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Steroid hormone masculinization of neural structure in rats: a tale of two nuclei

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Abstract

We review the mechanisms by which steroid hormones masculinize two different regions of the central nervous system (CNS) in rats. Although in both cases, androgens induce a male phenotype, the detailed mechanisms are remarkably different in the two models. In the spinal nucleus of the bulbocavernosus (SNB), testosterone must be present during the perinatal period to spare motoneurons and their target muscles from cell death. This masculinization of the SNB system is through activation of androgen receptors, because XY rats with a defective gene for the androgen receptor fail to develop a masculine SNB system. Interestingly, the motoneurons are spared by androgen, even though they themselves do not possess androgen receptors during the critical period for their survival. Thus, steroids can act on one part of the body to secondarily masculinize the CNS. In the posterodorsal aspect of the medial amygdala (MePD), testosterone can induce masculine development even in adulthood, indicating that there is no critical period for steroids to affect sexual differentiation of this system. In the case of the MePD, both estrogen receptors and androgen receptors appear to mediate testosterone's masculinizing influence on neural structure. The extended neural plasticity of the MePD may reflect annual "reorganization" of the brain in the seasonally breeding ancestors of laboratory rats.

Keywords: Sexual differentiation; Androgens; Estrogens; Spinal nucleus of the bulbocavernosus; Medial amygdala

In most mammalian species, the presence of a Y chromosome carrying the *sex-determining region of the Y* (*Sry*) gene causes the fetal gonad to develop into a testis, while the absence of a Y chromosome (in XX individuals) causes the fetal gonad to develop as an ovary. Steroid secretions from the fetal testes then play a crucial role in sexual differentiation, the process by which an individual develops either a male or a female body; androgens such as testosterone masculinize internal and external structures. In females, the relative absence of testicular steroids permits the internal and external genitalia to take the feminine configuration. The process by which individuals develop either a male or female body, sexual differentiation, is thus driven by testicular secretions that organize masculine development. Over 40 years ago, Phoenix et al. [1] proposed that the same steroidal signals that masculinize the structure of external genitalia may also organize the structure of the brain and thereby masculinize behavior. This conjecture has been amply confirmed. They further speculated that just as the genitalia must be exposed to androgen in early development to be fully masculinized (androgen treatment in adulthood has only very modest effects), the brain, too, may be sensitive to the masculinizing effects of steroids only during development.

Here, we will review two model systems in the central nervous system (CNS) that are masculinized by testosterone. One model, based in the spinal cord, conforms to the original notion of steroidal organization of the nervous system: Perinatal androgen permanently alters the number of neurons present. The other model, based in the brain, is also masculinized by androgen, but the system appears to be

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capable of responding to androgen throughout life, suggesting that some brain regions can be organized (or "reorganized") even in adulthood. Comparing these two systems will display a range of cellular mechanisms in which steroids can affect neural tissues and behavior.

Comparing these two systems will also point out a large gap in our current knowledge of how hormones direct sexual differentiation of the nervous system. Although we have known for some time that steroids engender a wide variety of neural sexual dimorphisms, we do not know which cell population is the initiating site of hormone action for any sexual dimorphism in the brain. Only in the spinal cord has progress been made in determining the primary site of androgen action for directing sexual differentiation of neural structure.

1. Are steroid hormones doing all the work?

As a prelude to that discussion, it is important to step back and ask whether there are other influences, independent of steroid hormones, that also direct sexual differentiation of the CNS. There have been several recent reports that are consistent with the idea that there may be direct genetic influences on sexual differentiation of the nervous system. Specifically, genes that are present only in males (because they reside on the Y chromosome) may be expressed in the developing CNS to induce masculine development. By extension, the expression of other genes may vary depending on whether an individual carries an XY or XX genotype, and these genes may direct developing neural tissues in either a masculine or feminine fashion. Of course, we already know that sex chromosomes affect CNS development—Y chromosomes lead to testes, which produce hormones that masculinize neural systems. The question is whether sex chromosomes also affect neural development without resorting to hormonally mediated mechanisms.

For example, Dewing et al. [2] report that the brains of male and female mice express different genes even at 10.5 days post-conception, "before any gonadal hormone influence." The limitation of this approach, of course, lies in that last phrase. How can one determine when gonads first exert an influence? The best one can say is that, given the knowledge and technical ability we have at present, we are not aware of any difference in hormonal output between ovaries and testes at this stage. However, it is always possible that the gonads are releasing hormones we are unaware of, or releasing known hormones in quantities too low to be detected by present methods.

Similarly, De Vries et al. [3] examined mice with anomalous genetic compositions, such as having a Y chromosome that was missing *Sry* (making an XY individual that appeared to be mostly female, including ovaries), or having two X chromosomes plus an *Sry* gene on an autosome (making an XX individual that appeared to be mostly male, including testes). For the most part, these animals confirm the importance of gonadal influences on CNS development. By and large, the sex of the gonads allowed one to predict the sex of the brain. There was an exception. The vasopressinergic fibers of the lateral septum, which are normally sexually dimorphic, were more masculine in "females" with a Y chromosome (but no *Sry*), than in females with two X chromosomes. Furthermore, behavioral differences could be discerned between males who carried *Sry* in its usual location on the Y and XX "males" with *Sry* on an autosome.

The limitation of this approach is that, whatever the genetic composition of these mice, the gonads carry the same chromosomes as the brain. Thus, it is always possible that the testes of an XX "male" carrying *Sry* on an autosome do not secrete exactly the same amounts and pattern of steroids as the testes of a normal XY male. For example, these XX males fail to produce functional sperm, so it is not far-fetched to think they might display subtle defects in male hormone secretion. Likewise, an XY "female" missing the *Sry* gene has visible ovaries, but it would be difficult to prove that those ovaries perinatally secrete the very same (low?) levels of hormone as normal ovaries do. One way to show that genes directly contribute to sexual differentiation of the brain without acting through gonadal hormones is to construct an animal in which the gonads and the brain have different genotypes (e.g., XY testes and XX brain), and compare them to animals with a single genotype (e.g., XY testes and XY brain). Thus, while it may be that "sex chromosome genes contribute directly to the development of a sex difference in the brain" [3], the case has not yet been proven if by "directly" one means independent of genetic influence upon gonadal secretions.

On the other hand, these studies indicate that gonadal steroid secretion accounts for the vast majority of sexual dimorphism in the mammalian CNS. At the very least, these experiments indicate that any direct (non-gonadal-hormone-mediated) influence of the sex chromosomes on neural sexual differentiation is either very subtle or non-existent in the systems examined so far. So, gonadal steroids are either doing all the work, or the great majority of the work, initiating the cascade of events required to make the brain masculine. With that validation of the importance of steroids for this process, let us see how hormones exert their effects in two model systems that are quite different from one another.

2. Spinal nucleus of the bulbocavernosus (SNB)

In the lower lumbar spinal cord of rodents is a collection of motoneurons that innervate the striated muscles bulbocavernosus (BC) and levator ani (LA), each of which attaches exclusively to the base of the penis. These motoneurons form the SNB, and, as you might expect, there are more SNB motoneurons in males than in females

[4]. Similarly, the SNB target muscle BC is entirely absent in adult female rats [5], while the LA is severely diminished [6]. This sexual dimorphism of SNB motoneurons and their target muscles appears to be completely dependent upon androgenic stimulation in males, because XY animals with a defect in the gene for the androgen receptor, while masculine in some regards, possess a very feminine SNB system [7]. Having established that androgen stimulation is necessary for masculine development of the SNB, a program of research has revealed what the hormones do to form a male system.

During fetal development, there is a time when the SNB system is sexually monomorphic. The muscles are present in both sexes and are approximately the same size [8]; the numbers of motoneurons in the SNB region are approximately equal [9]; and the neuromuscular junctions between motoneurons and the target muscle are functional [10]. However, starting just before birth, the muscles continue to grow in males, while shrinking in females [8], and the number of motoneurons in the SNB region declines only slightly in males, but quite precipitously in females [9]. Perinatal androgen treatment of females prevents the loss of muscle targets and motoneurons, while perinatal androgen deprivation of males causes the system to die [11,12].

Thus, the nature of the sensitive period for androgenic masculinization of the SNB system is made clear: If androgen arrives before the muscles and motoneurons have died, they will be spared; once the muscles and motoneurons have died, it is too late for androgen to have an effect. This seems to be an instance in which one can readily defend the idea that there is not only a "sensitive period" for steroid action on the system, but that there is also a "critical period". No amount of androgen can resurrect the system once it has died.

The single greatest success of the SNB system has been the feasibility of asking where androgens act upon the system to initiate masculinization. This is in stark contrast to our knowledge about sites of steroid action for sexual dimorphisms in the brain. Yet, even for the SNB system, what we have learned is primarily where androgen does not act to masculinize the system. One experiment demonstrated that even when the connections from the brain to the spinal cord were severed in newborn female rats, androgen treatment still spared the SNB system from involution [13]. This result showed that androgen did not act upon brain afferents to the SNB motoneurons to keep them alive, but that left many possibilities open. For example, androgen could act on some distant site to secrete another hormone, etc. The simplest remaining hypotheses were that either the muscles and/or the motoneurons were responding directly to androgen. There was a hint that androgens did not rescue the motoneurons directly, because during ontogeny, the SNB cells do not appear to express androgen receptor until after the first week of life [14], long after the period of apoptosis. Freeman et al. [15] confirmed this notion when they examined female rats that were mosaic for functional androgen receptors. These females possessed two X chromosomes, but only one of the two carried a gene for producing a functional androgen receptor. The other X carried a defective androgen receptor gene. Because of random X inactivation, somatic cells expressing one X would possess functional AR protein, while cells expressing the other X would possess dysfunctional AR protein. It turned out that SNB motoneurons carrying either X were just as likely to be spared by perinatal androgen treatment (Fig. 1). Thus, SNB motoneurons without the capacity to respond directly to androgen could still be spared by the hormone. So we know that neither brain afferents nor the SNB motoneurons themselves are the primary site of androgen action for sparing the system.

At this point, one might ask whether the androgen receptors found in the SNB motoneuron have any effect on the system. Making these same mosaic females permitted Watson et al. [16] to show that in adulthood, the androgen receptors in SNB motoneurons are responsible for causing their somata to enlarge. When they treated the mosaic females with testosterone in adulthood, it was only those SNB motoneurons using the functional androgen receptor gene that enlarged their somata. The SNB motoneurons using the defective (Tfm) version of the androgen receptor gene did not respond to adult androgen treatment. Thus, the ability of androgens to induce SNB motoneurons to expand their somata is a cell-autonomous response. Likewise, androgen's ability to decrease expression of calcitonin gene-related peptide (CGRP) in rat SNB motoneurons is also a cell-autonomous response, because only those motoneurons expressing wildtype androgen receptor suppressed CGRP expression in response to adult androgen; motoneurons expressing the defective androgen receptor expressed CGRP whether androgen was present or not [17]. As far as we know, these are the only examples in which investigators have determined at the cellular level the primary site of steroid action for influencing structure or function of the adult nervous system.

As for the question of the site of steroid action on the developing SNB, because developing Tfm motoneurons are as likely to be spared as wildtype motoneurons, androgen must be acting on some other cell population to maintain the system into adulthood. By elimination, these data would suggest that the target muscles BC and LA are the tissues that directly respond to androgenic hormones during development, and that this androgenic sparing of the target muscles indirectly causes the innervating SNB motoneurons to survive. There is some modest positive evidence for this scenario. Prenatal BC/LA muscles demonstrate a capacity to specifically bind radiolabeled androgen [18], so they show biochemical evidence of competence to respond to testosterone at the appropriate stage of development. However, just because the muscles possess AR at the right time does not mean that androgen is interacting with those particular AR proteins found in the muscles to spare the system. Furthermore, muscle is not composed of a unitary cell type.

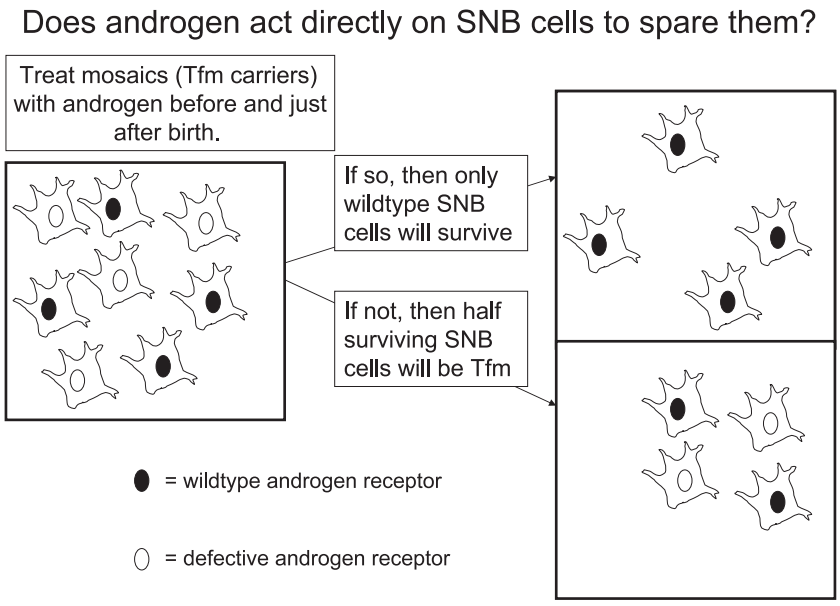

Fig. 1. When newborn female rats carrying a defective copy of the androgen receptor gene on one X are treated with testosterone during the perinatal period, they possess a partially masculinized SNB system. Immunocytochemical detection of the androgen receptor distinguishes those motoneurons using the defective (*Tfm*) version of the androgen receptor gene (depicted with a hollow, unstained nucleus) from those motoneurons using the wildtype androgen receptor gene (black nuclei). Of the two possibilities depicted on the right, the lower one was found, i.e., about half the surviving SNB motoneurons were Tfm [15]. This result demonstrates that androgen does not act directly on SNB motoneurons themselves to spare them from developmental cell death.

Muscles contain, in addition to muscle fibers, fibroblasts, Schwann cells, endothelial cells and proprioceptors. Interestingly, BC/LA muscles do not possess muscle spindles [19] (unpublished observations); however, it is still possible that androgen could act upon either fibroblasts or Schwann cells to maintain the muscles during the perinatal period. Examination of adult BC/LA muscles indicates that muscle fibers, fibroblasts, and endothelial cells, but not Schwann cells, possess AR [20]. But it is possible that during development Schwann cells also express AR. Thus, all we can say at present is that androgen may act upon the BC/LA muscles to maintain them, but we do not know which cell population within the muscles are responding to the hormone.

Having eliminated brain afferents and SNB motoneurons as the site of androgen action to spare the system, future experiments must attempt to manipulate AR expression in a tissue specific fashion. If one can create an animal that produces functional AR only in muscle fibers, then the SNB system would be spared only if muscle fibers are the critical site of androgen action. Alternatively, if molecular biological approaches were used to make a mouse that was missing the AR gene only in muscle fibers, then the SNB system should involute, despite androgen influence, if muscle fibers are the critical site for steroid action.

3. Medial amygdala

The medial amygdala receives input from secondary sensory neurons from the olfactory bulb and accessory olfactory bulb, which carry information about odors and pheromones. The medial amygdala, in turn, sends axons to a variety of targets, including the preoptic area. Thus, basic neuroanatomy suggests that the medial amygdala may play a role in reproduction in rodents, which rely heavily on olfactory and pheromonal cues for reproduction. Indeed, lesion and other manipulation studies confirmed a role for the medial amygdala in the regulation of reproductive behavior [21,22]. Moreover, in adult rats, there is a sexual dimorphism in the volume of the medial amygdala and in the size of neurons found there; these measures are larger in males than in females [23].

The medial amygdala presents four main divisions along the dorsal–ventral and anterior–posterior dimensions. The posterodorsal medial amygdala (MePD) offers especially distinct boundaries in Nissl stain and a particularly dense concentration of cells expressing androgen receptors and estrogen receptors [24]. We have therefore concentrated on the MePD as a model system for steroid-regulated neural plasticity, although the other quadrants of the medial amygdala are also steroid-sensitive.

The sex differences in the volume of the MePD and in the size of the neurons in this region can be entirely accounted for by circulating levels of androgens in adulthood. Castrating adult male rats results, 4 weeks later, in an MePD that is not significantly different from that of a normal female (Fig. 2). Conversely, treating an adult female rat with physiological levels of testosterone for a month causes the MePD to enlarge (both in regional volume and in the size of neurons) to a size seen in normal males [23].

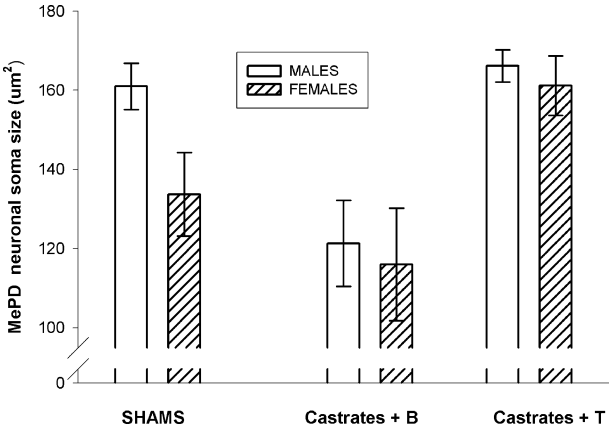

Fig. 2. The sexual dimorphism in the size of MePD neuronal somata (Sham surgery; left) can be reversed by manipulations of androgen. Castration of adult males causes the somata to shrink if the animals are given blank (B) capsules. Conversely, adult male or female rats treated with testosterone (T) for 4 weeks will possess enlarged MePD somata. There is a sex difference only among sham operated animals, while both male and female castrates treated with T have larger somata than castrates given blank capsules (all p's<0.05). The same pattern of results is seen in the regional volume of the MePD.

While the sexual dimorphism in the SNB system seems to be entirely due to stimulation of androgen receptors, in the MePD, it appears that both androgen receptors and estrogen receptors mediate masculinization of neural structure. In other neural systems, such as the sexually dimorphic nucleus of the preoptic area (SDN-POA), it is the aromatized metabolites of testosterone that interact with estrogen receptors to masculinize the nucleus perinatally [25]. In adult male rats that were castrated, soma size of neurons in MePD could be maintained by treatment with a non-aromatizable androgen (dihydrotestosterone) [26]. However, treatment with estradiol also maintained MePD soma size in castrate males. Furthermore, only estradiol treatment, not dihydrotestosterone treatment, was capable of maintaining MePD volume in castrates. These results suggest that testosterone normally stimulates both androgen receptors and estrogen receptors to masculinize the MePD, and indeed combined treatment with estradiol and dihydrotestosterone was more effective than either metabolite alone at maintaining MePD structures [26]. What remains to be determined is whether the changes in adult MePD regional volume are accompanied by changes in the number of neurons and/or glia. Likewise, no one has yet counted the number of neurons in the MePD of gonadally intact animals to see if there is normally a sex difference in neuronal number there.

Why is the masculine character of the MePD susceptible to changes in circulating androgen in adulthood? In some sense, this adult sensitivity to steroids seems to contradict the organizational model of hormonally regulated sexual differentiation in the CNS. On the other hand, one could regard the MePD as still following the spirit of the organizational hypothesis (testicular steroids masculinize the brain) with the interesting proviso that the sensitive period for organizing the MePD extends into adulthood. This extension of the sensitive period into adulthood is also seen in the birdsong brain nuclei of canaries [27]. The adult plasticity of the rat MePD

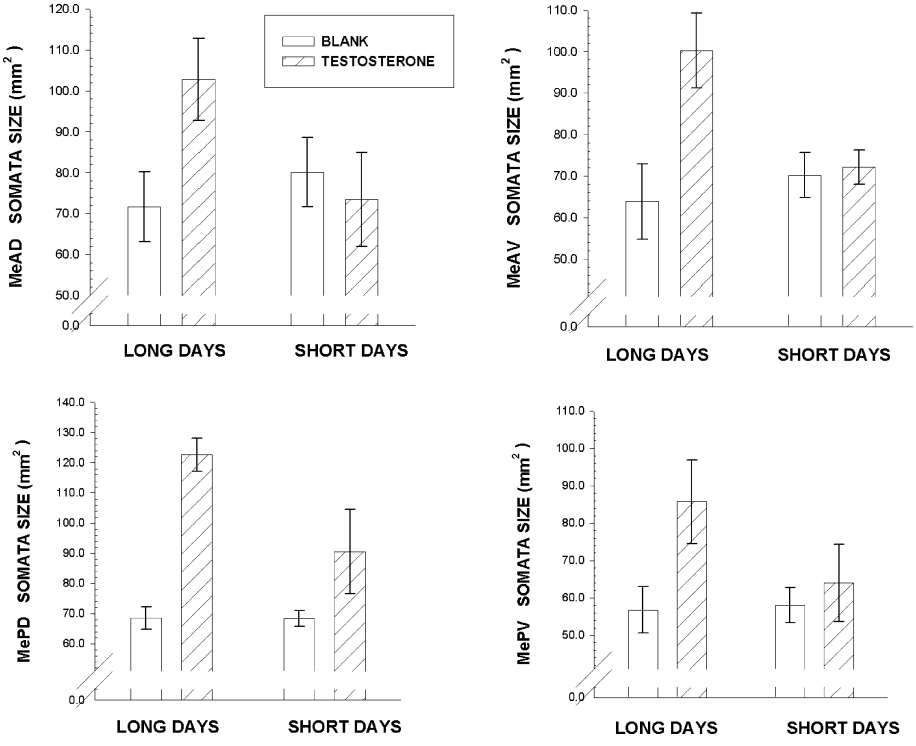

Fig. 3. Neurons in the four quadrants of the medial amygdala enlarge after androgen treatment of adult castrated male Siberian hamsters. However, the neurons will show this response to androgen only if the animals are kept in summer-like long days (all p's<0.01 except for MePV where p=0.07, two-tailed). In winter-like short days, the medial amygdala is refractory to androgen in these seasonally breeding rodents (all p's≥0.10).

probably reflects the fact that the ancestor to laboratory rats was a seasonal breeder, which is a characteristic that human husbandry has been selecting against for thousands of (rat) generations. For example, the seasonally breeding Siberian hamster (*Phodopus sungorus*) can be shifted from reproductive competence to reproductive quiescence by exposure to short day, winter-like photoperiods in the laboratory [28]. Moving adult male Siberian hamsters from long days to short days causes the MePD to shrink in regional volume and neuronal soma size. Moving such animals back into long days allows the MePD to expand in size again [29]. Because the MePD waxes and wanes in size in this seasonally breeding rodent, it seems likely that the brain nucleus showed this hormonally mediated reversibility in the seasonally breeding ancestor to laboratory rats. Although seasonal breeding has been selected out of the rats, the hormonal plasticity of the MePD remains.

Interestingly, the androgen induced plasticity of the hamster MePD can be modulated by environmental factors. We castrated adult male Siberian hamsters that had been reared in long days and then gave them either blank capsules or capsules containing testosterone. As expected, the MePD regional volume and neuronal soma size shrank in the males following castration, and testosterone treatment prevented this shrinkage but only if the animals were kept in long days. If after the surgery the animals were transferred to short days, the MePD shrank whether or not androgen was provided [30]. Thus, it appears that long days, in addition to maintaining the reproductive system, and therefore androgen levels, in adult male hamsters, also maintain the competence of the MePD to respond morphologically to the androgen (Fig. 3).

4. What do these studies tell us?

One lesson to be drawn from comparing the SNB and the medial amygdala is that different neural regions may be masculinized by steroid hormones in dramatically different ways. Hormones must act upon the SNB during development in order to masculinize motoneuron number and target muscle size. However, the MePD can be masculinized by androgen even in adulthood, and, as far as we know, this masculinization is complete. The SNB seems to be masculinized by the activation of androgen receptors, not estrogen receptors, while masculinization of the MePD is accomplished by activation of both types of receptors. In contrast, masculinization of the developing SDN–POA seems to rely on stimulation of estrogen receptors, not androgen receptors.

The SNB system has taught us that it is not easy to determine precisely which cells are responding to steroid hormones to initiate masculinization of the CNS. Adult SNB motoneurons possess androgen receptors, yet, their survival in development has nothing to do with those receptors. So even if the cells in a sexually dimorphic neural system possess steroid receptors, that fact may provide no information about the site where hormone acts to engender the dimorphism. The hormone may act on the nucleus itself to masculinize it, or it may act elsewhere, starting some chain of events that will later indirectly masculinize the nucleus. The MePD, for example, is sexually dimorphic and contains both androgen and estrogen receptors, but we know nothing about the cellular site(s) of steroid action that cause the MePD to grow.

On the other hand, the MePD has reminded us that not all sexual dimorphism in the nervous system is organized by perinatal steroid action. Adult hormonal fluctuations also affect the structure of song control nuclei in the brains of canaries, although it is not yet clear whether those dimorphisms can be entirely reversed by adult manipulations. This adult plasticity in canaries is probably related to their seasonal breeding and serves to remind us that the majority of vertebrate species in temperate regions are also seasonal breeders. We would do well to remember that some, but not necessarily all, neural plasticity in regions related to reproduction may have been selectively bred out of our convenient laboratory species. In practical terms, this means that no matter how dramatic a sexual dimorphism one finds in the CNS, it is still important to ask whether hormones act during development or adulthood to induce the sex difference.

The MePD has also taught us that environmental stimuli, such as photoperiod, can influence the responsiveness of the CNS to steroid hormones. This context-dependent hormone responsiveness of the nervous system may be more the rule than the exception. For example, in highly social species, one might expect that social interactions could have a significant influence on the brain's responsiveness to hormones. Future research might profitably attempt to find the environmental and experiential factors that modulate hormone responsiveness in other neural systems.

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