Published in final edited form as:
Horm Behav. 2009 May ; 55(5): 605–610. doi:10.1016/j.yhbeh.2009.03.008.

The Organizational Hypothesis and Final Common Pathways: Sexual Differentiation of the Spinal Cord and PeripheralNervous System

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Abstract

In honor of the 50th anniversary of the "organizational hypothesis," this paper reviews work on sexual differentiation of the spinal cord and peripheral nervous system. Topics considered include the spinal nucleus of the bulbocavernosus, the ejaculation center, the cremaster nucleus, sensory and autonomic neurons, and pain. These relatively simple neural systems offer ample confirmation that early exposure to testicular hormones masculinizes the nervous system, including final common pathways. However, I also discuss findings that challenge, or at least stretch, the organizational hypothesis, with important implications for understanding sex differences throughout the nervous system.

Introduction

Fifty years ago, Phoenix, Goy, Gerall and Young (1959) formulated what was to become known as the "organizational hypothesis." They observed that testosterone given to pregnant guinea pigs permanently altered the sexual behavior of the adult offspring, and proposed that hormones produced from the testes had organizing effects on the developing nervous system. Here, I examine the organizational hypothesis as it relates to sexual differentiation of 'final common pathways,' i.e., spinal motoneurons and the peripheral nervous system. Because the circuits involved are relatively simple, researchers studying sex differences in spinal and peripheral neurons have been able to address questions about the function of neural sex differences and the mechanism(s) of hormone action in ways that are often not possible for more complex systems in the brain. In the process, this work has revealed principles, as well as caveats, likely to be relevant to understanding sexual differentiation throughout the nervous system.

From a historical perspective, studies of sexual differentiation of the spinal cord and peripheral nervous system bring us full circle when thinking about how hormones change behavior. Although it seems almost self-evident today that gonadal hormones affect the developing brain, the idea was a significant shift in thinking when proposed in 1959. Phoenix et al. (1959) were cautious in the title of their landmark paper, referring to the effects of prenatally administered testosterone on the "tissues mediating behavior." Elsewhere within the paper, however, they make it clear that these tissues included the nervous system, and it is this that set the paper apart from prior work. For example, Young's group had previously performed very similar

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experiments to those described in Phoenix et al., (1959), and had demonstrated lasting suppression of feminine sexual behavior in rodents exposed to testosterone pre- or neonatally (Wilson et al., 1940, 1941). The interpretation of those earlier studies, however, focused on effects of perinatal androgens on peripheral structures such as the ovaries and uterus, with only vague references to "the behavioral mechanism." Similarly, Frank Beach and colleagues had demonstrated prior to Phoenix et al., (1959) that perinatal castration permanently decreased sexual behavior in male rats (Beach and Holz, 1946). The authors were struck, however, by the fact that effects of perinatal androgen manipulations on later sexual performance paralleled rather perfectly hormone effects on development of the penis (Beach and Holz, 1946; Beach et al., 1969). Thus, while not denying the possibility that androgens direct brain development, Beach and colleagues suggested that hormonal effects on peripheral structures should not be forgotten in attempts to explain some of the lasting effects of perinatal androgen exposure on later behavioral potential.

A perspective of 50 years validates both points of view. There have been numerous discoveries of permanent sex differences in neural structure or neurochemistry due to early effects of gonadal steroids, and it is undeniable that testosterone and its metabolites "organize," or permanently "sensitize" (a term preferred by Beach), neural areas. At the same time, some effects of steroids on the developing nervous system require hormone action at a distant site, a point that has been made most clearly by work on spinal and peripheral neurons, as described below.

The Spinal Nucleus of the Bulbocavernosus – *Importance of the target*

One of the earliest morphological sex differences found in the mammalian CNS concerned the spinal nucleus of the bulbocavernosus (SNB), a pool of motoneurons in the lower lumbar spinal cord of rats. SNB motoneurons project to striated muscles of the perineum, including the bulbocavernosus (BC) and levator ani (LA), which attach to the base of the penis and contract rhythmically during erection and ejaculation (Hart, 1972; Breedlove and Arnold, 1980; Schroder, 1980; Holmes and Sachs, 1994). Male rats have many more SNB cells than do females and the BC and LA muscles are absent or vestigial in females (Cihák et al., 1970; Breedlove and Arnold, 1980). Developmentally, SNB motoneurons and BC/LA muscles initially form in both sexes, but degenerate in females around the time of birth (Cihák et al., 1970; Nordeen et al., 1985; Jacob et al., 2006; 2008). A very similar sex difference and developmental profile is seen for motoneurons in the neighboring dorsolateral nucleus (DLN), which innervate the ischiocavernosus, a third muscle associated with the penis (Jordan et al., 1982; Sengelaub and Arnold, 1989).

The SNB and DLN neuromuscular systems have been the subject of more than 200 papers to date (see Sengelaub and Forger, 2008, for a recent comprehensive review), and the basic sex difference first found in rats has been extended to many mammalian species, including mice, gerbils, dogs, hyenas, monkeys, and humans[1] (Forger and Breedlove, 1986; Wee and Clemens, 1987; Ueyama et al., 1985; 1987; Ulibarri et al., 1995; Forger et al., 1996). Progress has been made possible in part because of the relative simplicity of the "wiring diagram" (all SNB and DLN cells project to the striated perineal muscles) and the accessibility offered by neurons with projection sites in the periphery.

The SNB system in many ways perfectly adheres to the organizational hypothesis: SNB motoneuron number is not altered by adult hormone manipulations (Breedlove and Arnold, 1981); the sex differences in SNB motoneurons and their target muscles can be eliminated by

[1]An interesting exception is seen in naked mole-rats, which lack sex differences in the perineal muscles and their innervating motoneurons (Peroulakis et al., 2002; Seney et al., 2006). This may be related to their unusual social structure. Naked mole-rats live in large colonies with only a few breeding individuals; the majority of colony members live their entire lives as non-reproducing subordinates.

treating female rats with testosterone during perinatal life (Breedlove et al., 1982); gonadal sex determines SNB development regardless of sex chromosomal complement (i.e., there is no apparent direct contribution of genes encoded on the sex chromosomes to the differentiation of this system; De Vries et al., 2002); and testosterone acts via the classical, intracellular androgen receptor (AR) to masculinize SNB cell number (Breedlove and Arnold, 1981). By contrast, an amendment to the organizational hypothesis, as originally formulated, had to be made to account for many sex differences in the rodent brain, where the aromatization of testosterone to estradiol is required for masculinization. Nonetheless, an interesting twist soon emerged regarding the site of hormone action in the development of the SNB neuromuscular system: several lines of evidence suggest that androgens do not act at the SNB motoneurons themselves to control their survival.

For example, ARs are not detected in SNB cells until after the critical, perinatal period when testosterone determines their fate (Fishman et al., 1990; Jordan et al., 1991; 1997). In addition, in female carriers of the Tfm mutation, which express a non-functional AR in about half of their cells, testosterone can rescue SNB motoneurons that themselves do not express functional receptors (Freeman et al., 1996). The target muscles of SNB cells, the BC and LA, do express AR during the critical perinatal period, and androgen-induced masculinization of the SNB can be prevented by application of the anti-androgen, flutamide, to the muscles (Fishman et al., 1990; Fishman and Breedlove, 1992). Thus, the target muscles of SNB motoneurons have been proposed to be the critical site mediating effects of androgens on SNB cell number. Testosterone is also thought to act at the target muscles to control the development of SNB dendritic trees, although in this case, estrogenic metabolites of the hormone are important (Nowacek and Sengelaub, 2006).

Muscles are a critical source of trophic factors for essentially all motoneurons, and exogenous trophic factors delivered to the perineum (the site of SNB axon terminals) rescues SNB motoneurons in females (Forger et al., 1993). Moreover, antagonists to such trophic factors, when injected into the perineum, block the sparing effect of androgens (Xu et al., 2001). Taken together, hormone action at the level of the muscle may lead to BC/LA survival and increased availability of trophic support for SNB motoneurons. Note that in this scenario, hormones from the testes have permanent effects on the nervous system (as predicted by the organizational hypothesis), but do so by acting at the periphery, much as suggested by Beach in his critique of the hypothesis (Beach et al., 1969).

The most recent wrinkle in the story comes from a clever study in which rats were created that express functional androgen receptors *only* in muscle fibers. Monks and colleagues crossed Tfm rats with transgenic rats expressing the wildtype human AR under the control of a striated muscle specific promoter (Niel et al., 2009). These animals express the transgenic, functional, AR only in striated muscle fibers, while non-functional AR is expressed in all other cell types. Surprisingly, the SNB system is not masculinized in the transgenic males, or in transgenic females treated with testosterone (Niel et al, 2009). This suggests that expression of AR solely in muscle fibers is not sufficient to spare the SNB system. It remains possible that some other cell type within the perineal muscles is the critical site of hormone action (for example, fibroblasts in the BC and LA muscles express AR; Monks et al., 2004), or that the sparing of SNB motoneurons requires hormone action at multiple sites, including at BC/LA muscle fibers.

Regardless of how the story ends, it makes for an interesting cautionary tale. Work on the site of hormone action in the brain has largely been limited to describing where particular hormone receptors are expressed. The SNB system suggests that we may be in for some surprises when hypotheses about sites of hormone action in the brain are put to the test.

The Ejaculation Center – *Sexually dimorphic spinal center with projections to the brain*

The culmination, one might even say the 'purpose,' of all male sexual behaviors is ejaculation. There are two phases to ejaculation: emission, which involves the secretion and accumulation of seminal fluids in the urethra and is under the control of the autonomic nervous system, and expulsion, in which smooth muscles of the urethra and the striated SNB target muscles participate in the forceful ejection of the urethral contents. Because ejaculation can occur in spinalectomized animals, or in humans with spinal cord injuries, it is clear that spinal circuits alone can control the behavior. Until quite recently, however, it was not known how ejaculation is triggered, or how coordination between the emission and expulsion phases is achieved. The discovery of an 'ejaculation generator' or 'ejaculation center' in the upper lumbar spinal cord of rats addresses some of these issues (Truitt and Coolen, 2002).

Previous work had identified a sexually dimorphic population of neurons dorsolateral to the central canal in spinal lumbar segments 3 and 4 that express galanin (GAL) and cholecystokinin (CCK) (Ju et al., 1987; Newton, 1992a,b; Phan and Newton, 1999). These GAL/CCK cells send ascending projections to the thalamus, as well as descending projections to the SNB region and to autonomic centers involved in ejaculation. They express the immediate early gene, *fos*, after ejaculation, but not after other components of male sexual behavior or vaginocervical stimulation in females (Truitt et al., 2003). Most tellingly, ejaculation is completely eliminated following the specific deletion of the GAL/CCK cells using a targeted neurotoxin, while other aspects of male sexual behavior are unaffected (Truitt and Coolen, 2002). This latter observation establishes the GAL/CCK cells in L3/L4 as an ejaculation center.

Recently, Sakamoto and colleagues (2008) identified a group of neurons in the region of the ejaculation center that express gastrin releasing peptide (GRP). GRP appears to be a crucial molecule controlling male sexual reflexes, as administration of a GRP receptor agonist partially restores penile reflexes in castrated male rats, and GRP-treated castrates actually exhibit more spontaneous erections than do intact controls (Sakamoto et al., 2008). Conversely, an antagonist to the GRP receptor results in a dose-dependent decrease in spontaneous ejaculations and penile reflexes in gonadally intact males (Sakamoto et al., 2008).

Males have many more GAL/CCK and GRP-expressing cells than do females within the ejaculation center and cell number in both cases is hyperfeminine in Tfm males lacking functional androgen receptors (Newton and Phan, 2006; Sakamoto et al., 2008). Treating females with testosterone in adulthood does not fully masculinize GRP immunoreactivity (Sakamoto et al., 2008), suggesting that there are organizational effects of androgens on GRP cell number or on the ability to respond to androgens with increased GRP expression in adulthood.

Thus, testicular androgens may act during early development to influence development of the external genitalia, the muscles associated with the genitalia (e.g., BC, LA, and ischiocavernosus), motoneurons innervating the penile muscles (e.g., SNB and DLN), and cells in higher spinal levels (e.g., the ejaculation center) that send projections to sites such as the SNB, autonomic centers involved in male sexual function, and the brain. It is not likely to end there. Although less well studied, the pre- and post-ganglionic autonomic neurons themselves may be sexually differentiated, and sensory input to the circuit may differ in males and females, as described in the next section.

Primary Sensory and Autonomic Neurons – *Apples and oranges*

Because males and females have different pelvic viscera (e.g., fallopian tubes and uterus in females versus seminal vesicles, vas deferens, and epididymis in males), there must almost by necessity be sex differences in the sensory and autonomic innervation of these structures.

Parasympathetic ganglia, in particular, are often found in close apposition to their target organs, so that organizational effects of steroids on the development of the internal reproductive organs would presumably result in vastly different innervation patterns. There has been little direct comparison of end organ innervation in males and females, perhaps because of this "apples and oranges" problem. Primary sensory neurons, with their cell bodies in dorsal root ganglia (DRG) that run along the vertebral column, and preganglionic sympathetic neurons, which reside in the spinal cord itself, can more readily be compared between the sexes. Although only a smattering of reports have done so, the results suggest that sex differences do exist, they can be of large magnitude, and in at least some cases are subject to organizational effects of testosterone.

For example, the hypogastric nerve provides sympathetic innervation to the bladder and penis and is involved in erection, ejaculation, and penile detumescence (deGroat and Booth, 1980). Using retrograde labeling, Nadelhaft and McKenna (1987) demonstrated that male rats have at least three times as many sympathetic preganglionic neurons projecting via the hypogastric nerve as do females, and a similar sex difference is found in guinea pigs (McLachlan, 1985). The mechanism(s) determining these sex differences is not known.

The DRGs providing sensory innervation to SNB target muscles and to the perineal skin are found in the 6^{th} lumbar and 1^{st} sacral spinal segments of rats; they are sexually dimorphic, with more primary sensory neurons in these ganglia in adult males than in females (McKenna and Nadelhaft, 1986). This sex difference is dependent on organizational effects of testosterone. Specifically, DRG cell number is similar in males and females on embryonic day 18; a sex difference emerges by postnatal day 10 and correlates with a greater number of dying (pyknotic) cells in the DRGs of females during this interval (Mills and Sengelaub, 1993). Treatment of females with testosterone propionate during perinatal life eliminates the sex difference in pyknotic cells and in DRG neuron number (Mills and Sengelaub, 1993). Thus, the SNB motoneurons, their target muscles, and primary sensory neurons projecting to the level of the SNB all follow a very similar, androgen-dependent developmental trajectory.

The Cremaster Nucleus – *It's not just androgens*

The cremaster neuromuscular system is still shrouded in some mystery but promises to stretch the definition of the organizational hypothesis. The cremaster muscle envelopes the testes and contractions of the muscle lift the scrotum to control testis temperature in mammals. The source of cremaster muscle fibers remains controversial, but the muscle forms postnatally as the testes descend from their embryonic position in the abdomen to reside in the scrotum (Hrabovszky et al., 2002). Motoneurons innervating the cremaster are found in the ventral horn of the first and second segments of the lumbar spinal cord (L1/L2), and male rats have three times as many cremaster nucleus (CN) motoneurons as do females (Newton, 1990). Castration of males on the day of birth prevents cremaster muscle formation and significantly reduces CN motoneuron number (Newton and Hamill, 1989).

So far, this sounds a lot like development of the SNB and DLN motor nuclei. There are several important differences, however. First, testosterone treatments during perinatal life do not masculinize the cremaster muscle or motoneurons of female rats (B. Newton, personal communication). In addition, the number of CN motoneurons is not female-like in Tfm rats (Barthold et al., 1994), and prenatal treatment with the anti-androgen, flutamide, causes only a slight reduction in CN motoneuron number in male rats or pigs (Barthold et al., 1996). Finally, and most tellingly, unilateral castration prevents formation of the ipsilateral cremaster and causes an ipsilateral reduction in CN motoneuron number; these effects are not reversed by replacing androgens at the time of surgery (Newton and Hamill, 1989). This last observation suggests that the presence of a mechanical signal from the testes, or possibly a paracrine factor

that is present at high enough concentrations only locally, is required for formation of the cremaster muscle and masculinization of CN cell number.

There is another aspect of this story that is interesting when considering the organizational hypothesis. The testes descend in two phases: first, from the abdomen to the inguinal canal, and second, from the inguinal canal into the scrotal sac. While androgens are important during the second phase, the first phase of testicular descent is crucially controlled by insulin-like factor 3 (INSL3; reviewed in Foresta et al., 2008). INSL3 is produced by the Leydig cells of the testes, and mice with a deletion of the genes that code for INSL3 or its receptor exhibit 100% cryporchidism (Nef and Parada, 1999; Overbeek et al., 2001). It is not known how, or whether, INSL3 affects cremaster motoneurons.

Thus, signals from the testes act early in development to control testicular descent and differentiation of the spinal CN. However, in a broadening of our usual conception of the organizational hypothesis, the testes may masculinize in this case via not only the endocrine secretion of androgens, but also via mechanical signals, paracrine actions of hormones, and the secretion of a protein factor.

Pain perception and the SNB revisited: *The organization of early experience?*

The perception of a painful stimulus and the response to it involves sensory receptors, primary sensory neurons, spinal neurons, and the brain. Sex differences in pain perception have been reported in a large number of studies, in both experimental animals and the clinic. In humans, pain thresholds and pain tolerance tend to be lower in women across a variety of sensory modalities, and activational effects of steroids, especially of estrogens, on pain perception are well established (reviewed in Craft, 2007). A handful of animal studies demonstrate that there are also likely to be organizational effects of gonadal steroids on pain (Sternberg et al., 1995; Cicero et al., 2002; Borzan and Fuchs, 2006). For example, male rats display a greater reduction in pain sensitivity to a given dose of morphine than do females; castration of males and treatment of females with testosterone on the day of birth reverses this sex difference (Cicero et al., 2002).

The mechanisms underlying organizational effects of testosterone on pain sensitivity are not known. One study points to sex differences in the spinal cord at an early age: in a slice preparation of postnatal rat lumbar spinal cord, lamina X neurons exhibit a greater electrophysiological response to exogenously applied galanin in males than in females (Phelan and Newton, 2000). As galanin has an antinociceptive role in the spinal cord, this might be one substrate for sex differences in nociception, and the fact that this sex difference is seen prepubertally suggests organizational effects.

A recent study suggests another way to think about organizational effects of gonadal steroids on adult responses to pain (or, for that matter, to other sorts of stimuli). La Prairie and Murphy (2007) measured adult pain responses of rats that were subjected to a mild peripheral injury at different postnatal ages. Females were found to be more vulnerable to the long-term consequences of neonatal injury, but not if the initial injury was delayed to postnatal day 14 (LaPrairie and Murphy, 2007). The authors suggest that higher levels of testosterone, and hence of estradiol, in newborn males may act as a neuroprotectant against neonatal injury. If so, then in this case testicular hormones act by gating the response to a painful experience during neonatal life. By virtue of differences in circulating hormone levels soon after birth, otherwise identical painful events might cause different responses in males and females. Similarly, developmental stressors have different effects on later behavior in males and females (reviewed in Weinstock, 2007); in the case of pain, however, organizational effects may well be present at the level of the peripheral nervous system or spinal cord.

Recent studies of sex differences in pain also offer a challenge to the organizational hypothesis, by identifying direct effects of sex chromosome complement on nociception. Gioisa et al., (2008a; 2008b) compared mice in which sex chromosomal complement was varied independent of gonadal type. In a hotplate tail withdrawal test, XX mice showed faster baseline latencies to withdraw the tail than did XY mice, regardless of gonadal type. This effect of sex chromosomes was seen in newborns as well as in adults that had been gonadectomized for several weeks prior to testing (Gioisa et al., 2008a; 2008b). As is the case for the other sex differences in pain, mentioned above, it is not known whether the effect of sex chromosomes on nociception is at the level of the peripheral nervous system, spinal cord, brain, or some combination of sites.

The SNB neuromuscular system also offers an example of how androgens may organize the nervous system by changing early experience. Rat dams treat their male and female offspring differently, such that they spend more time licking the anogenital region of male pups than of females (Moore and Morelli, 1979). This difference in maternal behavior is influenced by the hormonal condition of the pups, because if female pups are treated with testosterone, estradiol, or dihydrotestosterone on the day of birth, they subsequently elicit more anogenital licking from the mother (Moore, 1982). Several studies have examined the consequences of altering the amount of anogenital stimulation a pup receives during the first weeks of life. When maternal licking is experimentally reduced by making dams anosmic, male offspring exhibit decrements in sexual behavior, reduced numbers of SNB motoneurons, and a reduction in SNB dendritic trees in adulthood (Moore et al., 1992; Lenz and Sengelaub, 2006). Similarly, if pups are reared in isolation and provided varying levels of anogenital stimulation with a paintbrush, a reduction in SNB dendritic trees is seen in those males receiving a low level of stimulation (Lenz et al., 2008). Taken together, androgens may cause organizational effects on neuroanatomy by altering the tactile stimulation elicited from the mother.

Summary

Most of the emphasis in the field of sexual differentiation has been on organizational effects of gonadal steroids on the brain and behavior. By examining sex differences in final common final pathways, several shifts in thinking emerge: 1) sometimes organizational effects of hormones are on peripheral tissues (e.g., perineal muscles or pelvic viscera) that then affect the development of neural structures; 2) the brains of males and females receive differing input from their differentiated bodies and spinal cords (projections from the ejaculation center to the thalamus are just one example), so even in the absence of sex differences in the brain, one would predict sex differences in brain input; 3) the testes may be the source of differentiating signals other than androgens (e.g., INSL3); and 4) gonadal steroids may “organize” the nervous system by altering experience (e.g., by changing the maternal care received or the response to neonatal injury).

The paper by Phoenix et al. (1959) was a landmark because it posited that there must be changes in the nervous system when hormones permanently alter behavior. The authors also explicitly drew a parallel between the effects of testicular secretions on developing neural structures with effects on reproductive organs. They noted that, “suppression of the capacity for displaying lordosis was achieved with a quantity of androgen less than that required for masculinization of the external genitalia.” This was important at the time, because it demonstrated that masculinization of behavior was separable from masculinization of the genitalia. It also demonstrates that the authors gave serious consideration to the interpretation that hormones might masculinize the nervous system by acting at the periphery.

It is not known to what degree sex differences in the brain are due to sex differences in the periphery or spinal cord. Motoneuron number in the SNB can be masculinized when the spinal

cord is separated from the brain (Fishman and Breedlove, 1985), but to my knowledge, no studies have taken the reverse approach and examined brain sexual differentiation in spinalectomized animals. We generally think of end organs, such as the external genitals and reproductive organs, as passive recipients of instructions from the nervous system. Perhaps we should ask once again how much of brain differentiation depends on the periphery. This is a perspective that W.C. Young's group, including Phoenix et al. (1959), would have been familiar with (see Wilson et al., 1940; 1941).

Acknowledgments

Supported by NIH grants R01MH068482, K02MH072825, and NSF grants IOB-0344312 and IOS-0642050. I am grateful to Lique Coolen, Anne Murphy, and Bruce Newton for helpful discussions; any errors, however, are entirely my own.

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