REGULATION OF MOTONEURONAL CALCITONIN GENE - RELATED PEPTIDE BY TESTOSTERONE IN THE RAT SPINAL NUCLEUS OF THE BULBOCAVERNOSUS: AN IN VIVO IMMUNOCYTOCHEMICAL STUDY

by

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ABSTRACT

The course of mammalian sexual differentiation is largely under the control of the principal male gonadal steroid, the androgen testosterone. Despite the importance of androgens in the mediation of sexual differentiation, the molecular basis of androgenic activity in the central nervous system remains poorly characterized.

Calcitonin gene-related peptide (CGRP) has previously been reported to be regulated by testosterone in the Spinal Nucleus of the Bulbocavernosus (SNB). The locus of androgenic regulation of CGRP expression within SNB motoneurons remains unclear, as the SNB is a component of a highly androgen sensitive neuromuscular circuit, with multiple possible targets for androgenic action. The present research utilizes a mosaic paradigm to test the hypothesis that the intracellular androgen receptor of SNB motoneurons regulates CGRP expression directly in this cell population.

Females heterozygous for the testicular feminization (tfm) androgen receptor mutation were androgenized perinatally to rescue the SNB and were implanted at sixty days with silastic capsules containing testosterone or nothing. Previous work has established that this method yields females with
approximately half of the population of SNB motoneurons expressing functional androgen receptors. The remaining SNB motoneurons express non functional androgen receptors. This cellular mosaic of androgen receptors within the SNB allows for cellular analysis of androgen receptor activity in this neuronal population.

Mosaic females were sacrificed six to eight weeks following implantation and spinal sections containing SNB motoneurons were immunolabeled simultaneously for CGRP and androgen receptor. Testosterone was found to decrease the proportion of CGRP immunoreactive SNB motoneurons only in cells immunolabeled for functional androgen receptor.

This finding clearly demonstrates the necessity of functional androgen receptor in SNB motoneurons for androgenic regulation of CGRP. The absence of an effect for testosterone on cells not immunoreactive for androgen receptor challenges models of androgenic regulation of CGRP within the SNB based on retrograde influence of the SNB target muscles. The current report constitutes, to the best of my knowledge, the first unambiguous in vivo demonstration of the necessity of a steroid receptor for steroid induced alterations in gene expression in a defined cell population.

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Mammalian Sexual Differentiation

Mammalian sexual differentiation is largely a function of the hormonal milieu of the organism through development and in adulthood (MacLusky & Naftolin 1981). While genetic differences exist between the sexes (Maxson, 1997), their role in sexual differentiation remains unclear. The earliest event in mammalian sexual differentiation is genetic and to date represents the only clearly demonstrated direct genetic contribution to sexual differentiation (Arnold, 1996). An embryonic genetic signal, the sry gene product (sometimes referred to as the testis determining factor), initiates the formation of the male gonads, the testis (Lovell-Badge & Hacker, 1995).

In the absence of the sry gene product the genital primordium, the genital ridge, forms female gonads, the ovaries (Lovell-Badge & Hacker, 1995). Sry constitutes the minimum genetic signal for the formation of secretory testis and the development of a male phenotype (Koopman et al. 1991). In utero, the testis are secretory with little gametogenesis, while the ovaries are essentially non-secretory and highly gametogenic (Johnson & Everitt, 1995). While the female fetus is thought not to synthesize significant levels of endocrine hormones, it does experience significant levels of maternal (Weisz & Ward, 1980) and fraternal androgens (Meisel & Ward 1981). As a result, female fetuses do experience a variable degree of masculinization in utero (Gladue and Clemens, 1982).
The endocrine action of the testis during perinatal critical periods initiates epigenetic deviation from the female phenotype, which occurs in the absence of the testis secretory products: the androgen testosterone and Mullerian-inhibiting hormone (Lovell-Badge & Hacker, 1995). The developmental actions of testosterone represent a class of hormone effects referred to as “organizational” (Phoenix et al. 1959). During adulthood, gonadal steroids have been demonstrated to influence reproductive physiology and behaviour, although limited by the organism's developmental history of hormone exposure; this class of hormone actions is termed "activational" (Phoenix et al. 1959).

While the rigid dichotomization of steroid action into organizational and activational effects has been challenged (Arnold and Breedlove 1985), it remains a useful terminology. Despite the significant role androgens play in the organization and activation of sexual behaviour, the molecular mechanisms by which androgens exert their influence on the nervous system remain unclear.

Sex Steroid Biosynthesis

Sex steroids fall into four major classes, the androgens, the estrogens, the progestagens and the corticosteroids (Johnson & Everitt, 1995). The gonads and adrenals are differentially steroidogenic for these four classes. Steroidogenesis is

The gonads and the adrenals constitute the major steroidogenic organs (Johnson & Everitt 1995). Although both the male and female gonads synthesize progestagens, estrogens and androgens, they are differentially secretory for them. The androgen testosterone (T4) is the major endocrine factor secreted by the testis, while the estrogen 17β estradiol (E2) and the progestagen progesterone (P4) are the major secretory product of the ovaries. The adrenals primarily synthesize glucocorticoids, mineralocorticoids and the androgens androstenedione (A4) and dehydroepiandrosterone (DHEA).

Steroidogenesis in the gonads is regulated by anterior pituitary tropic hormones, the gonadotropins luteinizing hormone and follicle stimulating hormone. Anterior pituitary tropic hormone secretion is in turn regulated by hypothalamic releasing hormones, which are secreted into the pituitary portal blood by hypothalamic neurons traversing the neurohyphysis (Johnson & Everitt 1995). The production and secretion of hypothalamic releasing hormones is typically subject to negative feedback control by the endproducts, such as T4 or E2. The cellular mechanisms underlying negative feedback control of gonadotropin releasing hormone (GnRH) by the gonadal steroids is thought to be indirect, as hypothalamic GnRH containing neurons are thought not to contain steroid receptors (Sagrillo et al. 1996).
Steroid Hormone Distribution

Steroids are secreted into circulation and diffuse freely across cell membranes from the blood, but the rate of delivery of plasma steroids to target tissues is regulated by steroid binding proteins present in plasma, such as albumin, orosomucoid, corticosteroid-binding globulin and sex hormone- binding globulin (Hammond 1997). Sex hormone-binding globulin (SHBG) binds androgens with high affinity and SHBG bound androgen constitutes the vast majority of circulating androgen in male rats (Hammond 1997).

Androgens

The testicular androgen testosterone is the major endocrine factor mediating male sexual differentiation. Testicular function in ontogeny has been well studied in the rat (Weisz & Ward 1980). A spike in plasma testosterone is observed in male rats on prenatal day 18 and on postnatal day 1 (Weisz and Ward, 1980), corresponding to the perinatal critical periods for the masculinization of reproductive functions in castrated males or females by the administration of exogenous testosterone (Goy, 1970). Plasma T4 remains low until the onset of puberty at which point levels rise to and plateau at adult levels (Johnson & Everitt, 1995). Testosterone secretion by the testis is pulsatile and tonic but shows both circadian and ultradian rhythmicity (Desjardins et al. 1981).
Testosterone is synthesized in the Leydig cells of the testis as a consequence of LH receptor stimulation, which is thought to result in increased transport of cholesterol across the mitochondrial membrane via the steroid acute regulatory protein (Stocco 1997) and complexes with a testicular SHBG in Sertoli cells subsequent to secretion into the plasma (Hammond 1997). Circulating testosterone interacts with target tissues by activating androgen receptors directly or by being converted into active androgenic or estrogenic metabolites which then interact with their respective receptors (Martini et al. 1993).

The ovaries also synthesize and secrete the androgens testosterone, dihydrottestosterone (DHT), 3α-androstenediol (3α-diol) and 3β-androstenediol (3β-diol) in small amounts and variably in the estrous cycle and through development, which have marked antagonistic properties on estrus duration and receptivity in female rats (Erskine 1983). Androgen receptor function participates significantly in both sexual behaviour and reproductive capacity in female rodents (Gladue and Clemens, 1982, Lyon & Glenister 1980).

Although the gonads and the adrenal glands are the major source of steroid hormones, the central nervous system (CNS) has some steroidogenic capability. Many brain sites express 5-α reductase which forms androgenic metabolites of testosterone (Martini et al. 1993) and/or cytochrome P450 aromatase, which forms estrogenic metabolites of testosterone (Lephart 1996).
Early reports indicated, counterintuitively, that many of testosterone's masculinizing actions can be achieved with exogenous estrogen (Levine and Mullins Jr. 1964, Sodersten 1973, Emery & Sachs 1975), although the necessity of estrogen receptors for some testosterone activated masculine sexual behaviour is questionable due to the defeminized and partially masculinized sexual behaviour of alpha estrogen receptor null male mice (Ogawa et al. 1997, Rissman et al. 1996), as well as the possibility that exogenous estrogen acts indirectly, for example by altering adrenal action (Gorzalka et al. 1975).

Testosterone's estrogenic metabolite E2 has both genomic and non-genomic actions in the brain (McEwen, 1991, Wehling, 1997). Genomically, E2 acts through the intracellular estrogen receptor, which is found extensively in brain areas containing both aromatase activity and androgen receptors (Simerly et al 1990). Cytochrome P450 aromatase has been demonstrated to be under direct androgenic regulation in all areas examined having estrogen synthesizing capability with the exception of the amygdala (Abdelgadir et al. 1994).

In the CNS, testosterone's androgenic metabolites include DHT, testosterone's 5-α reduced metabolite, as well as A4, which is also synthesized in the adrenal glands. DHT activates the androgen receptor and is not readily aromatized to an estrogen and as such is useful in separating testosterone's androgenic and
estrogenic actions. DHT may also be metabolized to a number of androgens, such as 3α-diol and 3β-diol (Martini et al. 1993).

DHT is approximately 10 times more potent an activator of the androgen receptor (see below) than is T as measured by the mammary mouse tumor virus chloramphenicol acetyl transferase (MMTV-CAT) reporter gene in vitro (Deslypere et al. 1992). Although the MMTV-CAT gene is lacking an androgen response element, it has a potent glucocorticoid response element with which the androgen receptor is thought to interact (Tan et al. 1992). DHT has been widely implicated in the virilization of the male genitalia (Johnson & Everitt 1995). The role of DHT in the CNS remains unclear, although DHT and 3α-androstenediol are more potent inhibitors of luteinizing hormone secretion in rats than is testosterone (Martini et al. 1993).

**The Androgen Receptor**

While steroid hormones are endocrine factors and thus have a systemic distribution, they have discrete action on specific cell populations or “target tissues”. Sensitivity to steroids is conferred to target tissues by the presence of appropriate receptors in their composite cells (Beato, 1989). Steroids may also act as autocrine and paracrine factors, as some target tissues possess steroid metabolizing molecules. For example, T4 may be metabolized to E2 (Lephart 1996) as well as to other androgens (Martini et al. 1993). Steroid hormones may
effect neural tissue both genomically, through hormone receptors or non-
genomically, by interacting with membrane bound receptors as well as with

The androgen receptor belongs to the steroid hormone receptor superfamily,
which also includes such intracellular receptors as the estrogen and progesterone
receptors, as well as the thyroid hormone and retinoid receptors. Steroid hormone
receptors typically have three functional domains: a carboxy (COOH) terminus
ligand binding domain, a DNA binding domain and an amino (NH3) terminus factor
interacting domain (Evans 1988).

Intracellular steroid receptors may exist in unbound form within the cytosol or
within the cell nucleus of target cells. Unbound nuclear steroid receptors may
exist freely within the nucleus or bind DNA without altering gene transcription
(Truss and Beato, 1993). Upon binding ligand steroid, cytosolic receptors are
believed to dissociate from inhibitory complexes, translocate to the nucleus, bind
DNA and alter gene transcription (Truss and Beato 1993). The androgen receptor
is thought to be cytoplasmic when unbound and to translocate to the nucleus upon
binding ligand, based on cell fractioning studies and studies with specific agonists
and antagonists of the androgen receptor (Kemppainen et al. 1992).

Activated steroid receptors interact with specific regions of DNA, termed "hormone
response elements" (HRE's), and interact with the transcription initiation
complexes (TIC's), enhancing or repressing gene expression. The cascades of gene expression resulting from steroid hormone receptor activation may have phenotypic consequences of extremely long duration (McEwen, 1991). The specificity of HREs is not exact, and several receptor types may interact with the same HRE's. Further, the HRE's and the TIC's which are active vary according to cell type. The effects of activation of different steroid hormone receptors may therefore be comparable within certain cell types and the effects of activation of the same steroid hormone receptor may differ between cell types (Beato and Sanchez-Pacheco, 1996).

Although some steroid receptors may act as monomers (Glass, 1994), the androgen receptor binds HREs exclusively as a homodimer, requiring two ligand activated androgen receptor molecules to cooperatively induce DNA interaction (Wong et al. 1994). Unlike other steroid receptors, which seem to have high affinity for a unique HRE, the androgen receptor binds both glucocorticoid receptor response elements and progesterone receptor response elements in vitro (Kemppainen et al. 1992). PREs and GREs are commonly found in genes known to be under androgenic control (Tan et al. 1992).

Regulation of the androgen receptor in neurons.

In hamster motoneurons, and rat brain homogenates, androgen receptor mRNA levels have been reported to be regulated through the androgen receptor although
an endogenous source of estrogen appears necessary for this effect (Quarmby et al.
1990, Drengler et al. 1996b). In motoneurons of the spinal nucleus of the
bulbocavernosus (see below), an extremely androgen sensitive motoneuron pool,
androgen receptor immunoreactivity has been reported to be upregulated by
testosterone (Matsumoto et al. 1996). Target innervation as measured by
axotomy or grafting, has also been implicated in regulation of motoneuronal
androgen receptor levels, with intact connections to target muscles serving to
maintain androgen receptor levels (Yu & McGinnis 1986, Al-Shamma & Arnold

Anatomy of the androgen receptor in the CNS.

The anatomical distribution of androgen receptor has been comprehensively
described in the rat brain using androgen autoradiography (Stumpf & Sar 1976)
and in situ hybridization (Simerly et al. 1990). The highest levels of androgen
receptor mRNA are reported in the hypothalamus, notably in the preoptic area and
ventromedial nucleus, as well as in interconnected structures, such as the septum,
the hippocampus and the amygdala (Simerly et al. 1990). Moderate levels of
androgen receptor mRNA are found throughout the nervous system, particularly in
the brainstem. The distribution of androgen receptors have also been described
in the spinal cord, notably in motoneurons, which are variably sensitive to
androgens (Lumbroso et al. 1996).
Typically, females exhibit a similar distribution but a less intense labelling of androgen receptor, which has been demonstrated to be due to low circulating androgens (Drengler et al. 1996a, Matsumoto et al 1996). However, sex differences in cytosolic androgen binding in brain homogenates have been reported in long term gonadectomized rats (McGinnis & Katz 1996).

Mediation of androgenic activity by the androgen receptor.

Androgenic actions reported in the mammalian nervous system to date have been strictly genomic in nature. Some evidence has been presented (Frye et al. 1996) that the androgen 3-α androstenediol (3α diol), which binds weakly to the androgen receptor and has behavioural effects not blocked by flutamide administration, may interact with the GABA_A receptor. It seems likely that such mechanisms exist, although demonstrating such effects for testosterone, which binds strongly to the androgen receptor, have been elusive in neural populations. Androgen receptor mediated alterations in gene expression are thought to be cell type specific, which is to say that it is expected that different types of androgen sensitive neurons will express different genes in response to androgen receptor activation.

In vivo approaches to the study of androgen receptor function

The systemic nature of steroid hormone action has made demonstrations of receptor function difficult. Studies of androgenic activity in vivo have been largely
activational and systemic in nature, observing the alterations in gene expression associated with androgen removal through castration or administration of specific antagonists and the subsequent replacement of androgen through administration of exogenous T4 or DHT. An inherent difficulty with these approaches is identifying the site of steroid action. The CNS is vastly interconnected with a great number of target cell populations. Several demonstrations of indirect trophic actions of steroid hormones have been made, for example in the rat spinal nucleus of the bulbocavernosus (Freeman et al. 1996). Consequently, interpretations of the locus of action of a hormone cannot be made simply by correlating the hormonal state of the organism and an alteration in a cell population.

Androgen receptor agonists and antagonists.

Both flutamide and hydroxy-flutamide (OH-F) have been extensively used as specific antagonists of the androgen receptor. Hydroxy-flutamide has been reported to bind the ligand binding domain and induce translocation of the androgen receptor into the cell nucleus without altering gene transcription (Kemppainen et al. 1992). Flutamide has no specific antagonist activity in vitro, but is thought to be activated in vivo by metabolism to OH-F (Kemppainen et al. 1992). Cyproterone acetate has also been used as an anti androgen in developmental studies (e.g. Neumann 1966) although it has agonistic properties at the androgen receptor (Kemppainen et al. 1992).
The principle androgenic metabolite of T4, DHT, has been widely used as a specific agonist of the androgen receptor, which it activates strongly in vitro (Deslypere, 1992) and which has powerful virilizing effects in vivo (Martini et al. 1993). Further, DHT is not aromatized into an estrogenic compound, facilitating interpretation of its effects (Martini et al. 1993).

_Tfm_ mutation in rats.

The sequelae of androgen receptor failure in target tissues have been studied in animals expressing an androgen receptor mutation, the testicular feminization mutation (tfm). Early genetic studies established the tfm mutation to be X-linked (Lyon 1970). Homozygotes for the tfm mutation occur only in the male offspring of carriers of the tfm mutation as these males are rendered infertile due to androgen insensitivity. Carriers for the tfm mutation have partial androgen sensitivity, with individual cells of target tissues being either completely androgen sensitive or tfm affected (see below).

Karyotypic males expressing the tfm mutation (having the X^{tfm}Y chromosomal complement) develop secretory testis and high levels of circulating testosterone and LH (indicative of a failure of androgen receptor mediated HPG axis negative feedback control), yet express female secondary sex characteristics. The pattern of gonadotropin release (Goldman et al. 1975, Shapiro et al 1974) as well as sexual behaviour (Olsen 1979a, 1979b) of tfm affected males is, however,
defeminized and partially masculinized, an effect which is thought to be mediated by central metabolism of testosterone to estrogen, to which tfm affected males are thought to be fully sensitive (Olsen and Whalen, 1982).

The rat tfm androgen receptor results from a point mutation in the ligand binding domain (Yarborough et al. 1990) resulting in only residual androgenic activity in affected target tissues. One quantitative estimate of tfm androgen receptor activity has been made based on [3H]-T4 and [3H]-DHT binding assays of cytosolic fractions, which yielded androgenic binding 10-15% of normal (Naess et al. 1976). The extent to which this translates into functionality of tfm androgen receptors is somewhat ambiguous, although pharmacological doses of T4P or DHT (20ng/ml) induced inhibition of gonadotropin secretion in tfm affected male rats (Naess et al. 1976). The reduced functionality of the rat tfm androgen receptor is attested to by the absence of peripheral masculinization of tfm affected males.

**Systemic androgen manipulation.**

Androgenized females have often been used to study the sufficiency of androgens in various aspects of sexual differentiation (Goy, 1970), including normal patterning of male-typical mounting and intromission behaviour (Sachs et al. 1973), penile formation and ejaculation (Ward, 1969). Several proteins have been demonstrated to be regulated at the transcriptional level through action on the
androgen receptor (e.g. Berger, 1989, Argente et al. 1990, Zorrilla et al. 1990). These studies have relied on systemic delivery of DHT, leading to ambiguity concerning androgen's site of action. There have been no unambiguous in vivo demonstrations of the genomic effects of the androgen receptor within individual neurons, in the SNB or elsewhere in the central nervous system.

Microimplant approaches to localization of androgenic action.

The local administration of hormone through stereotaxically placed microimplants has been practiced for some time (Smith et al. 1977). This methodology relies on spatial restriction of hormone delivery without resolving the multiplicity of androgen sensitive cell types present, complicating interpretation. For example, one such study has demonstrated androgen receptor mediation of γ-aminobutyric acid decarboxylase 67 expression due to microimplantation of DHT in the male rat anterior preoptic area (Grattan et al. 1996). While this provides strong evidence for a local effect of androgen receptor activation in the anterior preoptic area resulting in alteration in GABA synthesis, it does not constitute a demonstration that androgen receptors within GABAergic neurons are mediating this effect, as cellular resolution of androgen receptors and GABA synthesis is not possible employing this methodology.
Androgen receptor mosaicism.

Mammalian females (XX sex chromosome complement) undergo a process of random X inactivation embryonically (Lyon, 1961). During the embryonic preimplantation blastocyst stage, the trophectoderm selectively inactivates paternal X chromosomes while the inner cell mass retains activity in both X chromosomes (Monk & Harper, 1979). The inner cell mass undergoes random X chromosome inactivation (Lyonization) with the onset of cellular differentiation. Lyonization is thought to be complete by the onset of gastrulation (Monk & Harper, 1979).

The consequence of Lyonization is a phenotypic mosaic of cells, with approximately half of all cells expressing maternal genes and approximately half of all cells expressing paternal genes residing on the X chromosome. As the androgen receptor gene is carried on the X chromosome, females heterozygous for the tfm mutation are genetically mosaic for functional androgen receptors in target tissues.

Genetically mosaic tissues are useful as discriminators of locus of androgenic influence on gene expression, a problem which is particularly evident in studies of the central nervous system, where structures are vastly interconnected and effects of systemic testosterone manipulation could be attributed to androgenic influence.
on a number of androgen sensitive structures. A mosaic cell population allows \textit{in vivo} comparisons within individual animals, between neighboring cells differing only in activity of the androgen receptor.

Androgen receptor mosaicism has been used previously as a tool for studying androgen receptor mediated events by generating $X^{tfm}X^{sxr}$ mice heterozygous for both the sex reversal mutation ($sxr$) and the $tfm$ mutation (Drews et al. 1988, Breedlove 1986). The sex reversal mutation is a translocation of the distal short arm of the Y chromosome onto an X chromosome, as a result, the genes necessary for testicular development and spermatogenesis (including $sry$) are expressed, and a masculine phenotype results (Cattanach et al. 1971). These animals have secretory testis associated with varying degrees of masculinization and mosaic androgen receptor expression. Breedlove (1986) used these mosaics to localize the trophic action of testosterone on SNB motoneurons in the course of development. Drews and others (1988) used $X^{tfm}X^{sxr}$ mice to demonstrate androgen receptor mediated differences in epididimal cell morphology as well as the presence of estrogen receptors in both wild type and $tfm$ cells of the epididimis.

One salient difficulty with the $tfm$ sex reversal mutation cross is the variability in endogenous androgens. The interindividual variation in testicular function complicates both qualitative and quantitative analysis.
The spinal nucleus of the bulbocavernosus as a model system of androgenic action

The developmental actions of testosterone, being the primary gonadal androgen, result in several central nervous system sexual dimorphisms, or morphological differences between males and females (Breedlove 1992). One such dimorphism is the spinal nucleus of the bulbocavernosus (SNB), which is present in adult males and virtually absent in adult females (Breedlove & Arnold 1980). Sexual dimorphisms homologous to the SNB are present in rodents and primates, including humans (Onuf 1899).

The Spinal Nucleus of the Bulbocavernosus (SNB) has a number of advantages as a model system for the molecular basis of androgenic action on the central nervous system: it is easily accessible, is comprised of a small number of large motoneurons and it has a relatively simple and well delineated function essential to sexual behaviour. Further, androgenic effects on the SNB are thought to be mediated directly through the androgen receptor as 80 - 90% of SNB motoneurons are androgen sensitive as estimated by autoradiography (Breedlove and Arnold, 1983b) and immunocytochemistry (Freeman et al. 1995) but none accumulate radiolabeled 17-β estradiol (Breedlove and Arnold, 1983b), or express α-estrogen receptor mRNA (Simerly et al. 1990). This pattern of steroid sensitivity (i.e. very androgen sensitive but not estrogen sensitive) facilitates
interpretation of testosterone’s mechanism of action.
Figure 1.

A perineal dissection including the bulbocavernosus muscles of an adult female rat having been exposed perinatally to testosterone.

a). penis

b). medial bulbocavernosus

c). lateral bulbocavernosus

d). colon

(levator ani muscle hidden underneath colon)
Figure 2.

Lumbar spinal cord cross section including SNB (arrows) from a female rat androgenized perinatally.

brightfield photomicrograph 20X magnification (scale bar = 500 microns)
Anatomy of the SNB.

The SNB is a pool of motoneurons spanning lumbar segments 5 and 6 as well as occasionally extending into sacral segment 1 of the rat spinal cord. The SNB innervates the perineal muscles, including the base of the penis. The perineal muscles innervated by SNB motoneurons are: the sexually dimorphic medial and lateral bulbocavernosus (BC) and levator ani (LA) muscles as well as the sexually monomorphic external anal sphincter. The BC and LA muscles are considered functionally and anatomically related and will thus be referred to as the BC/LA muscle complex (see figure 1, figure 2).

Sexual dimorphism of the SNB.

Several estimates of SNB motoneuronal number and size have been made using various methodologies and with varying results (Breedlove and Arnold 1981 Sachs et al. 1985 Sengelaub et al 1989). Generally, estimates employing stereology, an unbiased system for cell number estimation, have arrived at mean values of 160-200 motoneurons in males, 50 motoneurons in females and 30 in tfm affected males (Breedlove & Arnold 1981 Sengelaub et al. 1989a). As the BC muscle is absent in females and the presence of LA muscle in females is a matter of controversy- being virtually or entirely absent- SNB motoneurons in these groups are thought to innervate the external anal sphincter, which is present in both sexes.
Steroid sensitivity of SNB motoneurons.

Autoradiographic estimates of SNB steroid binding indicate that in the adult male rat, roughly 95% of SNB motoneurons accumulate testosterone and virtually none (5%) accumulate estrogen (Breedlove and Arnold 1980), whereas in a non-dimorphic motoneuron pool, the retrodorsolateral nucleus, fewer (80%) accumulate testosterone. Androgen receptor immunoreactivity, a method of estimation which includes unbound androgen receptors, likewise finds a greater proportion of SNB motoneurons (82%) than RDLN motoneurons (59%) to be androgen sensitive (Freeman et al. 1995).

SNB function.

The SNB has been directly implicated in the sexual behaviour of the male rat, mediating the efficacy of ejaculation (Hart 1983). The bulbocavernosus (BC) and levator ani (LA) have been implicated in both the formation and removal of the seminal plugs which male rats adhere to the cervix following ejaculation, as BC or LA lesions eliminate cups (intense erections) and significantly increase the number of intromissions necessary to remove seminal plugs and significantly decrease the strength of plug adherence (Sachs 1982, Wallace and Hart 1983). These functions are critical to effective sperm transport, intermale sperm

Hormonal regulation of the SNB developmentally.

Surprisingly, androgen does not appear to act directly on SNB motoneurons to rescue them from programmed cell death, or apoptosis, which occurs in the absence of perinatal testosterone (Nordeen et al. 1985). The inference that testosterone does not act directly on SNB motoneurons to rescue them from apoptosis is drawn from a number of lines of evidence.

SNB motoneurons do not express androgen receptors until after the critical period for this trophic action has passed (see below), whereas the BC/LA muscles are androgen sensitive perinatally, excluding a direct action of the androgen receptor within SNB motoneurons, and supporting a role for the BC/LA muscles in this process (Jordan et al. 1997, Fishman et al. 1990). Removal of supraspinal SNB afferents does not influence SNB development (Fishman & Breedlove 1985). Elimination of SNB motoneurons has no effect on BC/LA development (Fishman & Breedlove 1988) but removal of BC/LA muscles (Kurtz et al. 1992), or local delivery of flutamide to the BC/LA (Fishman & Breedlove 1992) prevents SNB survival.
Androgenic influence on the SNB resulting indirectly from androgenic activity on the BC/LA muscle is clearly demonstrated developmentally, as the SNB is spared from apoptosis by the trophic actions of testosterone on the bulbocavernosus and levator ani muscles during a critical period bounded by embryonic day 18 and postnatal day 7 in the rat (Nordeen et al. 1985, Ward et al. 1996, Forger et al. 1992, Freeman et al. 1996).

As adults, tfm affected males have little or no detectable SNB (Breedlove & Arnold 1981, Sengelaub et al. 1989a), similar to adult wild type males administered the specific androgen receptor antagonist flutamide perinatally (Breedlove and Arnold, 1983a). A direct action of the androgen receptor is necessary, therefore, for the sexual dimorphism present in the SNB. The sufficiency of androgen for the formation of the SNB dimorphism is demonstrated by administering exogenous androgen to females during this critical period. Karyotypic females (XX chromosomal complement) receiving exogenous testosterone during this period develop a perineal musculature and concomitant motoneuronal innervation resembling that of males (Sachs and Thomas 1985, Ward et al. 1996, Freeman et al. 1996).

Although perinatal administration of testosterone is sufficient for the formation of a male typical SNB, androgen receptor activation alone is insufficient for this to occur. DHT treatment alone results in an atypical pattern of organization of motoneurons innervating the BC and LA muscles, a result which may be related to
the pharmacological doses of DHT used (Breedlove 1985, Sengelaub et al.,
1989b). Similarly, perinatal administration of exogenous testosterone serves to
decrease SNB motoneuron number in intact male rats (Sachs & Thomas, 1985).

**Hormonal regulation of the SNB in adulthood.**

Systemic alterations in plasma T4 (e.g. castration and T4 replacement) result in
alterations in SNB motoneuronal size (Breedlove and Arnold, 1980). Somal size
in motoneurons is thought to be an index of protein synthesis (Sato, 1994),
leading to the inference that T4 acts to increase protein synthesis in SNB
motoneurons.

Several alterations in mRNA levels resulting from castration and reversed by
testosterone replacement have been documented in the SNB. Specifically,
mRNA levels of calcitonin gene-related peptide (Popper & Micevych 1990)
cholecystokinin (Popper, Abelson, Micevych, 1992), B-tubulin (Matsumoto et al.
1993) and connexin 32 (Matsumoto et al. 1991) have been identified as being
regulated by systemic testosterone manipulations. Further, calcitonin gene-
related peptide synthesis has also been reported to be regulated by systemic
testosterone manipulations (Popper & Micevych 1989). Localizing these effects
of androgen to the androgen receptor within SNB motoneurons has remained
problematic, however (Popper, Abelson, Micevych 1992, Popper, Abelson,
Localizing androgenic effects to SNB motoneurons.

The primary site of action of androgen’s effects on SNB motoneurons cannot be inferred with systemic manipulations of androgen activity. The SNB is but one target for androgenic action. Both efferent (Jordan et al, 1997, Fishman et al. 1990, Dube et al. 1975 Popper, Ulibarri and Micevych 1992) and afferent (Monahan & Breedlove 1991) structures are androgen sensitive and retrograde androgenic influences from peripheral structures on SNB motoneurons have been well documented (Freeman et al 1996).

Androgen receptor mosaicism in the SNB.

Androgenized female rats heterozygous for the tfm mutation (X\text{tfm}X\text{wt}) have been generated in order to study androgen receptor mediated events in the SNB (Freeman et al. 1996, Watson et al. 1996). Females were androgenized with exogenous T4 during the perinatal critical periods. The substitution of exogenous T4 for the sxr mutation employed by Breedlove (1986) has made the mosaic less subject to interindividual variability in circulating androgen, facilitating quantitative analysis of the SNB androgen receptor mosaic. Androgenized carriers of the tfm mutation were found to have significantly fewer SNB motoneurons than androgenized wild type female littermates. Further, mosaic animals had
approximately half as many androgen sensitive SNB motoneurons as androgenized wild type female littermates (Freeman et al. 1996).

The fact that tfm motoneurons persist in the SNB in adulthood provides a direct demonstration that androgenic regulation of SNB motoneuron apoptosis occurs outside of the SNB. When taken in the context of evidence implicating the muscle but not descending afferents to the SNB in the regulation of SNB motoneuron apoptosis, this observation provides compelling evidence limiting apoptotic regulation of the SNB to the BC/LA muscles.

In another study of mosaicism of SNB motoneurons, Watson and others (1996) demonstrated that androgenic regulation of SNB motoneuronal somal size is regulated directly within the SNB motoneurons. As somal size is thought to be an index of translational activity within motoneurons (Sato 1994), this result is suggestive of a direct alteration of protein synthesis due to the androgen receptor within SNB motoneurons (Sato, 1994). It is yet undetermined which proteins are being regulated directly by testosterone in SNB motoneurons.

**Calcitonin gene-related peptide in the SNB**

Calcitonin Gene-Related Peptide is a member of the calcitonin related peptide multigene family, which includes those peptides encoded on the calcitonin genes (Zaida et al. 1990). CGRP is a 37 amino acid protein whose existence was
predicted from the cloning of the calcitonin gene, which has a sequencing suggestive of alternative splice patterns (Amara et al. 1982) and was later discovered in neural tissue (Rosenfeld, 1983). CGRP is encoded on the same gene as calcitonin and arises through alternative splicing of the calcitonin gene in neurons (Cote et al. 1990). Two distinct forms of CGRP are encoded on the two calcitonin genes, alpha CGRP and beta CGRP. The nucleotide sequencing of the two isoforms is sufficiently different to show low hybridization of mRNA probes (Popper & Micevych 1990). Antisera directed against synthetic CGRP fragments is variably reactive towards alpha and beta CGRP isoforms. CGRP 1, the antibody used in this study, shows 100% reactivity with alpha CGRP and 78% reactivity with beta CGRP (Penninsula). An unrelated peptide, amylin also shows a 46% sequence homology with CGRP isoforms, although neither mRNA probes or antibodies cross react significantly with this peptide or its mRNA transcript.

**Anatomy of CGRP in the spinal cord.**

CGRP immunoreactivity has been described in brain (Rosenfeld et al., 1983) and spinal cord (Gibson et al. 1984) for a number of species, including rat. CGRP immunoreactivity is found in sensory neurons and motoneurons, and has considerable overlap in distribution with Substance P (Gibson et al. 1984). In the lumbar spinal cord, CGRP immunoreactive fibers are observed in the dorsal horns and dorsal to the central canal, presumably being sensory afferents, and many
motoneurons are immunoreactive in the ventral horn (Gibson et al. 1984, Popper and Micevych, 1989a).

CGRP is thought to be universally but differentially expressed in motoneurons, as colchicine pretreatment or neuromuscular blockade, two techniques for enhancing visualization by substrate accumulation, result in virtually all somata or neuromuscular junctions, respectively, being immunoreactive (Csillik et al. 1993). The accumulation of CGRP at the endplate by muscular blockade may provoke increased CGRP transcription, however (Popper, Abelson & Micevych 1992). CGRP immunoreactivity has been described in SNB motoneurons as well as in neuromuscular junctions in the bulbocavernosus muscle in the male rat (Popper & Micevych 1989a).

Function of CGRP in motoneurons.

CGRP has been implicated as a neurotransmitter and myotrophic factor in motoneurons (Zaidi et al. 1990). In vitro, CGRP potentiates numerous functions in striated muscle relevant to cholinergic synaptic transmission and plasticity. For example, CGRP induces postsynaptic nicotinic receptor subunit synthesis, potentiates presynaptic quantal acetylcholine release, induces postsynaptic acetylcholinesterase activity, encourages spontaneous embryonic myotube electrical activity and prevents disuse induced sprouting in adulthood (New &

**Regulation of CGRP expression within SNB motoneurons.**

Castration of male rats results in an increase in the proportion of SNB motoneurons which are CGRP immunoreactive and testosterone administration decreases this proportion to control levels (Popper & Micevych 1989b). This androgenic regulation of CGRP expression in SNB motoneurons has been determined to be transcriptional in nature (Popper et al. 1990).

Identifying the primary location where androgen acts to regulate CGRP in SNB motoneurons has proven problematic. Injection of BC/LA muscle extracts from both castrated male rats and intact male rats into intact male rats results in an increase in CGRP immunoreactivity in SNB motoneurons. Injection of BC/LA muscle extracts from castrated male rats but not intact male rats into intact male rats results in an increase in CGRP mRNA, a result which persists after protease treatment and heat-inactivating of the extracts (Popper, Abelson & Micevych 1992). While these results are suggestive of indirect androgenic action on SNB CGRP expression through effects of the SNB target muscles, it is difficult to determine what bioactive constituents of the extract could be mediating this effect, as similar results were obtained for mRNA levels with an inactivated extract and with axotomy of the pudendal nerve. Further, the experimental design of that study
does not preclude the possibility of androgenic regulation of CGRP within SNB motoneurons.

**Ontogeny of CGRP in the SNB.**

SNB motoneurons have a developmental course distinct from other motoneuron pools within the lumbar spinal cord (see 3.3 above). CGRP immunoreactivity is also delayed and sexually dimorphic in the SNB motoneurons (Forger et al. 1993). Forger and others (1993) conclude that the ontogeny of CGRP production makes a role for CGRP in the regulation of apoptosis in the SNB unlikely. This conclusion is puzzling considering the results obtained. Concomitant with a two to threefold reduction in SNB motoneuron number during the first week postnatally (Nordeen et al. 1985), there is a 4 fold increase in the proportion of CGRP immunoreactive SNB motoneurons. The developmental pattern of CGRP immunoreactivity in SNB, therefore, corresponds to the predicted pattern if CGRP is presumed to influence apoptosis in SNB motoneurons.

**Summary**

A prominent sexual dimorphism exists in the rat SNB, being present in males and virtually absent in females. The SNB innervates the perineal BC/LA muscles which play an essential role in male copulation. Several lines of evidence suggest that this sexual dimorphism is established indirectly in the course of
development, with testosterone acting on the BC/LA muscles perinatally, which then rescue SNB motoneurons from apoptosis.

In adults exposed perinatally to testosterone (intact males or androgenized females), 80-90% of SNB motoneurons are androgen sensitive. The function of the intracellular androgen receptors found in adult SNB motoneurons is unknown. Somata of SNB motoneurons enlarge in response to adult exposure to testosterone, suggesting an increase in gene expression.

Several candidate proteins have been identified as being regulated by testosterone in SNB motoneurons at the transcriptional level. It is not known whether these proteins are being regulated by the androgen receptor within SNB motoneurons or whether the observed alterations in mRNA levels result from actions of testosterone at the BC/LA muscles or elsewhere in the nervous system.
METHODS

The specific hypothesis tested was that the androgen receptor within SNB motoneurons regulates CGRP expression. The following experiment assessed the effects of systemic testosterone on SNB motoneuronal expression of CGRP which is attributable to direct action of testosterone on these cells.

The approach taken was to compare neighboring SNB cells in tfm mosaic animals both in the presence of chronic systemic testosterone and in its absence. It was hypothesized that only SNB cells which are androgen sensitive (as determined by androgen receptor immunocytochemistry) would show a decrease in CGRP expression, (as measured by CGRP immunocytochemistry) in the presence of systemic testosterone. To test this hypothesis, the proportion of androgen sensitive SNB cells immunoreactive for CGRP was compared to the proportion of tfm SNB cells immunoreactive for CGRP in both testosterone treated and untreated mosaic animals.

Animals

All animals were obtained from our breeding colony. Sixteen mosaic animals were generated by the breeding of known carriers of the tfm mutation. Carriers were identified by the presence of internal testis in pups of previous litters.
Identified carriers were placed with a sexually vigorous male until copulatory plugs were seen and the appearance of plugs was taken as the day of conception, embryonic day 0 (Em0). Pregnant carriers received daily injections of 2mg testosterone propionate (TP) s.c. in oil from day Em16 until day Em21. The prenatal injections of TP serve to maximize SNB motoneuron survival (Ward et al. 1996) as well as distinguish between wild type females, whose nipple lines are completely masculinized by prenatal testosterone (Goldman et al. 1976) and mosaic females, who form a partial nipple line, due to the presence of androgen insensitive nipple tissue in tfm mosaic animals. As exogenous perinatal testosterone administration inhibits vaginal delivery, litters not delivered by Em22 were removed by cesarean section from treated carriers under ether anesthesia. Pups delivered by cesarean section were cross fostered to a lactating wild type female having delivered within four days prior. Carriers undergoing cesarean section were immediately euthanized via a lethal dose of sodium pentobarbital. Pups were injected with 1mg T4P s.c. dissolved in 0.1 ml corn oil on postnatal days 1 and 3 (Po1 and Po3) in order to maximally masculinize SNB motoneurons (Ward et al. 1996).

Animals were identified at day Po30 according to the following system of phenotypic markers (see Table I). All identification was verified by dissection of reproductive organs at the end of the experiment.

Androgen manipulation in adulthood
Nine mosaic animals received 2-20 mm silastic implants (1.57 mm inner diameter, 3.18 mm outer diameter) packed with crystalline testosterone (Steraloids) at 60 days of age and eleven mosaic animals received 2 - 20 mm empty silastic implants placed subcutaneously between the scapulae, under ether anesthesia. Testosterone implants of this size yield high physiological doses of plasma T4 in castrated male rats (Smith et al. 1977).

After 4-6 weeks, the implants were removed under ether anesthesia. After a further 24 -36 hours, animals received either 0.2 (n=11) or 2 (n=9) mg hydroxy flutamide (OH-F) in propylene glycol subcutaneously, which binds to the androgen receptor and induces nuclear translocation of the androgen receptor without altering gene transcription (Wong et al. 1994), facilitating immunocytochemical analysis. All animals were perfused 4-6 hours following OH-F injection.
Table 1.

**Method of identifying pup karyotype by phenotypic markers.**

<table>
<thead>
<tr>
<th>Presumed pup karyotype</th>
<th>Testis</th>
<th>Nipples</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tfm</em> affected male</td>
<td><em>X^{tfm} Y</em></td>
<td>undescended</td>
</tr>
<tr>
<td>Mosaic female</td>
<td><em>X^{tfm} X^{wt}</em></td>
<td>absent</td>
</tr>
<tr>
<td>Wild Type female</td>
<td><em>X^{wt} X^{wt}</em></td>
<td>absent</td>
</tr>
<tr>
<td>Wild Type Male</td>
<td><em>X^{wt} Y</em></td>
<td>external</td>
</tr>
</tbody>
</table>
Perfusion and tissue preparation

Animals received an overdose of sodium pentobarbital (approximately 46 mg intraperitoneally). On achievement of deep anesthesia, as measured by the disappearance of deep reflexes, animals were perfused transcardially with 200 ml ice cold phosphate buffered (pH = 7.4) physiological saline (PBS) over 20 minutes followed by 200 ml ice cold 4% paraformaldehyde - PBS (pH = 7.4) over 20 minutes. Spinal cords were removed by dissection of the spinal column rostral to the pelvis and caudal to the last 2 ribs followed by extrusion into 4% paraformaldehyde for 2 hours of post fixation at 4°C. Tissue was then transferred to 20% sucrose - PBS at 4°C until the spinal cords sank. Tissue was then sectioned coronally at 50 μm on a freezing microtome. Spinal sections corresponding to L5-6 and S1 were sequentially collected into three eppendorf tubes containing de Olmos solution (propylene glycol based cryopreservant previously demonstrated to preserve antigens over long periods of time - Watson et al. 1986) and stored at -20°C until ICC was performed. Only every third SNB sections (i.e. one eppendorf tube) collected from each animal was used for immunocytochemistry and data collection.

Immunocytochemistry

Tissue in de Olmos solution was reconstituted in tissue wells with five 10 minute washes in PBS solution containing 0.1 % gelatin and 0.3% Triton-X100 (Sigma)
and sections were sequentially double labeled for androgen receptor and CGRP as follows. Free floating sections were incubated with PBS-GT containing 10% normal goat serum (NGS) for 1 hour at 24°C to prevent non-specific secondary antibody binding. Androgen receptor immunoreactivity was then assessed using the rabbit polyclonal primary antibody PG21 directed against the 21 amino acid COOH epitope of the rat androgen receptor (gift of Gail Prins, University of Chicago). PG21 has been previously characterized in the rat SNB, and SNB AR-IR has been shown to discriminate wild type and tfm affected neurons (Freeman et al. 1995). Tissue was incubated at 4°C for 36-48 hours under constant agitation on a mixing platform in PG21 primary antiserum diluted in PBS-GT containing 1% NGS at a dilution of 1:3000. After rinsing, tissue was incubated with biotinilated goat anti-rabbit antiserum (Vector Laboratories) at 1:250 dilution in PBS-GT for one hour at 24°C. Following rinsing, tissue was incubated with avidin-biotin peroxidase complex (Vectastain elite kit, Vector Laboratories) for 1 hour at 24°C. Peroxidase (and hence androgen receptor immunoreactivity) was detected by activating the chromagen 3’ 5’ diaminobenzidine (DAB) with hydrogen peroxide in the presence of nickel chloride in Tris buffer (pH = 7.2), resulting in a blue-black label. Tissue was thoroughly rinsed of DAB solution and CGRP immunocytochemistry begun.

CGRP immunoreactivity was assessed with rabbit polyclonal antisera generated against synthetic rat CGRP (Penninsula). Sections were again incubated in 10% NGS for 1 hour at 24°C following which sections were incubated for 48 hours in
CGRP antisera at 1:16000 dilution at 4°C under constant agitation on a mixing platform. Sections were rinsed and incubated with biotinilated goat anti-rabbit secondary antisera in PBS-GT (1:250 dilution, Vector Laboratories) for 1 hour at 24°C. Following rinsing, sections were incubated in avidin-biotin complex (Vectastain elite kit, Vector Laboratories) for 1 hour at 24°C, rinsed and visualized with DAB without nickel enhancement, yielding a red-brown label. Sections were thoroughly rinsed of DAB solution, mounted on gelatin coated slides, and coverslipped with Permount (Anachemia Science) following dehydration through graded alcohols and clearing in xylene.

**Identification of CGRP-IR and AR-IR cells**

Sections were analyzed under a light microscope (Nikon Optiphot -2) at 200X magnification by an experimenter blind to experimental condition. SNB cells were rated as being lightly = 1 or darkly = 2 immunoreactive for CGRP and/or AR (see table II). Cells rated as immunoreactive for either or both proteins were drawn and labeled using a camera lucida attachment. A second experimenter analyzed tissue from six randomly selected animals as a reliability check.

Following identification of immunoreactive SNB cells, coverslips were soaked off in xylene and Neutral Red counterstain was applied in order to identify SNB cells unlabelled by either antiserum and to visualize cells labelled for AR alone. Cells
without either label were identified as *tfm* CGRP-IR negative. All cells identified by Neutral Red were drawn using a camera lucida attachment.

RESULTS

Analyses

All analyses were conducted on SPSS standard version (release 7.5.1) using the general linear model function for omnibus ANOVA and T-tests for planned comparison. Only mosaic animals having ten or more cells of each type (AR-IR positive and AR-IR negative) were included in statistical analyses. Four animals were excluded under this criterion. Further, only cells rated as darkly immunoreactive for CGRP and/or AR were classified as positive, for analysis purposes. Cells rated as lightly or not immunoreactive for CGRP and/or AR were classified as being negative, for analysis purposes.

Interrater reliability

Interrater reliability coefficients were established to be 0.98 for AR negative, CGRP immunopositive cells and 0.997 for AR positive, CGRP immunopositive cells (p<0.05).
Table 2.

Method of identification of immunolabeling of CGRP and AR and designation of cell type.

<table>
<thead>
<tr>
<th>Immunolabel</th>
<th>Cell Type</th>
<th>Microscopic appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR/CGRP-IR</td>
<td><em>wt</em></td>
<td>blue-black nucleus, red-brown soma</td>
</tr>
<tr>
<td>CGRP-IR</td>
<td><em>tfm</em></td>
<td>unlabeled nucleus, red-brown soma</td>
</tr>
<tr>
<td>AR-IR</td>
<td><em>wt</em></td>
<td>blue-black nucleus, pink soma after counterstain</td>
</tr>
<tr>
<td>no label</td>
<td><em>tfm</em></td>
<td>unlabelled nucleus, pink soma after counterstain</td>
</tr>
</tbody>
</table>
Figure 3.

A. **Cells rated as immunolabeled for CGRP and/or AR.** a.) darkly immunolabeled for androgen receptor but not CGRP (AR-IR) b.) darkly immunolabeled for CGRP but not androgen receptor (CGRP-IR) c.) darkly immunolabeled for both CGRP and androgen receptor (CGRP/AR-IR).

*brightfield photomicrograph 200X magnification (scale bar = 50 microns)*
Effects of hydroxy flutamide dose on androgen receptor identification

Animals receiving 0.2 mg (n=9) and animals receiving 2mg (n=7) did not differ in the mean number of rated androgen receptor positive SNB nuclei (independent samples T-test, df= 16, t= 2.03, p>0.05).

Degree of observed mosaicism

The mean proportion of SNB cells immunoreactive for androgen receptor was found to be 49.8%, ranging from 30% to 70%. No difference in mean proportion of SNB cells immunoreactive for androgen receptor was observed between treatment groups (independent samples T-test df = 14, t= -0.47, p >0.05).

Number of SNB cells observed

The mean number of SNB cells observed in one third of spinal segments S1, L5 and L6 was found to be 56.25 (range = 81 - 32, standard deviation = 15.46). This agrees very well with previous reports of SNB cell number in androgenized mosaic females (Freeman et al. 1996).
Effects of cell type and testosterone exposure on CGRP immunoreactivity

Animals were assigned four scores corresponding to four possible outcomes for SNB motoneurons: number of CGRP-IR cells, number of AR-IR cells, number of CGRP/AR-IR cells and number of cells unlabelled by either antisera. Analyses were carried out by cell type (as determined by androgen receptor immunoreactivity) and by experimental group to which the animal was assigned (T or B implanted). The proportion of AR-IR positive cells also immunoreactive for CGRP were compared to the proportion of AR-IR negative cells immunoreactive for CGRP within each animal.

2 by 2 ANOVA (Treatment by Cell Type) within/between subjects comparison, revealed a significant effect for cell type (df = 1,14, F=10.179, p<0.05). Planned comparisons of group means (paired samples T-test) revealed that a lesser proportion of wild type (AR-IR) cells were darkly immunoreactive for CGRP only in the testosterone implanted group (df = 5, t=4.633, p<0.05) but not the blank implanted group (df = 9, t = 1.230, p>0.05) (see figure 4.).
Figure 4.

Effects of testosterone treatment on CGRP-IR in SNB motoneurons mosaic for androgen insensitivity.
DISCUSSION

Locus of androgenic regulation of SNB CGRP

It seems clear from the present study that the androgenic regulation of CGRP expression within SNB motoneurons is mediated locally in the adult rat. Functional androgen receptors within SNB motoneurons are necessary for the reduction in SNB motoneuronal CGRP expression associated with circulating androgen. This constitutes the first in vivo demonstration of androgen receptor dependence of gene expression localized to a specific neural population. The involvement of androgen receptor within SNB motoneurons in regulation of CGRP expression runs contrary to the prevailing line of inquiry and as such is novel. Additionally, the present study provides a demonstration of the utility of the mosaic paradigm in elucidating androgen dependent intraneuronal regulation of gene expression.

As continued androgen exposure is necessary to maintain low levels of CGRP mRNA (Popper & Micevych 1990), the most parsimonious interpretation of the present result is a genomic effect of the androgen receptor in down-regulating CGRP gene expression. This hypothesis is strengthened by the finding that in the trigeminal nucleus, P4 increases CGRP expression (Moussaoul et al. 1996).
As previously noted, the progesterone receptor appears to share a HRE with the androgen receptor. Nevertheless, the possibility that CGRP is significantly downstream from the genomic effects of the androgen receptor in SNB motoneurons remains untested.

The results of this study challenge the sufficiency of androgenic action on the BC/LA in the regulation of CGRP levels in the SNB. While previous work, reporting effects for extract injection resulting in elevated CGRP mRNA levels in the SNB (Popper & Micevych 1989, Popper, Ulibarri & Micevych 1992, Popper, Abelson & Micevych 1992), suggests that the BC/LA response may be sufficient to increase SNB CGRP gene expression, it is not clear that this type of regulation is physiological. Further, the inconsistency of results employing the extract injection technique prevents simple interpretation of the reported findings. Muscle may be involved in the regulation of CGRP levels, although the present study indicates that functional androgen receptors in SNB motoneurons are a necessary requirement for this to occur.

The finding that only SNB cells containing functional androgen receptors show a reduction in CGRP expression in the presence of testosterone suggests that in the mosaic model, muscle effects are trivial. It is important to note, however, that the methodology employed in the current study does not distinguish between SNB motoneurons innervating the BC/LA and SNB motoneurons innervating the external anal sphincter. It is conceivable that regulation of SNB CGRP
expression is distinct in this muscle and that skews in connectivity could obscure an effect of the BC/LA muscles on SNB CGRP expression.

An interpretation consistent with both findings is that the androgen receptor in SNB motoneurons has a permissive role in a process of androgenic regulation of CGRP involving androgenic action on muscles. As the developmental action of androgen on the SNB is mediated through the BC/LA muscles, and paralysis of muscles leads to increased CGRP expression in SNB motoneurons (Popper & Micevych 1989), such a mechanism is plausible. As no resolution of muscular events was made in the present study, and as all animals employed in the extract injection studies (Popper & Micevych 1989, Popper, Abelson & Micevych 1992, Popper, Ulibarri & Micevych 1992) were intact males, and therefore the vast majority of SNB motoneurons had functional androgen receptors which were activated by circulating androgen, this interpretation has not been tested.

In general, the mosaic animals employed in this studied displayed a degree of SNB androgen receptor mosaicism which agreed well with previously observed results (Freeman et al. 1996). Half (mean = 49.8 %) of SNB cells were immunoreactive for androgen receptor. While slightly higher than the roughly 35% AR positive SNB neurons observed by Freeman et al. 1996, it remains significantly less than the roughly 75% AR positive SNB neurons reported in wild type littermates of mosaic animals (Freeman et al. 1996). The method of identifying androgen receptor positive cells likewise proved consistent across
dose (hydroxy flutamide doses = 0.2 mg or 2 mg). These observations support the accuracy of the method of androgen receptor identification and hence mosaic cell type classification employed in this study.

The overall proportion of SNB motoneurons rated as immunoreactive for CGRP departed significantly from the values reported elsewhere (see figure 3). Although testosterone treatment reduced the number of SNB neurons expressing CGRP by approximately one half in both Popper and Micevych's (1989) study and in the androgen sensitive population in our study, we observed fewer labeled cells overall. This difference can be attributed to a number of factors: 1) differences in the method of identifying immunolabeled neurons, 2) intrinsic differences in androgenic responses in mosaic tissues and 3) intrinsic differences between males and androgenized female models.

While no clear criteria for rating CGRP immunolabeled cells are reported by Popper and Micevych (1989), it is conceivable that differences in immunostaining methodology (for example, in the anti-CGRP antibody employed) or in the method of rating cells as immunoreactive might exist. As Popper and Micevych used Long-Evans rats as subjects whereas the animals in the present study were King-Holtzmann / Wistar crosses, a strain difference is a possibility. In a developmental study of CGRP immunoreactivity in the SNB conducted by other investigators for example, roughly 65% of male SNB motoneurons were reported
to be CGRP immunoreactive at 60 days (Jordan et al. 1993), versus the roughly 40% reported by Popper and Micevych (1989).

Physiological consequences of direct regulation of CGRP expression within SNB motoneurons

Questions pertaining to the ultimate causation of androgen receptor regulation of SNB CGRP expression are difficult to address. Many functional roles for CGRP in neuromodulation of cholinergic transmission (Liou & Fu 1995, Hodges-Savola & Fernandez 1995) and maintenance of synaptic integrity (New & Mudge, 1986, Tsujimoto & Kuno 1988, Changeux 1991, Lu et al. 1993) have been put forward, and discerning a unitary role for CGRP in the neuromuscular junction is elusive.

CGRP appears to modulate functional parameters of neuromuscular transmission. A neuromodulator such as this could be important in potentiating the restoration of functional transmission at the neuromuscular junction in infrequently used muscle groups. It appears that CGRP expression in muscles is inversely related to the frequency of use, or activational history (Blanco et al. 1997). Studies of CGRP expression in motoneurons innervating constitutently active muscles, such as the diaphragm, indicate low CGRP expression, whereas muscles frequently active (such as the soleus) are innervated by motoneurons expressing intermediate levels of CGRP. The highest levels of CGRP expression are found in the
muscles which are infrequently active, such as the BC/LA which is devoted to sexual behaviour.

Artificially inducing inactivity, by paralyzing the BC muscle increases motoneuronal CGRP mRNA synthesis in the SNB (Popper, Ulibarri & Micevych 1992). That CGRP appears to regulate nicotinic receptor expression in muscle by a signal transduction pathway distinct from that with which cholinergic transmission does so (Fontaine et al. 1987) strengthens the argument that CGRP may potentiate synaptic integrity in infrequently active muscles.

If such a role for CGRP in neuromuscular transmission proves accurate, then androgen serves, paradoxically, to decrease the synaptic integrity at neuromuscular junctions specific for reproduction. This paradox may be resolved by considering how variation in androgen levels and activity in the SNB might occur ecologically in the rat.

Male rats which are not actively breeding, such as rats undergoing seasonal attenuation of reproduction, would presumably have lower activity in the SNB-BC/LA system and also lower circulating testosterone values (Kamel et al. 1975). Male rats frequently breeding would have frequently active SNB-BC/LA systems and high testosterone levels. In this way, androgen decreasing CGRP expression in the SNB would be economical. In periods of high sexual activity, transmission at the SNB-BC/LA neuromuscular junction would be relatively frequent and the
trophic action of cholinergic transmission presumably sufficient to mediate synaptic integrity. In periods of low sexual activity, transmission at the SNB-BC/LA neuromuscular junction would be infrequent, but increased CGRP expression in SNB motoneurons due to lowered circulating androgen would potentiate both cholinergic transmission (Lu et al. 1993) and the trophic actions of cholinergic transmission (New & Mudge 1986, Tsujimoto & Kuno 1988).

Presynaptic CGRP, then, may play a role in the maintenance of neuromuscular synaptic integrity. In this case, variation in androgen levels resulting from reproductive behaviour would then enact an adaptive response in CGRP expression in SNB motoneurons. Low levels of androgen resulting from periods of relative sexual inactivity would increase CGRP expression presynaptically and thus contribute to synaptic integrity. Essentially, androgenic regulation of CGRP within SNB motoneurons would serve to keep the BC/LA system ready for use.

Androgenic regulation of CGRP in the nervous system

The finding that CGRP is under direct control of the androgen receptor in SNB motoneurons raises the possibility that androgen regulates CGRP levels in other motoneuron pools, and in other neural populations. Androgen mediated cellular events may not be as dramatic in other neural populations as the SNB is remarkable in androgen sensitivity and absence of estrogen sensitivity.
Other motoneuron pools show varying degrees of androgen sensitivity, but the proportion of androgen sensitivity in these motoneuron pools is less than that of the SNB (see above). The sexual dimorphism present in the SNB is remarkable for motoneuron pools, as such, it seems unlikely that androgen would be a major regulator of CGRP expression in motoneurons innervating muscles whose activity is not contingent on androgen.

If it is assumed that androgen regulates CGRP in the same manner in all motoneuron pools, CGRP expression does not correspond in the expected way to degree of androgen sensitivity. If the SNB is taken as the most androgen sensitive motoneuron pool, it also shows the highest levels of CGRP expression (Blanco et al. 1997), which does not agree with the hypothesized attenuation of CGRP expression in motoneurons by androgen. It could be, however, that androgenic regulation of CGRP is similar in other motoneuron pools, but that other factors regulating CGRP expression (Durham et al. 1997) would obscure androgen's effects.

Steroid regulation of CGRP has been described in the hypothalamus and thalamus of mammals. Studies in the MPOA and periventricular nucleus have indicated that, in female rats, estrogen increases CGRP expression (Yuri & Kawata 1992). Similarly, testosterone appears to increase hypothalamic CGRP expression in male mice, although it is not clear that this is not a consequence of
aromatization, nor is this effect consistent in trigeminal or cervical spinal cord areas examined (Moussaoul et al. 1996). Doubtless CGRP is regulated by steroids, but the nature of this regulation is not obviously conserved across neural populations.

**Mechanism of androgen receptor regulation of CGRP expression**

The present study indicates that the androgen receptor in SNB motoneurons is necessary for androgenic regulation of CGRP but it does not discriminate at what level this regulation is occurring within the cell. CGRP could be significantly downstream from the primary genomic effects of the androgen receptor. No definitive ARE has been identified, and possible HREs in the calcitonin gene promoter regions have not been described, making interpretation of *in vivo* assessments of CGRP regulation difficult.

**Summary**

The present results indicate that SNB CGRP is regulated directly through the androgen receptors within SNB motoneurons. Indirect methods of determining the primary site of androgenic action in the regulation of SNB CGRP have lead to misleading results, which may represent artifact of the method of testing. No support was found for the model of BC/LA muscles regulating SNB CGRP in response to systemic testosterone.
The mosaic paradigm provides a powerful tool for the analysis of steroid receptor action *in vivo*. The present study provides a demonstration of its utility in studies of gene expression. Localizing steroid action has traditionally been difficult and addressed very indirectly. The mosaic paradigm allows for a direct test of these concerns.
LIST OF ABBREVIATIONS USED

% = percent
°C = degrees centigrade
3α-diol = 3α-androstenediol
3β-diol = 3β-androstenediol
μm = microns
[3H] = tritiated
A4 = androstenedione
ANOVA = analysis of variance
AR = androgen receptor
ARE = androgen response element
B = blank silastic implant
BC = medial and lateral bulbocavernosus muscles
BC/LA = medial and lateral bulbocavernosus muscles and levator ani muscles
CGRP = calcitonin gene-related peptide
cm = centimeters
CNS = central nervous system
COOH = carboxyl
DAB = 3’ 5’ diaminobenzidine
DHEA = 5-androstene-3β-ol-17-one, dehydroepiandrosterone
DHT = dihydrotestosterone
DNA = deoxyribonucleic acid
$E_2 = 17\beta$-estradiol

$E_m = \text{embryonic day}$

$ER = \alpha$-estrogen receptor

$GABA = \gamma$-aminobutyric acid

$HRE = \text{hormone response element}$

$ICC = \text{immunocytochemistry}$

$IR = \text{immunoreactive}$

$IR^+ = \text{displaying immunoreactivity}$

$IR^- = \text{displaying no immunoreactivity}$

$L = \text{lumbar segment of spinal cord}$

$LA = \text{levator ani muscle}$

$m$-RNA = messenger ribonucleic acid

$ml = \text{milliliters}$

$mm = \text{millimeters}$

$\text{MMTV-CAT} = \text{mammmilary mouse tumor virus chloramphenicol acetyl transferase}$

$\text{MPOA} = \text{medial preoptic area of the hypothalamus}$

$\text{NGS} = \text{normal goat serum}$

$\text{NH}_3 = \text{amino}$

$\text{OH-F} = \text{hydroxy flutamide}$

$P_4 = \text{progesterone}$

$PBS = \text{phosphate buffered saline}$

$PBS-GT = 0.3 \text{ percent triton X-100, 0.1 percent gelatin phosphate buffered saline}$
Po = postnatal day
PRE = progesterone response element
RDLN = retrodorsolateral nucleus
S = sacral segment of spinal cord
SHBG = steroid hormone binding globulin
SNB = spinal nucleus of the bulbocavernosus
sxr = sex reversal mutation
T = testosterone filled silastic implant
T4 = testosterone
T4P = testosterone propionate
\( tfm \) = testicular feminization mutation
TIC = transcription initiation complex
X = X chromosome
\( X^{wt} \) = wild type X chromosome
\( X^{tm} \) = X chromosome with the \( tfm \) mutation
Y = Y chromosome
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IMAGE EVALUATION
TEST TARGET (QA-3)

1.0  1.1  1.25  1.4  1.6

1.0  1.1  1.25  1.4  1.6

150mm

6"

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