (Perez et al., 1996). A similar increase is evoked by androgen treatment, but does not require synaptic contact with the muscle. Aaxonotomy induces cell death in this system, but the effect is mitigated by androgen (Perez and Kelley, 1997). Thus for androgen-induced motorneuron rescue, as was the case for androgen-induced myogenesis, androgen can substitute for the loss of the synaptic partner.

Unlike the control of cell number, androgen regulation of muscle fiber type does not depend on intact synaptic contacts. Whether denervated or innervated, all muscle fibers in juveniles are switched from slow to fast twitch by 3 weeks of androgen treatment. Finally, we have not been able to evaluate the role of the synaptic contact in establishing synaptic strength during development because an intact synapse is required for the assay itself (postsynaptic recordings in muscle fibers). Nonetheless, the presence of estrogen receptor expression in laryngeal muscle, but not motorneurons, suggests the possibility of a retrograde signal from muscle to motor neuron that changes synaptic efficacy.

During development in males, the period of ontogenetic cell death for laryngeal motoneurons largely precedes the period of muscle fiber addition, although there is some overlap during the first 6 months PM. There are no sex differences in laryngeal muscle fiber...
number during metamorphosis or immediately thereafter, although there are already marked sex differences in motor number from tadpole stage 36 on. Thus, unlike the SNB system previously discussed, it is very unlikely that sex differences in number of muscle fibers are responsible for sex differences in number of motoneurons. In the SNB system, motoneurons do not express the AR during the period of ontogenetic cell death and can be rescued by local application of androgen to the LA and BC muscle; these observations suggest strongly that the action of androgen on motoneurons is via its targets in muscle (see previous discussion). In *X. laevis*, on the other hand, both cell types express the receptor from very early stages, and it is likely that androgen has some direct effects on each tissue type.

Organizational and Activational Effects of Steroids: A Reevaluation in the *Xenopus* Vocal System

Since the very influential paper by Phoenix and colleagues (1959), sexual differentiation of the neural substrates for reproductive behaviors has been viewed as reflecting two kinds of hormonal effects—organizational and activational. Our increasing knowledge of the cellular and molecular actions of steroid hormones allows us to determine how these effects are achieved. One distinction between the two is permanence; organizational effects are irreversible, whereas activational effects are reversible. In the *X. laevis* vocal system, the effects of androgen on cell number and type are organizational. If the testes are removed, masculinization halts but is not reversed. Once laryngeal muscle fiber number and type are masculinized, these features cannot be altered by androgen ablation; males castrated for 3 years retain a fully masculinized fiber and motor neuron number and a full complement of fast-twitch laryngeal muscle fibers. The switches that permit laryngeal muscle to respond to androgen—thyrroxine and prolactin secretion—are flipped during late metamorphic and early juvenile stages and the relevant programs can then be carried out if testicular androgens are present. There is little or no cell death in laryngeal muscle during these stages, so muscle fibers, once formed, are maintained. The change from fast- to slow-twitch fibers is also a permanent one. One possibility is that this permanence is due to the fusion of LM-expressing myoblasts with existing muscle fibers; given that the fibers are maintained, laryngeal muscle fiber type could be maintained permanently. Another possibility (and these are not mutually exclusive) is that once exposed to prolactin and then androgen, the LM gene is constitutively expressed in laryngeal muscle.

Omnogenic cell death is a common feature of motor neuron development in all vertebrates; approximately one-half of the neurons that are initially generated die during the short period when axons are contacting their targets. There is some evidence that neurons lose this vulnerability with time. For example, in the chick, if motoneurons are prevented from dying by the blockade of synaptic transmission and muscle activity, a much larger complement of motoneurons can be maintained after the chicks hatch (Oppenheim, 1984). Thus the permanence of androgen rescue from omnogenic cell death may be due to an autonomous program that regulates the vulnerability in motoneurons.

In contrast, the effects of estrogen on synapse type are clearly activational. Castrated adult females lose their strong synapses, which can be restored by estradiol treatment. The cellular processes that regulate transmitter release apparently require prolonged and maintained exposure to estrogen; once withdrawn, the enhancement of synaptic efficacy involutes. One possibility is that estrogen enhances the expression of a synaptic vesicle protein isoform or channel isoform that increases the probability of vesicle release. If that protein were relatively short-lived and its transcription required continued occupancy of an estrogen response element the requirement for continuous estrogen would arise. Although this is a completely speculative scenario, it does illustrate the kinds of molecular mechanisms that might be involved in the transitory and reversible nature of organizational effects.

Another distinction between organizational and activational modes is their developmental time frame. Organizational effects generally operate within a limited developmental window (the sensitive period); if this is not present, there may be some ability to rescue the process for a short time (the critical period). After the critical period closes, however, the chance to change the program for sexual differentiation has been lost. Does the masculinization of vocal motoneurons and muscle in *X. laevis* have these sorts of time windows for endocrine
action? If so, what cellular events are responsible for their close?

Muscle fiber addition in males is generally complete by 6 months PM (Martin et al., 1990). Masculine number of muscle fibers, however, can be produced by a testis transplant even in adult females (Watson et al., 1993). Androgen (DHT) treatment does induce muscle fiber addition in juvenile females, although not in adults. Together these observations suggest a sensitive period centered around early juvenile stages (0–6 months PM), but no limit on the critical period. For males, the pool of available laryngeal myoblasts and the rate of muscle fiber addition probably control the duration of this time period. In females, responsive myoblasts apparently remain, perhaps as stem cells, and can be reactivated by a testis transplant. The numbers of muscle fibers achieved in these females do not exceed those of males, again suggesting an inherent limitation on the cellular precursor pool.

The control of the number of motoneurons should have marked sensitive and critical periods because dead neurons are removed and neurogenesis is limited to very early tadpole stages (before the gonads form). Nonetheless, the observation that a testis transplant can increase laryngeal axon numbers even in adult females suggests some remaining plasticity. One possibility is that interneurons are reactivated as motoneurons or that the medulla contains a pool of immature cells capable of axonal growth to the larynx in response to the testis transplant. Testing these alternatives requires the development of a specific set of cell markers and the ability to follow axon outgrowth following testis transplants. The ability of females to produce male songs was not apparent until at least 10 months after they received the testis transplant. When, during this period, the related cellular changes, more axons and muscle fibers, occurred is not known.

Sensitive and critical periods open as well as close. The X. laevis vocal system provides considerable insight into the timing of these periods. The opening of the sensitive period for muscle fiber addition is established by thyroxine secretion. The larynx is sensitive to thyroxine (and to androgen if it is first exposed to thyroxine) from very early tadpole stages and the opening of the sensitive period is thus not due to an intrinsic limit on the responsiveness of the tissue but rather to a limitation on when the signal itself is produced.

There may not be a critical period for thyroid hormone action. When tadpoles are treated with PTU, the iodination of tyrosine is blocked and they do not progress through metamorphosis. These animals are completely insensitive to exogenous androgen in terms of laryngeal growth (Robertson and Kelley, 1996). Once the PTU is removed, however, even after 1–2 years, tadpoles resume metamorphosis and can respond to androgen.

The sensitive period for the androgen regulation of muscle fiber type is opened by prolactin secretion (evoked, in turn, by prior exposure to thyroxine). As was the case for fiber number, fiber type does not have a critical period. Hypophysectomy prevents androgen-induced muscle fiber type transformation in juveniles, but the block can be relieved by exogenous prolactin (Edwards et al., 1999).

In summary, then, the vocal system of X. laevis permits the identification of cellular and molecular events that underlie the phenomena of sensitive and critical period for a sexually differentiated behavior. These events include hormone–hormone interactions (steroids, pituitary, and thyroid hormones), cell–cell interactions (motoneurons and muscle) and tissue-specific patterns of differentiation and gene expression. The molecular events associated with these processes are largely unknown, but the system permits their identification and provides a framework in which gene expression can be directly tied to sex-specific behavioral expression.

IV. OVERARCHING PRINCIPLES OF SEXUAL DIFFERENTIATION ILLUSTRATED BY NEUROMUSCULAR SYSTEMS

The study of these relatively simple neuromuscular systems suggests several principles that may well apply to sexually differentiated systems in the CNS. First, steroids can affect a wide spectrum of cellular events. During development and adulthood, steroids influence a remarkably broad range of processes, from stimulating cell proliferation to influencing cell fate and differentiation, including the prevention of ontogenetic cell death. The second lesson is that steroids can act on many different levels of the same system. For example, androgen affects the survival of the SNB system during
development by acting on the muscles, but acts on
the motoneurons to affect their somata and later on
the muscles again to affect motoneuronal dendrites.
The widespread distribution of steroid receptors and
the ability of receptor-expressing cells to influence
synaptic partners suggest that all steroid effects are
likely to include multiple sites of action. Data from
the rodent SNB and Xenopus vocal systems also sup-
port the concept that steroids normally interact with
other hormones and growth factors. Some examples in
Xenopus include the roles of thyroxine and prolactin
in establishing androgen competence. In the SNB, an-
drogen appears to be altering a neurotrophic factor that
can interact with the CNTFR to maintain motor neuron
numbers in males.

The extensive interdependence of synaptic partners
in the developing nervous system turns out to be well
modeled in these developing steroid-dependent neu-
romuscular systems. The motoneurons maintain the re-
 sponsiveness of the muscle targets to hormones, and
the target muscles also affect hormone responsiveness
of the motoneurons. Androgens, for example, can sub-
stitute for the influence of the nerve on the muscle and
for the influence of the target muscle on the motor neu-
ron. This interdependence complicates the cellular and
molecular analysis of hormone effects, but also provides
strong evidence that these neuromuscular system can
display the same kinds of synaptic–target interdepen-
dence as those present in developing CNS. The mutual
dependence of motoneuron and muscle target is also a
reminder that natural selection has favored systems in
which the various components continuously monitor
and affect one another, an important concept for anyone
trying to understand the neural control of behavior.

In early conceptions of steroid modulation of behav-
ior and the nervous system, developmental (organiza-
tional) effects were viewed as qualitatively different
from adult (activational) effects (Phoenix et al., 1959).
Data from Xenopus and rodent neuromuscular systems
remind us that activational effects of steroid hormones
can be as profound as those occurring earlier in de-
velopment. These observations echo a growing consens-
sus in the field of neurosciences generally—that the
adult nervous system retains a remarkable plasticity.
The cellular and molecular events that underlie the
remodeling of the adult nervous system in response
to steroid hormones—and the role played by behav-
ior in this remodeling—is an exciting area for current
studies.

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IV. Development of Hormone-Dependent Neuronal Systems


65. What Neuromuscular Systems Tell Us about Hormones and Behavior


