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PROJECTIONS OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS TO SEXUALLY DIMORPHIC LEVELS OF SPINAL CORD: A NEUROPHYSIN-CONTAINING, ESTROGEN-CONCENTRATING PATHWAY

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By

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ABSTRACT

PROJECTIONS OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS TO SEXUALLY DIMORPHIC LEVELS OF SPINAL CORD: A NEUROPHYSIN-CONTAINING, ESTROGEN-CONCENTRATING PATHWAY

By

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The study of neural circuits containing steroid sensitive neurons is essential to the understanding of the mechanisms by which steroids alter neuronal function to result in complex behaviors. A model that has been widely used in the study of steroid sensitive neurons, the spinal nucleus of the bulbocavernosus (SNB), is a sexually dimorphic motor nucleus in the lower lumbar spinal cord that innervates the striated bulbocavernosus (BC) muscle. The BC is responsible for penile reflexes in the male rat, which are important in ensuring pregnancy in females during mating. The identification and characterization of afferents to the SNB aids in the understanding of the neural circuity involved in reproductive behavior.

The paraventricular nucleus of the hypothalamus (PVN) plays a role in the modulation of penile reflexes. In addition, the PVN has been shown to project to all levels of spinal cord, including lumbar levels, making the PVN a possible afferent to the SNB. The results of the present studies demonstrate that the PVN projects to

levels of lumbar spinal cord known to contain the SNB. In addition, neurons of the lateral parvocellular (lp) subnucleus of PVN, that project to this level of spinal cord, contain neurophysin (NP), the coproduct of oxytocin and vasopressin. NP-containing fibers and putative terminals are found in the region of the SNB and appear to contact the soma and proximal dendrites of SNB motoneurons, including those SNB motoneurons retrogradely identified as BC-innervating. Electrolytic lesions that destroy the lp subnucleus of PVN abolish NP-containing fibers in the region of the SNB, suggesting that the PVN is the source of these NP fibers. Furthermore, a subpopulation of neurons in the lp subdivision of PVN that project to the region of the SNB concentrates radiolabelled estradiol.

These results strongly suggest that the PVN projects to the SNB motor nucleus and that either oxytocin or vasopressin, or both, is used in this pathway. In addition, results of the present experiments are consistent with the hypothesis that steroid sensitive neural circuits exist, in which all nuclei in the circuit contain neurons that are steroid concentrating.

To

my husband
Mark Hudson Wagner

and

my family
Kenneth and Joan Konen
and
William and Jean McMahon

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LIST OF ABBREVIATIONS

3V = third ventricle

acc = accessory nucleus

AH = anterior hypothalamus

ap = anterior parvocellular subnucleus of PVN

Arc = arcuate nucleus

BC = bulbocavernosus muscle

cc = central canal

DAH = dorsal area of the hypothalamus

DLN = dorsolateral nucleus

DM = dorsomedial nucleus of the hypothalamus

dp = dorsal parvocellular subnucleus of PVN

E = estrogen

fx = fornix

ic= internal capsule

IC = ischiocavernosus muscle

LH = lateral hypothalamus

lp (pvpo) = lateral parvocellular subnucleus of PVN

mp = medial parvocellular subnucleus of PVN

mt = mammillothalamic tract

nc = nucleus circularis

NP = neurophysin

opt = optic tract

OXY = oxytocin

Pef = perifornical nucleus

pm = posterior magnocellular nucleus of PVN

PVN = paraventricular nucleus

pvpo (lp) = posterior subnucleus of PVN

Rch = retrochiasmatic area

Re = Reunien's thalamic nucleus

SNB = spinal nucleus of the bulbocavernosus

SON = supraoptic nucleus

st = stigmoid nucleus

T = testosterone

VMH (VMN) = ventromedial nucleus of the hypothalamus

VP = vasopressin

ZI = zona incerta

INTRODUCTION

Steroids have a dramatic effect on the behavior of animals, including humans. It is now widely accepted that these steroids act within the central nervous system to alter the function of specific neural circuits, which in turn influence behavior. The study of these steroid sensitive neural circuits is crucial to the understanding of hormonal control of behavior and reproductive physiology.

A model that has been widely used in the study of steroid sensitive neurons is the spinal nucleus of the bulbocavernosus (SNB). This motor nucleus serves as an excellent model for several reasons: First, in the rat, the SNB has a relatively small number of darkly staining, morphologically distinct motoneurons and occupies a distinct location, thereby making it easy to identify. Secondly, the function and characteristics of these motoneurons are well documented. Thirdly, and most importantly, motoneurons of the SNB concentrate steroids and are dependent on steroids during both development and adulthood.

The identification and characterization of afferents to the SNB motor nucleus aids in understanding the function of steroid sensitive neural circuits. The present study describes a projection from the paraventricular nucleus (PVN) of the hypothalamus to the SNB. This pathway is in part oxytocinergic and/or

vasopressinergic and the PVN neurons involved in this pathway concentrate steroid hormones.

The SNB/DLN Neuromuscular System: Anatomy and Function

The perineal muscles controlling penile reflexes in the rat, the bulbocavernosus (BC) and the ischiocavernosus (IC), are extremely important in male reproductive success (Sachs, 1982; Hart and Melese-d'Hospital, 1983). Following excision of these muscles, males can demonstrate the gross motor patterns of mating behavior, but their ability to impregnate females is markedly impaired (Sachs, 1982). The BC and IC muscles are innervated by axons from motor nuclei located in L5-L6 of the spinal cord, the spinal nucleus of the bulbocavernosus (SNB) and the dorsolateral nucleus (DLN), respectively (Schroder, 1980; Breedlove, 1984; McKenna and Nadelhaft, 1986).

Development of the SNB and DLN

The SNB and DLN are sexually dimorphic, being present in the male and reduced in the female, with females having approximately 1/3 the number of cells as males (Breedlove and Arnold, 1981). This sexual dimorphism is the result of the presence or absence of steroids during development. During prenatal life, the SNB of the female contains the same number of motoneurons as the SNB of the male (Nordeen et al, 1985) and the female's SNB motoneurons make functional synapses with the target musculature (Rand and Breedlove, 1987). However, in the female, these motoneurons undergo naturally occurring cell death in the absence of endogenous testosterone (T) (Nordeen et al, 1985), whereas in the male, SNB neurons remain, resulting in a sex difference in the number of cells in this nucleus

in adulthood. When female rats and mice were exposed to T during pre- or postnatal life, the number of SNB motoneurons was significantly increased relative to that of untreated females (Breedlove and Arnold, 1983a; Wagner and Clemens, 1989). Perinatal treatment with the anti-androgen, flutamide, and/or castration on the day of birth, significantly reduced the number and size of SNB motoneurons in the adult castrate as compared to an intact male rat (Breedlove and Arnold, 1983b) and mouse (Wagner and Clemens, 1989).

SNB motoneurons of the adult male rat possess large and elaborate dendritic arbors whose development is regulated by androgens. The growth of SNB dendrites has been described as biphasic in that dendrites reach extensive lengths by the fourth week of life and then retract, to reach their adult lengths by seven weeks of age. Both the original exuberant outgrowth and the dendritic retraction are under androgenic control (Goldstein et al, 1990).

The anatomical specificity of the SNB and DLN neuromuscular system is affected by the presence of steroids during development (Breedlove, 1985). Dihydrotestosterone (DHT), the reduced metabolite of T, given to females prenatally, resulted in the masculine development of the BC musculature, yet the number of SNB cells was markedly reduced (feminine) (Sengelaub et al, 1989). This reduced number of SNB cells was due to the failure of DHT to prevent cell death in the SNB. Interestingly, the BC muscles that developed in these DHT treated females were innervated anomalously by motoneurons in the DLN. Motoneurons in the DLN normally innervate the IC muscle. Because the number of SNB motoneurons prior to the occurrence of cell death was lower in DHT treated females

than that in normal females, Sengelaub et al suggested that DHT affected the cell migration that is known to occur in this neuromuscular system and deviated BC-innervating motoneurons from their normal medially-oriented path.

Steroid Effects on the SNB in Adulthood

Neurons of the SNB are sensitive to steroids in adulthood. Castration of adult male rats resulted in a decrease in the soma area of SNB motoneurons but did not alter cell number (Breedlove and Arnold, 1981), whereas in the mouse, castration resulted in a decrease in both cell size and cell number (Wee and Clemens, 1987). Androgens also regulate the dendritic morphology of SNB motoneurons during adulthood, with castration resulting in a dramatic reduction in dendritic length (Kurz et al, 1986).

The synthesis of a neuropeptide by SNB motoneurons is altered by the presence of T during adulthood. SNB motoneurons of the adult male rat synthesize the neuropeptide calcitonin gene-related peptide (CGRP) (Popper and Micevych, 1989). CGRP is thought to be a trophic factor produced by motoneurons to regulate the synthesis of nicotinic acetylcholine receptors in target muscle cells (New and Mudge, 1986). Castration in adulthood resulted in a decrease in the number of SNB motoneurons containing CGRP (Popper and Micevych, 1989), as well as the number of cells producing CGRP mRNA and the amount of CGRP mRNA per cell (Popper and Micevych, 1990). Interestingly, the effect of castration on CGRP production in the SNB of mice is in the opposite direction (Wagner et al, submitted).

The effects of steroids on SNB neurons is presumably through nuclear steroid receptors and indeed, motoneurons of the SNB concentrate radiolabelled steroids.

These motoneurons appear to possess receptors for T and DHT, but seem to lack receptors for estrogen (E). Hormone accumulation in the SNB is sexually dimorphic: more cells in the male concentrate steroids than do cells in the female (Breedlove and Arnold, 1983c). Interestingly, in spite of the many dramatic effects of steroids on the SNB neuromuscular system during development, SNB motoneurons do not accumulate either T, DHT or E during pre- or early postnatal life (Fishman et al, 1990). These authors have suggested that steroid effects during this time take place via steroid action on the target musculature. However, other techniques to detect steroid receptors (e.g. immunohistochemistry or *in situ* hybridization) will need to be performed before this surprising finding can be confirmed.

Penile reflexes, which appear to be controlled by the SNB/DLN neuromuscular system, are also androgen-dependent (Hart, 1967, 1973; Rodgers and Alheid, 1972; Davidson et al, 1978; Bradshaw et al, 1981), with castration reducing the incidence of penile reflexes and T replacement restoring this function to intact levels. The extremely short latency in which T can restore penile reflexes (6-12h) has led some to suggest that T is acting at the level of the CNS rather than on peripheral tissues, such as the perineal muscles (Gray et al, 1980; Hart et al, 1983).

PVN, Oxytocin and SNB Function

Oxytocin (OXY) influences male sex behavior in the rat. When administered intraperitoneally (i.p.) or intracerebroventricularlly (i.c.v.), OXY significantly shortened both the latency to ejaculation and the postejaculation interval (Arletti et al, 1985). OXY levels in cerebrospinal fluid (CSF) increased to twice basal levels 5 minutes after ejaculation in the male rat and reached 3 times basal levels 20 minutes

after ejaculation. Discrete electrolytic lesions to the lateral and posterior PVN abolished this ejaculation-associated increase in CSF OXY, and resulted in prolonged mount and intromission latencies, while reducing postejaculatory intervals (Hughes et al, 1987).

The PVN appears to play a role in male copulatory behavior. However, the PVN may be involved in the function of the SNB/DLN neuromuscular system as well. OXY is a potent inducer of penile responses, as measured in a freely moving male rat in the absence of a female (Argiolas et al, 1986).

One site of action of OXY's influence on penile responses appears to be the PVN. Microinfusion of OXY into PVN or into the third ventricle increased the occurrence of penile responses (Argiolas et al, 1986; Melis et al, 1986). This effect was blocked by the administration (i.c.v) of the OXY antagonist, $d(CH_2)_5$Tyr(Me)-[Orn^8]vasotocin (Argiolas et al, 1987b). Furthermore, OXY's effects on penile responses following administration of OXY into the third ventricle were abolished by electrolytic lesions of PVN (Argiolas et al, 1987a).

Besides OXY, other neurotransmitters, or their agonists, have been shown to affect penile reflexes by actions at the level of the CNS. These include dopamine, alpha-melanocyte stimulating hormone (α-MSH) and adrenocorticotropin hormone (ACTH) (Ferrari et al, 1963; Argiolas et al, 1987a; Bitran et al, 1988; Pehek et al, 1988). ACTH and α-MSH appear to have effects at a site other than the PVN. Although one site of action for the effects of dopamine on penile reflexes is the medial preoptic area of the hypothalamus (MPOA) (Hull et al, 1986; Bitran et al, 1988), dopamine's effects after i.c.v. administration are prevented by lesions of the

PVN (Argiolas et al, 1987a). The POA is innervated by dopaminergic axons of the incertohypothalamic tract (A14) (Bjorklund et al, 1975) and the POA sends projections to the PVN (Silverman et al, 1981; Chiba and Murata, 1985). Therefore, dopamine may act on cells in the POA that project to PVN cells, which in turn influence penile reflexes. In support of this idea are the findings that the stimulation of penile reflexes, as well as male copulatory behavior, by apomorphine i.c.v. was blocked by the i.c.v. administration of the OXY antagonist $d(CH_2)_5Tyr(Me)$-$[Orn^8]$vasotocin (Argiolas et al, 1987b; 1989). Furthermore, OXY and apomorphine administered together did not produce an increase in the occurrence of penile reflexes above that of either substance alone (Melis et al, 1989) leading the authors to suggest that apomorphine induces penile erection by releasing OXY in the CNS. These findings suggest that PVN is involved in the regulation of penile reflexes and that OXY plays an important role in this system.

Anatomy of the PVN

The PVN is a heterogeneous region consisting of several subdivisions that are cytoarchitectonically distinct and can also be characterized by their projections (Armstrong et al 1980; Swanson and Kuypers, 1980). It has long been known that PVN projects to the neurohypophysis (Bargman and Scharrer, 1951), and these efferents arise from three magnocellular subnuclei of the PVN, the anterior, medial and posterior magnocellular subnuclei (Armstrong et al, 1980; Swanson and Kuypers, 1980). However, the PVN also projects to other areas within the CNS including the brainstem and spinal cord (Kuypers and Maisky, 1975; Conrad and Pfaff, 1976; Hancock, 1976; Saper et al 1976; Swanson, 1977; Ono et al, 1978; Hosoya, 1980;

Schwanzel-Fukuda et al, 1984). PVN cells that project to cervical and thoracic regions of the spinal cord originate in four parvocellular subnuclei, the anterior parvocellular (ap), the medial parvocellular (mp), the dorsal parvocellular (dp) and the lateral parvocellular (lp) also referred to as the posterior subnucleus of PVN (pvpo) (Armstrong et al, 1980; Swanson and Kuypers, 1980).

Axons projecting to the spinal cord leave the PVN in two major fiber bundles. One tract leaves the PVN dorsally and runs caudally, dorsal to the third ventricle to reach the mesencephalic central gray (Luiten et al, 1985). The second tract leaves the PVN medially and caudally and runs dorsal to the medial forebrain bundle. At the pontine level both fiber tracts join the lateral lemniscus, continuing caudally into the medulla, terminating in the lateral reticular nucleus, nucleus ambiguous, parvocellular reticular nucleus, nucleus of the solitary tract, dorsal motor vagus nucleus and area postrema. At spinal cord levels these fibers travel through the lateral funiculus the entire length of the cord, but send out lateral projections that terminate in the intermediolateral cell column (IML), where they are thought to regulate sympathetic function (Luiten et al, 1985; Saper et al, 1976).

The PVN receives substantial afferent input from limbic structures such as the lateral septal nucleus and the subicular cortex, and from the subfornical organ (Silverman et al, 1981). The medial amygdala and OVLT contribute smaller inputs. PVN receives hypothalamic input from the medial and lateral preoptic areas, suprachiasmatic nucleus, ventromedial nucleus, arcuate nucleus, retrochiasmatic and lateral hypothalamic areas.

Neurophysin, Oxytocin and Vasopressin

A major function of the PVN is the synthesis and release of OXY and vasopressin (VP) into the posterior pituitary (Brownstein et al, 1980). However, neurons in the parvocellular subdivisions of PVN also synthesize these neuropeptides and presumably release them within the CNS (Armstrong et al. 1980).

The synthesis of OXY and VP is fairly well understood (See Gainer et al, 1987 for review). The genes which encode these two neuropeptides are essentially identical in structure, both having three exons, which contain the amino acid sequences for the precursors. These exons are separated by two introns. The first exon contains the nucleotide bases encoding the signal peptide, followed by the nonapeptide, then a three amino acid spacer which contains the signal for the endoprotease cleavage of the precursor and then the first nine amino acids of the N-terminus of the neurophysin (NP). The second exon contains the amino acid positions 10-76 of the NP, while the third exon contains the C-terminus of this protein. This C-terminus is followed by a single arginine that separates the NP from a C-terminal 39 amino acid glycopeptide in the VP precursor. The OXY precursor contains only the extra arginine. The presence of this glycopeptide sequence is the only apparent structural difference between the OXY and VP genes.

Following translation of these genes into mRNA, the initial post-translational modifications occur in the rough endoplasmic reticulum and Golgi apparatus. These modifications form the precursor molecule. The consequent endoproteolytic cleavages form the individual peptides, OXY or VP and the coproducts, OXY-associated NP and VP-associated NP, respectively. This last step of the postranslational processing

appears to occur within the neurosecretory vesicles and it has been demonstrated in the pituitary that NP is released from terminals along with OXY or VP (Johnston et al, 1975).

Oxytocin and the Lumbosacral Spinal Cord

PVN has been shown to be a critical region for the effects of OXY on penile reflexes. PVN is known to contain OXY binding sites (Brinton et al, 1984) and cells in the dorsal and lateral (posterior) parvocellular subnuclei of PVN that project to upper levels of spinal cord, produce OXY (Armstrong et al, 1980; Sawchenko and Swanson, 1982). These two findings support the idea that OXY may have its effects on penile reflexes through actions in the PVN, as well as through terminals of PVN neurons that reach the spinal cord.

In fact, OXY fibers have been identified throughout the spinal cord, including lumbar levels. In ventral horn, the density of OXY fibers, identified by the presence of NP-I (OXY-associated), is highest in lumbar segments compared with all other levels (Swanson and McKellar, 1979). OXY levels in lumbosacral cord, as measured by radioimmunoassay, are 152% of that in thoracic cord and 274% of cervical cord levels (Valiquette et al, 1985). OXY levels in spinal cord, as measured by radioimmunoassay, drop dramatically after PVN lesions (Lang et al, 1983; Hawthorn et al 1985), suggesting that PVN is the major source of OXY in the spinal cord.

PVN and Gonadal Steroid Hormones

The hypothesis that PVN may play a role in a steroid sensitive neural circuit involving the SNB is supported by the fact that both the magnocellular and parvocellular regions of this nucleus contain cells that concentrate radioactively

labelled T and its metabolites (Sar and Stumpf, 1975; Stumpf et al, 1975), as well as cells that produce mRNA for androgen and estrogen receptor proteins (Simerly et al, 1990). Furthermore, certain characteristics of PVN have been shown to be steroid sensitive. The incidence of electrical coupling between PVN neurons in rats, as measured by dye-coupling, is affected by gonadal steroids, with castration reducing the number of coupled cells (Cobbett et al, 1987). This is similar to the finding that the incidence of gap junctions between SNB motoneurons, quantified with electron microscopy, is T dependent (Matsumoto et al, 1988a). In addition, the morphology of PVN neurons (i.e. nuclear size, nuclear shape) is affected by neonatal castration in mice (Perez-Delgado et al, 1987). Electrophysiological studies have demonstrated that the firing rate of tonically firing (oxytocinergic) neurons of the PVN in anesthetized male rats is increased 2 days after systemic injections of T, but not E (Akaishi and Sakuma, 1985).

The synaptic input onto SNB motoneurons is affected by gonadal steroids with castration reducing the number of synapses on SNB somata and proximal dendrites (Leedy et al, 1987; Matsumoto et al, 1988b). T replacement restored the number of synapses in as little as 48 hours in long-term castrated male rats (Leedy et al, 1987). While SNB motoneurons concentrate androgens, these terminals may also originate from steroid-sensitive cells elsewhere in the CNS. Results demonstrating that the PVN is androgen-sensitive are consistent with the idea that PVN cells may be one source of these SNB afferents.

Summary

Pharmacological studies provide a basis for suggesting that the PVN and OXY play a role in the function of the SNB/DLN neuromuscular system. Furthermore, cells in PVN project to all levels of spinal cord, contain T and E receptors and are steroid sensitive. The following experiments describe a direct projection from PVN to the SNB that is NP-containing. Furthermore, the results indicate that the cells of origin of this direct projection concentrate E.

EXPERIMENT 1: PROJECTIONS OF THE PVN TO THE LOWER LUMBAR SPINAL CORD: RETROGRADE STUDIES

The findings suggesting that PVN plays a role in the modulation of SNB/DLN function could mean that PVN affects these motoneurons indirectly (e.g. via brainstem sites) or that PVN monosynaptically modulates SNB/DLN motoneuron activity.

It has been well documented that PVN projects directly to the spinal cord. The majority of studies have demonstrated, using retrograde tracing techniques, that PVN projects to cervical and thoracic levels of the spinal cord (Hancock, 1976; Saper et al, 1976; Ono et al, 1978; Hosoya and Matsushita, 1979; Swanson and Kuypers, 1980), while only a few studies have demonstrated that neurons of the PVN project directly to lumbar segments (Hosoya, 1980; Schwanzel-Fukuda et al, 1984). However, the latter studies injected large regions of lumbar spinal cord rather than just the sexually dimorphic regions, L5-L6.

The purpose of Experiment 1 was to identify the PVN as a possible source of input to the SNB and to describe the distribution of PVN neurons projecting to this level of lumbar cord by injecting the retrograde tracers wheatgerm agglutinin conjugated horseradish peroxidase (WGA-HRP) or Fluorogold into the region of the SNB and DLN (L5-L6).

METHODS

General Methods

Animals used in all experiments were Sprague-Dawley albino rats ranging in age from 60-100 days. Animals were housed in wire mesh cages in a 14:10 light:dark cycle with food (Wayne rodent blox) and tap water ad lib. Anesthesia used in all surgical procedures was pentobarbital at a dose of 60mg/kg administered intraperitoneally.

EXPERIMENT 1A: INJECTIONS OF CT-HRP INTO BC/IC MUSCLES

Since the dendritic tree comprises 70-90% of the receptive field of motoneurons (Gelfan et al, 1970), it was necessary to determine that the volume of WGA-HRP injected into the region of L5-L6 would be sufficient to include the SNB and DLN and their dendritic trees. To ensure this I filled these motoneurons and their dendrites were filled with cholera toxin-bound horseradish peroxidase (CT-HRP) (List Biological Laboratories). In two males, injections of 1.0μl each of CT-HRP were made into both sides of the BC and IC muscles. After 48 hours animals were perfused with approximately 200ml of 0.87% saline containing 0.14% heparin, in 0.1M phosphate buffer, (pH7.4) at 4°C, followed by 500ml of 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M phosphate buffer (pH7.4) at 4°C. Spinal cords were removed and postfixed for 2-3 hours. Tissue was cryoprotected in 20% sucrose in 0.1M phosphate buffer (pH7.4) overnight, then sectioned in the coronal plane at 40μm on a freezing microtome. The CT-HRP was visualized using the TMB method (Mesulam, 1976). Sections were mounted onto gelatin coated slides and viewed, photographed and drawn under darkfield illumination.

EXPERIMENT 1B: INJECTIONS OF WGA-HRP INTO THE SPINAL CORD

Animal Preparation

Males were anesthetized and either castrated or sham-castrated 2 weeks prior to injection of WGA-HRP into the spinal cord. Females were anesthetized and ovariectomized 4 days prior to injection and then received 0.25μg estradiol benzoate (EB) (i.m.) 2 and 3 days prior to the spinal cord injection. This hormone treatment was done to control for cyclic variations in estrogen during the estrous cycle.

Spinal Cord Injections

For spinal cord injections, animals were anesthetized and a laminectomy was performed at the level of the lumbosacral enlargement. The meninges were slit with a #11 scalpel blade and a glass micropipette (inner diameter = 30-40μm) was visually guided, either stereotaxically-held or hand-held, into the cord directly adjacent to the dorsal artery and lowered 1.0-1.5mm. A volume of 0.5-0.8μl of wheatgerm agglutinin conjugated HRP, (gift from Drs. M. Weiss and T. Youngstrom, Michigan State University) was slowly pressure injected. Injections were aimed at the midline of the ventral horn and were of a volume sufficient to include the SNB and the DLN and the majority of the dendritic arbors of both the motor nuclei.

Tissue Preparation and Histology

After a survival time of 4-5 days, animals were perfused and tissue was prepared as in Experiment 1A. The hypothalamus was sectioned at 40μm in the coronal plane and the region of the lumbosacral enlargement was sectioned at 40μm longitudinally to allow better examination of the rostro-caudal extent of the injection

site. Some cords were sectioned coronally to evaluate spread of WGA-HRP throughout the ventral and dorsal horns.

The WGA-HRP was visualized using the TMB method (Mesulam, 1976). Alternate 40μm sections were mounted onto gelatin coated slides and left unstained or were counterstained with 1% Neutral red (pH 4.5). Tissue was viewed and photographed under darkfield illumination. The cytoarchitectonic subdivisions were determined in counterstained sections according to Armstrong et al (1980) and Swanson and Kuypers (1980). Labelled cells in PVN were counted in alternate 40μm sections. Total PVN cell counts were compared across groups (i.e. intact males, castrated males and females) using a one-way analysis of variance, (ANOVA; $p<0.05$).

EXPERIMENT 1C: INJECTIONS OF FLUOROGOLD INTO SPINAL CORD

Injections of 0.5μl of 2% Fluorogold (Fluorochrome, Inc.) were made into L5-L6 of intact males. This retrograde tracer was used to verify the findings using WGA-HRP as a tract tracer. Laminectomy was performed as in Experiment 1B and the micropipette was stereotaxically placed directly adjacent to the dorsal artery and lowered 1.0-1.5mm. Following a survival time of 1-2 weeks animals were perfused with 0.87% saline followed by 10% formalin in 0.1M phosphate buffer, pH7.4. Brain and spinal cords were removed and postfixed overnight. Tissue was cryoprotected overnight in 20% sucrose in 0.1M phosphate buffer (pH 7.4) and both brain and spinal cord were sectioned at 40μm in the coronal plane on a freezing microtome. Alternate sections were left unstained or were counterstained with thionin. Tissue was viewed and photographed using epifluorescence.

RESULTS

Ten animals were successfully injected with WGA-HRP into the L5-L6 region of the spinal cord (Figure 1). These consisted of 6 males, (3 intact, 3 castrated) and 4 females. Only animals in which the center of the injection site was within the rostro-caudal extent of the SNB were evaluated. In most injection sites the effective area of uptake included all of ventral horn and most of dorsal horn. White matter was usually excluded. The effective injection site covered the dendritic extent of motoneurons innervating both the BC and IC muscles, (Figure 2).

In all animals evaluated labelled cells were found in PVN. These cells were located in the anterior parvocellular (ap), dorsal parvocellular (dp) and medial parvocellular (mp) subnuclei of PVN, (Figures 3 & 4), defined here according to Swanson and Kuypers (1980). Some cells were also seen in the posterior magnocellular subnucleus (pm), but these cells were small and were most likely parvocellular neurons, (Figures 3 & 4). In pm, magnocellular and parvocellular neurons are intermixed (Swanson and Kuypers, 1980). Labelled cells were also seen in the posterior parvocellular subnucleus of PVN (pvpo), (Figures 3 & 5), defined here according to Armstrong et al (1980). This subnucleus has been also been designated the lateral parvocellular subnucleus (lp) by Swanson and Kuypers (1980) and is referred to as both pvpo and lp in the present document. Most of these neurons were quite large, (approximately 30μm x 10μm) and these neurons were elongated with most being oriented along their mediolateral axis (Figure 5B).

The total number of WGA-HRP labelled cells in the PVN of each animal is shown in Table 1. There were no statistically significant differences in the number

Figure 1. Camera lucida drawings of longitudinal sections through the lumbosacral spinal cord of intact males (S-4,5,8), castrated males (C-3,4,7) or females (F-2,4,5,6) showing the location of WGA-HRP injection sites in the region of the SNB. The dark stippled areas indicate the centers of the injection sites where the TMB reaction product was heaviest. The lighter stippled areas indicate the spread of the TMB reaction product. Black dots indicate the location of SNB motoneurons.

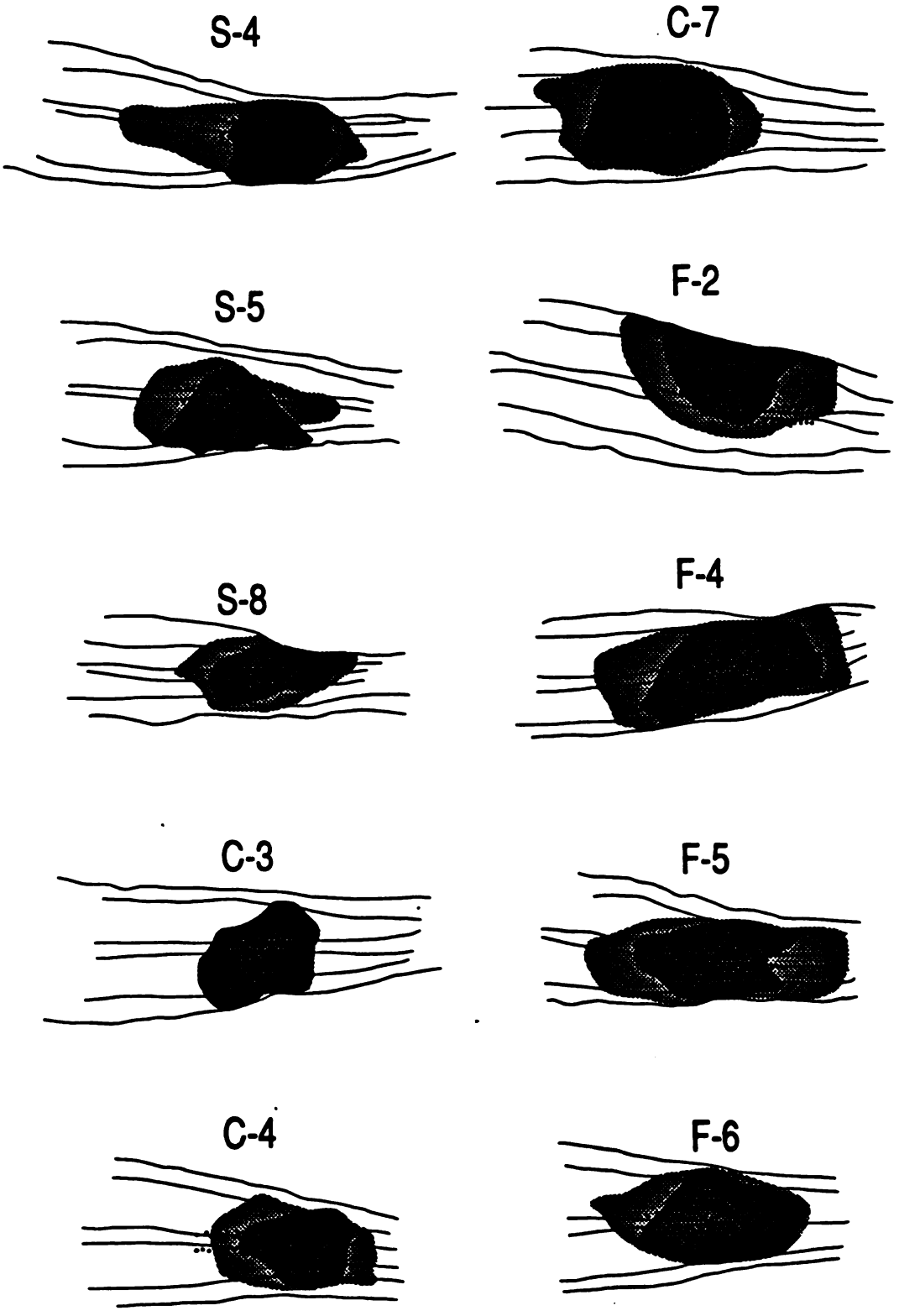

Figure 1

Figure 2. A) an example of the location and size of the WGA-HRP injection site in a coronal section through lower lumbar spinal cord spinal cord drawn over a camera lucida drawing of CT-HRP filled SNB/DLN motoneurons and their dendrites. **B)** darkfield photomicrograph of SNB and DLN motoneurons and their dendrites filled with CT-HRP following injections into both sides of the BC and IC muscles demonstrating the dendritic extent of this neuromuscular system. cc = central canal; SNB = spinal nucleus of the bulbocavernosus; DLN = dorsolateral nucleus. Bar = 500μm.

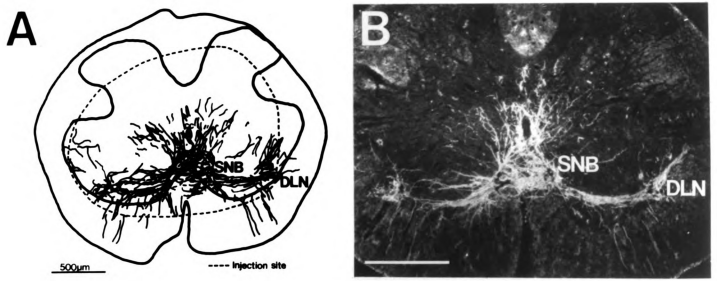

Figure 2

Figure 3. The distribution of WGA-HRP labelled cell bodies in the hypothalamus of a male rat following an injection of WGA-HRP into the lower lumbar spinal cord. See List of Abbreviations.

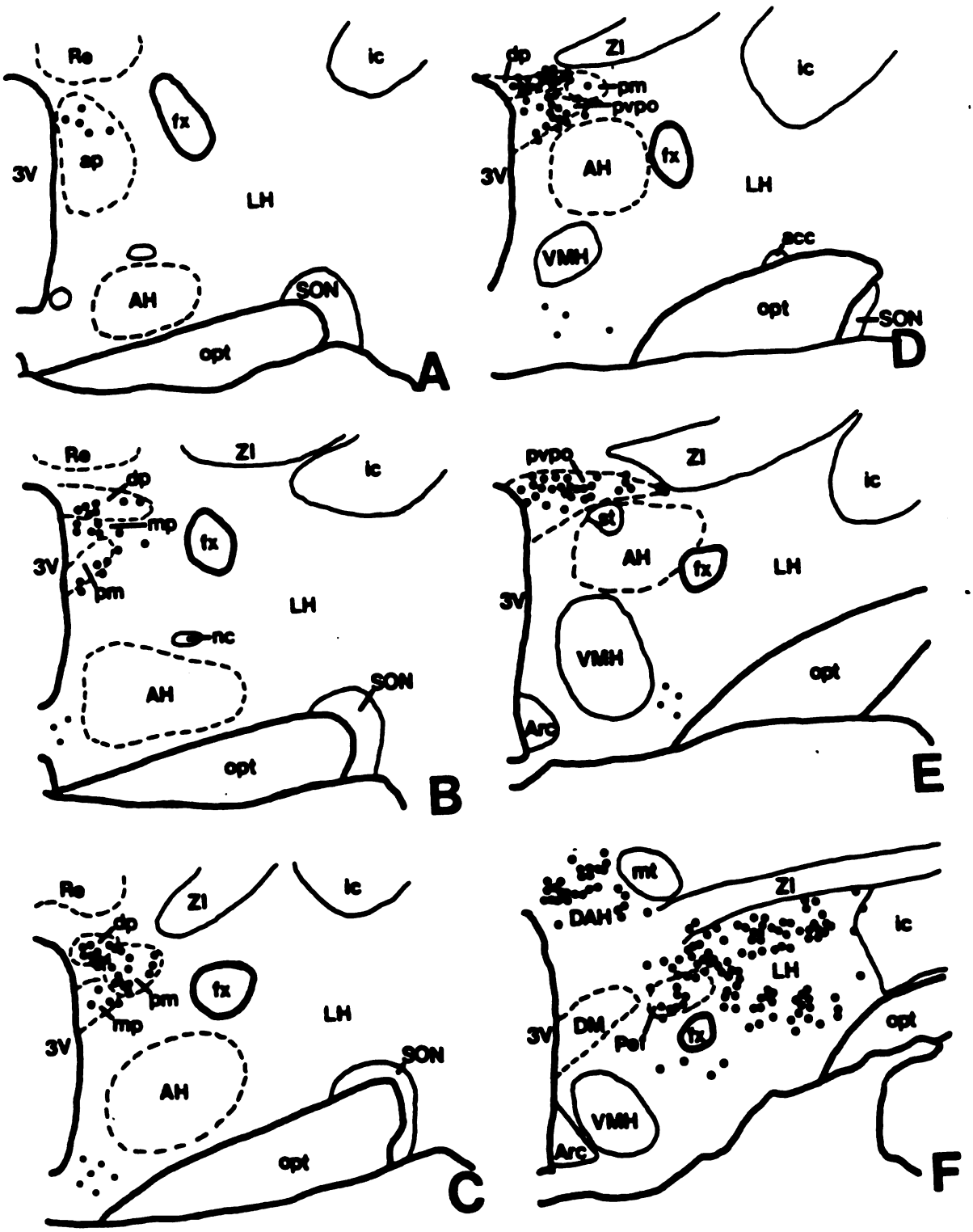
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Figure 3

Figure 4. WGA-HRP labelled neurons in the paraventricular nucleus (PVN) of a male rat following an injection of WGA-HRP into the lower lumbar spinal cord in **A)** anterior parvocellular subnucleus (ap) **B)** dorsal parvocellular (dp) and medial parvocellular (mp) subnuclei **C)** dp, mp, and posterior magnocellular (pm) subnuclei. 3V = third ventricle. Bar = 100μm.

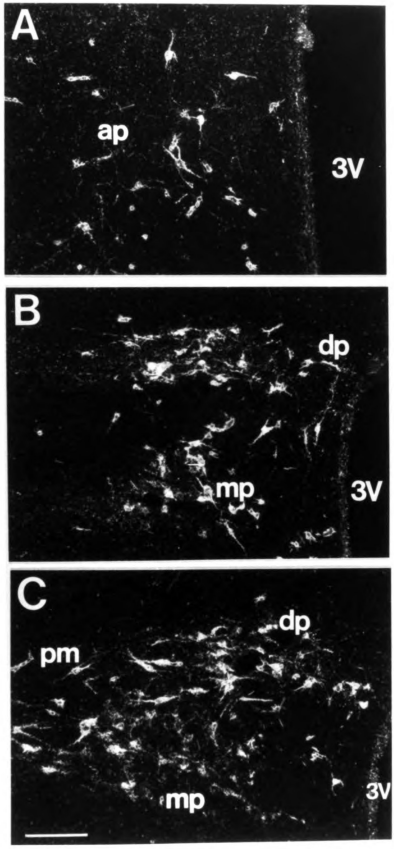

Figure 4

Figure 5. WGA-HRP labelled neurons in the paraventricular nucleus (PVN) of the hypothalamus in a male rat following an injection of WGA-HRP into the lower lumbar spinal cord in **A)** dorsal parvocellular (dp), medial parvocellular (mp) and posterior subnucleus (pvpo) or lateral parvocellular subnucleus **B)** dp and pvpo. Cells in pvpo are quite large and are oriented along their mediolateral axis. 3V = third ventricle. Bar = 100μm.

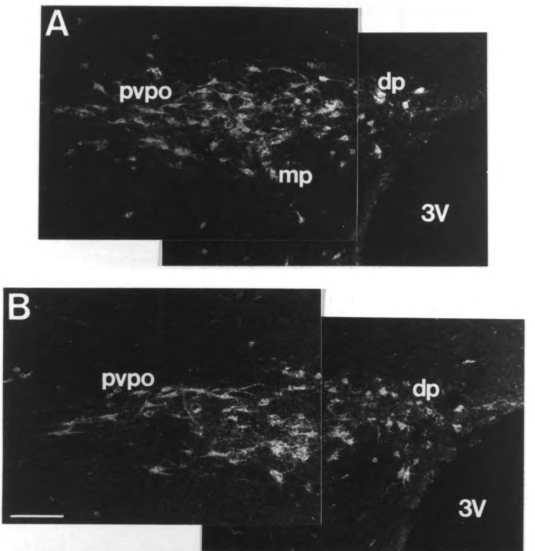

Figure 5

Table 1

The Individual and Group Mean Number of WGA-HRP Labelled Cells in the Paraventricular Nucleus Following an Injection of WGA-HRP into the Lower Lumbar Spinal Cord of Sham-Castrated Males (**SHAMS**), Castrated Males (**CASTRATES**) or **FEMALES**.

SHAMS		**CASTRATES**		**FEMALES**	
S-4	329	C-3	216	F-2	593
S-5	488	C-4	256	F-4	730
S-8	373	C-7	731	F-5	663
				F-6	348
MEAN	**396.7**		**401.1**		**583.5**
(s.e.m.)	**(47.4)**		**(165.4)**		**(83.3)**

of labelled cells between any of the groups, ($F(2,7)=0.99$). Injections that were more rostral ("misses") resulted in a larger number of neurons in PVN than did injections that were more caudal.

Labelled fibers could be traced out of anterior and medial parvocellular regions ventrally, just medial to the fornix into the anterior hypothalamus. Fibers leaving the dorsal parvocellular region extended both ventrally, just medial to the fornix, as well as laterally just dorsal to the fornix, then ventrally. Fibers leaving the pvpo region extended quite far laterally, dorsal to the fornix (Figures 4 & 5).

A large number of labelled cells was seen caudal to the PVN in the region of the lateral hypothalamus (LH), (Figure 3F). The majority of these cells were found medial to the internal capsule (ic) and ventral to the zona incerta (ZI). A few cells were seen on the most ventral border of the ZI but no cells were found within the ZI. Neurons were clustered just dorsal to the fornix in the perifornical area (Pef), (Figure 3F). Occasionally a few cells were seen within the fornix.

No cells were found within the ventromedial nucleus (VMH), the arcuate nucleus (Arc) or the dorsomedial nucleus (DM) but quite a few cells were found just medial to the mammalothalamic tract (mt) in the dorsal area of the hypothalamus (DAH), (Figure 3F). Occasionally a few small but distinct cells were seen in the retrochiasmatic area (Rch), (Figure 3B,C). No cells were seen in the preoptic area, the supraoptic nucleus or the suprachiasmatic nucleus.

Similar results were found with Fluorogold. Labelled cells were found in the same PVN subnuclei as well as in LH (Figure 6) and DAH. The distribution and number of Fluorogold labelled cells were similar to that seen following WGA-HRP

Figure 6. Fluorogold labelled cells in **A & B)** the paraventricular nucleus of the hypothalamus or **C & D)** the lateral hypothalamus following an injection of Fluorogold into the lower lumbar spinal cord. A & B) Bar = 50μm; C & D) Bar = 25μm.

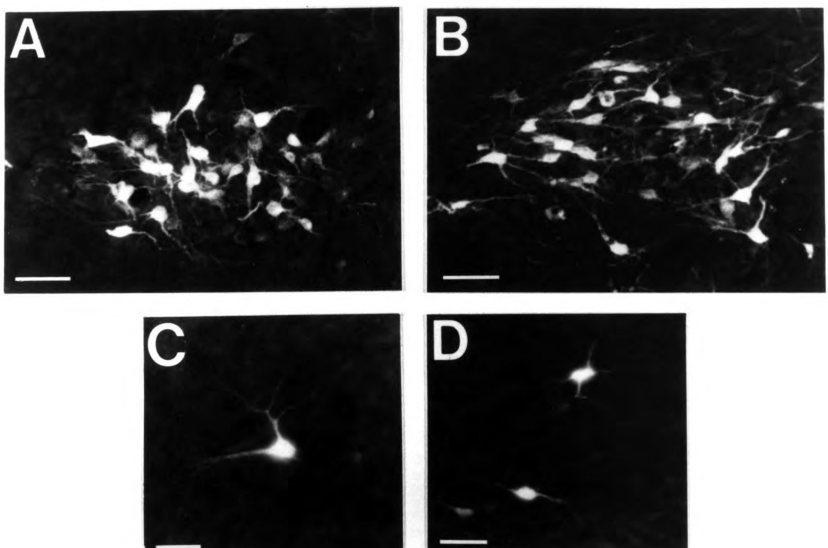

Figure 6

injections. Labelled cells in PVN demonstrated differing morphology depending on their location in various subnuclei, (Figure 6A,B). Some labelled cells in LH were quite large with varying morphology, (Figure 6C,D).

DISCUSSION

The results of Experiment 1 demonstrate that neurons of the PVN project to lower lumbar spinal cord levels (L5-L6), which contain the sexually dimorphic motor nuclei, the SNB and DLN. These spinally projecting PVN neurons are found in the anterior, medial and dorsal parvocellular as well as the posterior magnocellular subnuclei and in posterior PVN (pvpo or lp). In addition, some neurons in the LH and DAH were found to project to L5-L6.

The distribution of PVN neurons that project to L5-L6 is similar to that reported to project to cervical and thoracic levels of spinal cord and higher lumbar levels (Armstrong et al, 1980; Swanson and Kuypers, 1980; Schwanzel-Fukuda et al, 1984). However, the number of neurons projecting to L5-L6 is smaller than that projecting to higher levels of spinal cord. The distribution of labelled PVN neurons was similar whether WGA-HRP or Fluorogold was used. This suggests that the labelling seen in HRP animals was not due to trans-synaptic transport of the WGA-HRP.

The location and distribution of WGA-HRP labelled fibers leaving PVN on the way to the spinal cord is similar to that reported by Luiten et al (1985) who used PHA-L injections into PVN to anterogradely fill axons leaving PVN. These authors describe two major fiber bundles leaving PVN. One tract leaves the PVN dorsally

and runs caudally, dorsal to the third ventricle to reach the mesencephalic central gray. The second fiber tract leaves the PVN medially and caudally and runs dorsal to the medial forebrain bundle.

The projections from PVN and other hypothalamic nuclei (LH and DAH) to the L5-L6 region of spinal cord in females that do not have the SNB/DLN neuromuscular system, may involve reproductive functions specific to females. The pudendal nerve, which originates in segments L5-S1 of the spinal cord innervates structures important in both female copulatory behavior and parturition (Pacheco et al, 1989). OXY has been shown to have central effects on sexual receptivity in female rats (Caldwell et al, 1986). Electrophysiological evidence demonstrates that tonically firing (oxytocinergic) neurons of the PVN receive input from the uterus (Akaishi et al, 1988), and the present results suggest that reciprocal connections may exist. Furthermore, projections from PVN to lumbar and sacral levels of spinal cord, undoubtedly, regulate non-reproductive sympathetic functions in both males and females as they do in cervical and thoracic levels.

Although the number of WGA-HRP labelled PVN neurons was almost identical between castrated and sham-castrated males, the number of labelled cells in females was consistently, but not significantly, higher than in males. However, it is not clear if this was due to a greater projection from PVN to lumbar spinal cord or if this was simply the result of a shorter distance of transport for the WGA-HRP in the smaller female, allowing more cells to be labelled in a given survival time.

The possible function of projections to lower lumbar spinal cord from more caudal regions of hypothalamus, LH and DAH, is unclear. These areas project to

cervical and thoracic regions as well, where they terminate in IML (Saper et al, 1976). Interestingly, 90-100% of neurons in LH that project to thoracic levels of spinal cord contain α-MSH and 50-90% of neurons in DAH that project to thoracic spinal cord contain tyrosine hydroxylase (Cechetto and Saper, 1988). Both α-MSH and dopamine have been shown to have effects on penile responses (Ferrari et al, 1963; Argiolas et al, 1987a; Bitran et al, 1988; Pehek et al, 1988).

No retrogradely labelled cells were seen in the POA. This is significant in light of findings demonstrating that dopaminergic agonists and antagonists are able to alter penile reflexes when infused into the POA. The present results demonstrate that these effects must be indirect effects on SNB/DLN motoneurons.

EXPERIMENT 2: IDENTIFICATION OF NEUROPHYSIN-CONTAINING NEURONS OF THE PVN THAT PROJECT TO THE LOWER LUMBAR SPINAL CORD

The PVN has been implicated as a site involved in the function of the SNB/DLN neuromuscular system (Argiolas et al, 1986; 1987a; Melis et al, 1986) and Experiment 1 has demonstrated that the PVN sends a direct and monosynaptic pathway to this region of the spinal cord. A likely candidate for a neurotransmitter used in this pathway is OXY. The projection from the PVN to cervical and thoracic levels of spinal cord has been shown to contain NP, the coproduct of OXY and VP (Armstrong et al, 1980). OXY levels in the spinal cord, as measured by radioimmunoassay, were dramatically reduced by electrolytic lesions of PVN, suggesting that PVN is the major source of OXY in the spinal cord (Lang et al, 1983; Hawthorn et al, 1985). OXY levels, when measured in all levels of spinal cord, were found to be dramatically higher in lumbar segments than in cervical or thoracic segments (Valiquette et al, 1985).

The purpose of Experiment 2 was to determine if NP is contained in neurons of the PVN that project to the region of the SNB (L5-L6) and if so, to describe the distribution of these neurons within the PVN. This was accomplished using double fluorescent tract tracing-immunohistochemical techniques. Antisera against NP, which recognizes both OXY-associated NP and VP-associated NP was used in these experiments because the projection from PVN to upper spinal cord has been shown

to contain both OXY and VP, although OXY appears to be the major contributor (Sawchenko and Swanson, 1982; Cechetto and Saper, 1988).

METHODS

Spinal Injections and Histology

Injections of 0.5-0.8μl of Fluorogold were made into the region of the SNB in 7 males as in Experiment 1C. Animals were allowed to survive for 1-2 weeks and were then perfused with 100ml of 0.87% saline (4°C) followed by 300ml of 10% formaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. Brains and spinal cords were removed, postfixed for 2-3 hours, and then cryoprotected overnight in 20% sucrose in 0.1M phosphate buffer (pH 7.4) at 4°C.

Spinal cords were sectioned on a freezing microtome at 50μm in the longitudinal plane. Alternate sections were mounted on gelatin coated slides and were either counterstained with thionin or were left unstained. The hypothalamus was sectioned at 30μm in the coronal plane into 0.87% saline in 0.05M tris buffer (pH 7.4) at 4°C. Alternate sections were mounted onto gelatin coated slides and were counterstained with thionin.

Immunohistochemistry

The second set of alternate sections was processed for immunohistochemistry using antisera against NP (Chemicon International, Inc.) according to the protocol detailed in Appendix A. The immunohistochemistry was performed using Rhodamine-tagged avidin (Vector Labs, Inc.) to allow the detection of both Fluorogold labelled cells and NP-containing cells.

Following immunohistochemistry, sections were mounted onto gelatin coated slides, dehydrated in 100% ethanol for 1 minute and cleared in xylene for 2 minutes. Slides were coverslipped with a non-autofluorescent mountant (DPX, Fluka Chemika).

Mapping and Quantification

Two black and white photographs (PanX, Eastman Kodak, Inc.) were taken of each section through the PVN for each animal using epifluorescence, one at 480nm wavelength to visualize the Fluorogold and one at 570nm wavelength to visualize the rhodamine, indicating the presence of NP. The distribution of Fluorogold labelled cells was drawn on acetate sheets overlaying the photographs. The acetate sheets were then overlayed on the photographs of the NP and the distribution of NP-containing cells was drawn. Those cells appearing to contain Fluorogold and rhodamine were confirmed as double labelled cells on the microscope using epifluorescence.

Using adjacent thionin stained sections as reference sections the distribution and number of Fluorogold labelled, NP-containing and double labelled cells within the PVN subnuclei were determined.

RESULTS

Three males were successfully injected with Fluorogold into the lumbosacral spinal cord. Only those males in which the center of the injection site was within the rostro-caudal extent of the SNB (Figure 7) were used for mapping and quantification. A subset of the neurons in the PVN that were retrogradely labelled with Fluorogold

Figure 7. Schematic drawings of longitudinal sections through the lumbosacral spinal cord showing the location of the Fluorogold injection site in the region of the SNB in individual animals (NPFG-5, 6 & 16) that were subsequently analyzed for neurophysin immunoreactivity in PVN. The dark stippled area represents the center of the injection site where the Fluorogold was most intense. The lighter stippled area represents the spread of the Fluorogold. The black dots represent the location of SNB motoneurons.

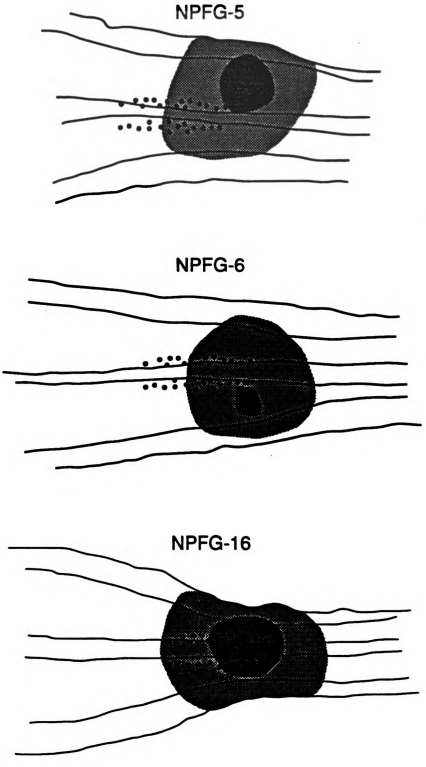

Figure 7

following an injection into the lumbosacral spinal cord also contained NP (Figure 8). The distribution of Fluorogold labelled, NP-containing and double-labelled cells within the PVN (Animal # NPFG-5) is shown in Figure 9. The distribution of the Fluorogold labelled cells was similar to that found in Experiment 1. The distribution of NP-immunoreactivity was similar to other reports (Armstrong et al, 1980) although the number of labelled cells is probably underestimated using the rhodamine-tagged avidin technique. This will be discussed below.

The quantification of double labelled cells within the PVN is summarized in Table 2. Approximately 17% of all Fluorogold labelled cells in the PVN also contained rhodamine. Almost 70% of these double labelled cells were located in the lateral parvocellular subnucleus (lp or pvpo). Almost 30% of the Fluorogold-labelled cells in lp also contained NP. The raw data for the quantification of double labelled cells are found in Appendix B.

DISCUSSION

The results of Experiment 2 demonstrate that approximately 20% of the neurons in PVN that project to lower lumbar levels of spinal cord contain NP, the coproduct of OXY and VP. The majority of the NP-containing neurons projecting to L5-L6 were found in the lp subnucleus.

These results are similar to findings from experiments examining projections from PVN to cervical and thoracic levels of spinal cord, in which it was found that NP and OXY were contained in these projections. Approximately 20-25% of PVN

Figure 8. A) Fluorogold labelled neurons in the lateral parvocellular subnucleus of PVN following an injection of Fluorogold into the lower lumbar spinal cord. **B)** Neurophysin-immunoreactive neurons in the lateral parvocellular subnucleus. Filled arrows indicate neurons that contain Fluorogold and neurophysin-immunoreactivity (NP-ir). Open arrows indicate neurons that contain either Fluorogold (A) or NP-ir (B) but not both. Blood vessels (v) are marked for reference. Bar = 25μm.

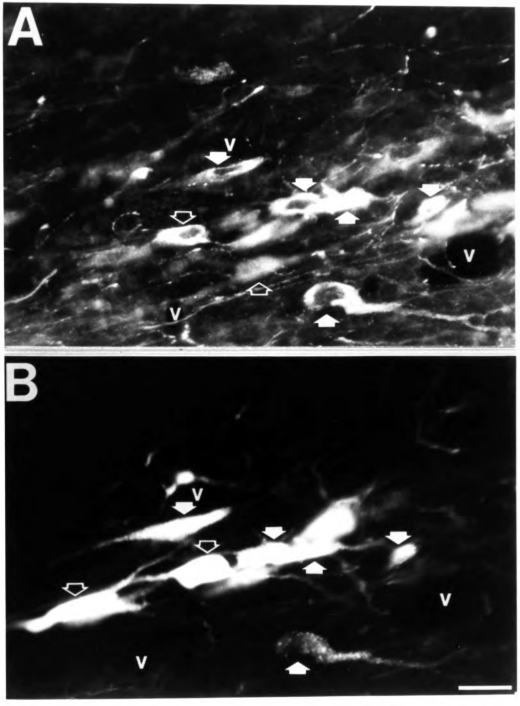

Figure 8

Figure 9. Camera lucida drawings of sections through the PVN showing the location of subnuclei (**LEFT**) and the distribution of Fluorogold-labelled cells (**SQUARES**) following an injection of Fluorogold into the lower lumbar spinal cord, neurophysin-containing cells (**CIRCLES**) or double-labelled cells (**TRIANGLES**) within these subnuclei (**LEFT**) from anterior (**A**) to posterior (**C**). The gray stippling indicates areas in which neurophysin labelling was extremely heavy. See List of Abbreviations.

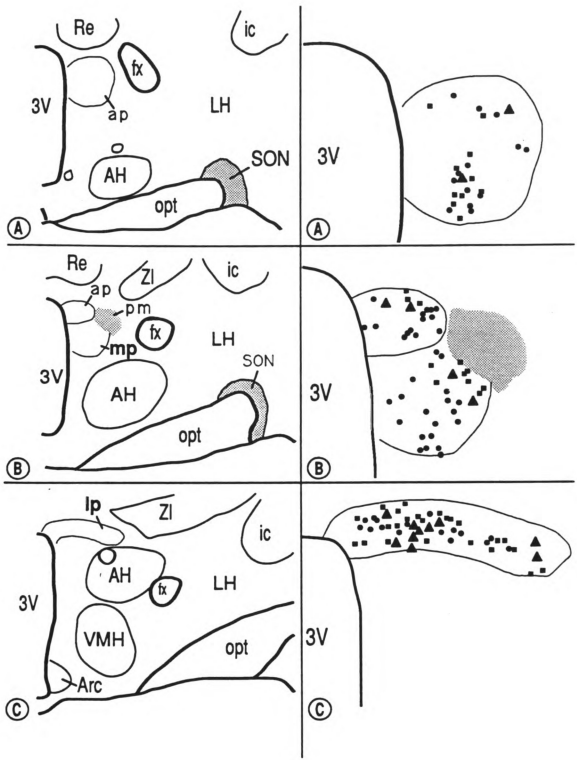
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Figure 9

Table 2

The Mean (s.e.m.) Percent of Paraventricular Nucleus Neurons that Project to the Lower Lumbar Spinal Cord and Contain Neurophysin Immunoreactivity.

% OF ALL FG CELLS CONTAINING NEUROPHYSIN	% DOUBLE LABELLED THAT ARE IN EACH SUBNUCLEUS*					% OF FG CELLS IN EACH SUBNUCLEUS* THAT CONTAIN NEUROPHYSIN				
	ap	dp	mp	pm	lp	ap	dp	mp	pm	lp
17.0 (3.6)	1.3 (0.9)	15.7 (2.1)	6.7 (1.6)	8.9 (2.3)	67.5 (3.0)	4.9 (2.5)	10.4 (2.7)	7.6 (1.5)	15.9 (3.0)	27.7 (6.3)

* ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; pm=posterior magnocellular; lp=lateral parvocellular

neurons that project to thoracic levels contain OXY and 25-35% contain VP (Cechetto and Saper, 1988).

The distribution of Fluorogold labelled cells in the present experiment was similar to that seen in Experiment 1. The distribution of NP-immunoreactivity within the PVN was similar to that described previously (Armstrong et al, 1980; Cechetto and Saper, 1988), however the number of NP-containing cells may have been underestimated in the present experiment. Immunohistochemistry for NP using the rhodamine-tagged avidin detection method may result in fewer labelled cells because an amplification step is lacking in this method as compared to the DAB method. This may reduce the amount of "signal" per cell. This, in addition to the possibility that the detectability of the fluorescent rhodamine may be less than that of the dark brown DAB reaction product, may result in the underestimation of NP cells as compared with studies using DAB. However, the result of 20% of neurons that project to lumbar levels contain NP is similar to the 20-35% of neurons projecting to thoracic levels containing OXY or VP found by Cechetto and Saper (1988) using double fluorescent labelling techniques.

The projection from PVN to lower lumbar spinal cord contains NP, but undoubtedly contains other peptides as well. Approximately 10% of the PVN neurons that project to thoracic levels of spinal cord contain enkephalin (Cechetto and Saper, 1988) and substance P is found in the parvocellular regions of PVN (Ljungdahl et al, 1978). Both met-enkephalin and substance P are also found in fibers and terminals in the region of the SNB (Micevych et al, 1986).

EXPERIMENT 3: NEUROPHYSIN-CONTAINING FIBERS IN THE LOWER LUMBAR SPINAL CORD

EXPERIMENT 3A: IDENTIFICATION OF NEUROPHYSIN-CONTAINING FIBERS AND PUTATIVE TERMINALS IN THE SNB OF MALES

NP- and OXY-immunoreactive fibers have been found in all levels of spinal cord, including lumbar levels. These fibers travel through the lateral funiculus and are found in the marginal zone (lamina I), in lamina X dorsal to the central canal and in the intermediolateral cell column (Swanson and McKellar, 1979). The density of NP-I (OXY-associated) fibers in the ventral horn is substantially greater in lumbar segments than in cervical, thoracic or sacral levels. This is consistent with findings demonstrating that OXY levels in lumbar cord, as measured with radioimmunoassay, are 152% of that in thoracic cord and 274% of cervical cord levels (Valiquette et al, 1985).

PVN appears to play a role in the function of the SNB/DLN motor nuclei. Because there is a NP-containing projection from the PVN to lower lumbar spinal cord and the OXY/NP innervation of the ventral horn at lumbar levels is relatively dense, the present study examined the hypothesis that the PVN sends NP-containing projections directly to SNB/DLN motoneurons. This hypothesis was tested by examining the distribution of NP-immunoreactive fibers in the lumbosacral spinal cord of the rat.

METHODS

PART 1: Neurophysin in the Lower Lumbar Spinal Cord of Males

Tissue Preparation and Histology

Five males were over-anesthetized and perfused intracardially with approximately 100ml of 0.87% saline (4°C) followed by approximately 300ml 10% formaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. Spinal cords were removed, postfixed overnight at 4°C and then cryoprotected overnight in 20% sucrose in 0.1M phosphate buffer (pH 7.4) at 4°C. Cords were sectioned at 30μm on a freezing microtome in the coronal plane (n=4) or the longitudinal plane (n=1) and were collected into 0.87% saline in 0.05M tris buffer (pH 7.4) at 4°C.

Immunohistochemistry

Sections were then processed for immunohistochemistry using antisera against NP using the ABC/DAB detection method according to the protocol in Appendix A. Following immunohistochemistry, sections were mounted onto gelatin coated slides, allowed to air dry and counterstained with thionin. Sections were viewed, drawn and photographed under both brightfield and darkfield illumination. Anatomy of the lower lumbar spinal cord was determined according to Molander et al (1984).

PART 2: Neurophysin Fibers on Retrogradely Identified Motoneurons

Muscle Injections

Two males were anesthetized and the bulbocavernosus muscles were exposed. Bilateral injections of 1.0μl of 4% Fluorogold were made using a Hamilton syringe with an attached glass micropipette (inner diameter 30-40μm). Each injection was

made in several smaller injections in various parts of the BC muscle to maximize the number of terminals exposed to the Fluorogold. Following injection, the incision was closed and the animals were allowed to recover.

Tissue Preparation and Histology

Following a survival time of 1 week animals were perfused, as in Part 1. Spinal cords were removed, postfixed and cryoprotected as in Part 1. Cords were sectioned on a freezing microtome at 30μm in the coronal plane into 0.87% saline in 0.05M tris buffer (pH 7.4) at 4°C.

Immunohistochemistry

Sections were then processed for immunohistochemistry using antisera against NP using the rhodamine-tagged avidin detection method according to the protocol in Appendix A. Following immunohistochemistry, sections were mounted onto gelatin coated slides, allowed to air dry and then dehydrated in 100% ethanol for 1min, cleared in xylene for 2min and then coverslipped with DPX mountant. Sections were viewed and photographed using epifluorescence.

RESULTS

PART 1: Neurophysin in the Lower Lumbar Spinal Cord of Males

Fibers containing NP were found in several regions of the lower lumbar spinal cord of the male (Figure 10). Fibers were seen in the region dorsal to the central canal in laminas X and IV. Many fibers and putative terminals were found in the region of the intermediolateral cell column and fibers cut in cross-section could be

Figure 10. Camera lucida drawings showing the distribution of neurophysin-containing fibers in coronal sections through the lumbosacral spinal cord of a male rat from most caudal (**A**) to most rostral (**E**). Neurophysin fibers in the ventral horn in the region of the SNB are shown in (**C**).

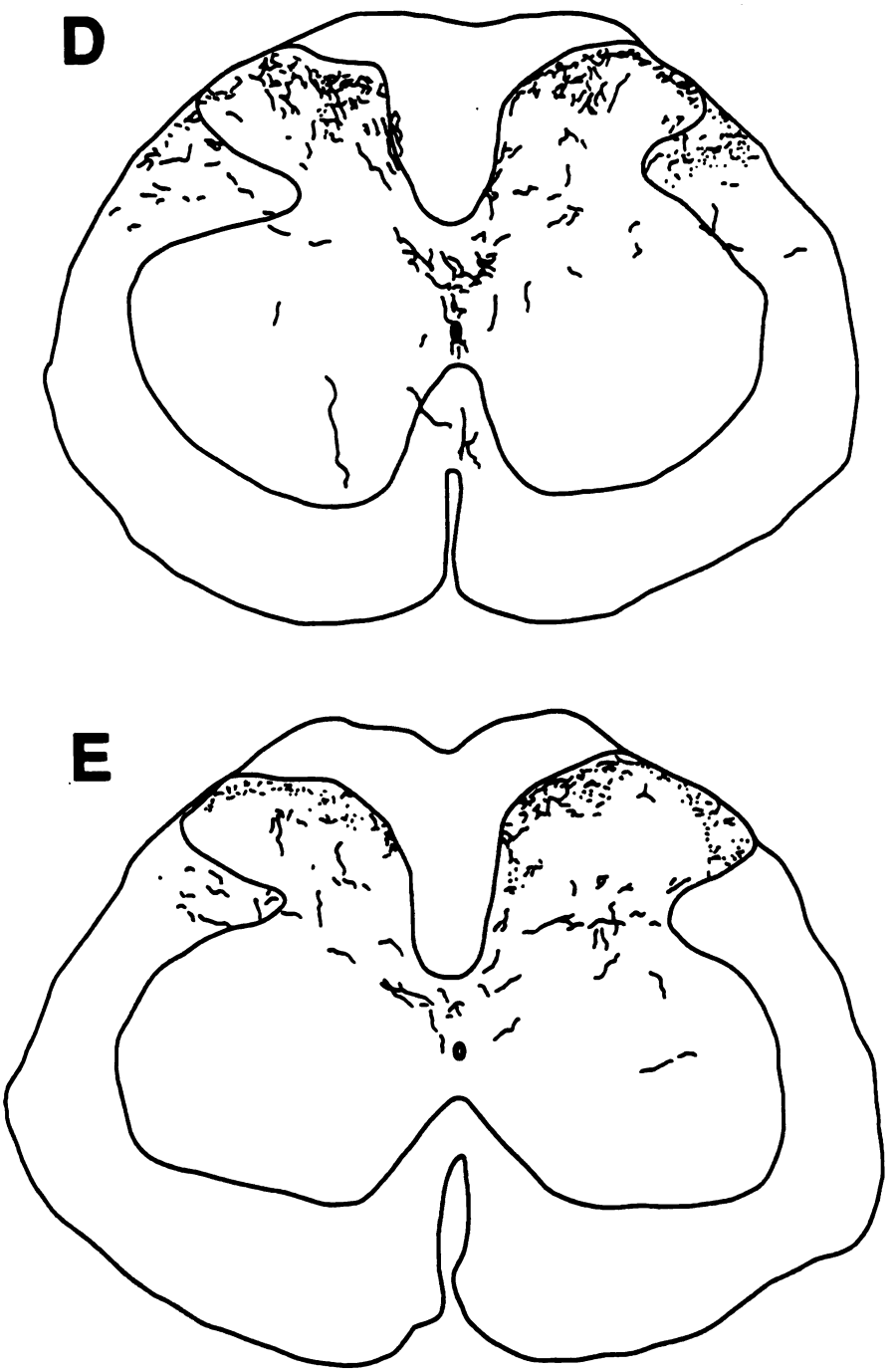

Figure 10 (cont'd)

seen in the lateral funiculus. In addition, quite a few fibers were seen in the apex of the dorsal horn in laminas II and III.

Rarely were fibers found in the ventral horn, with the exception of the region of the SNB (Figures 10C & 11). Many fibers, appearing to enter the ventral horn by running adjacent to the central canal, were found among the motoneuron cell bodies of the SNB (Figure 11). The fibers in this region of the ventral horn were located only within the rostro-caudal extent of the SNB motor nucleus (Figure 12), suggesting that these fibers play a role specific to the functioning of this nucleus. Fibers were rarely found in the region of the DLN, although occasionally a few short fibers were found in this nucleus and appeared to contact motoneurons in this nucleus. In contrast to what was predicted in Experiment 1, few fibers were found in the region of the dendritic bundles that run between the SNB and DLN (See Figure 2).

NP fibers and putative terminals surrounded and appeared to contact SNB motoneuron cell bodies and proximal dendrites (Figure 13). Many of these terminals were *en passant* terminals on fibers running in contact with the perimeter of the cell body. Occasionally, a proximal dendrite appeared to be encircled by NP fibers and terminals. In general, it appeared that some motoneurons within the SNB were heavily covered with NP fibers while others received almost no contact.

Interestingly, NP fibers within the region of the SNB occasionally formed balls or "swirls" of terminals (Figure 14). Although it cannot be determined at the light level, these swirls may be surrounding a process of some kind. This may be what is seen in cross-section in Figure 13B. Another interesting finding was that NP fibers

Figure 11. Neurophysin-containing fibers in the region of the SNB under darkfield illumination. cc = central canal. Bar = 100μm.

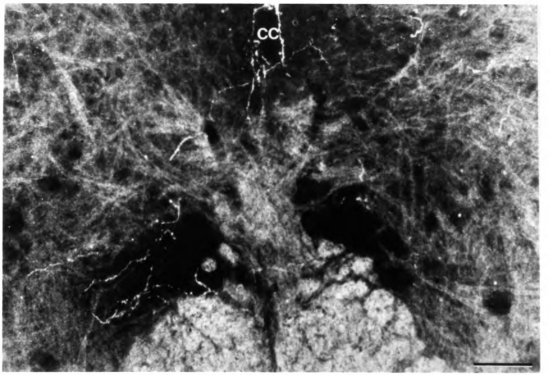

Figure 11

Figure 12. Camera lucida drawing showing the distribution of neurophysin-containing fibers in a longitudinal section through the lumbosacral spinal cord of a male rat demonstrating that neurophysin-containing fibers in the ventral horn are located specifically within the rostro-caudal extent of the spinal nucleus of the bulbocavernosus (SNB). Black dots represent the location of motoneurons.

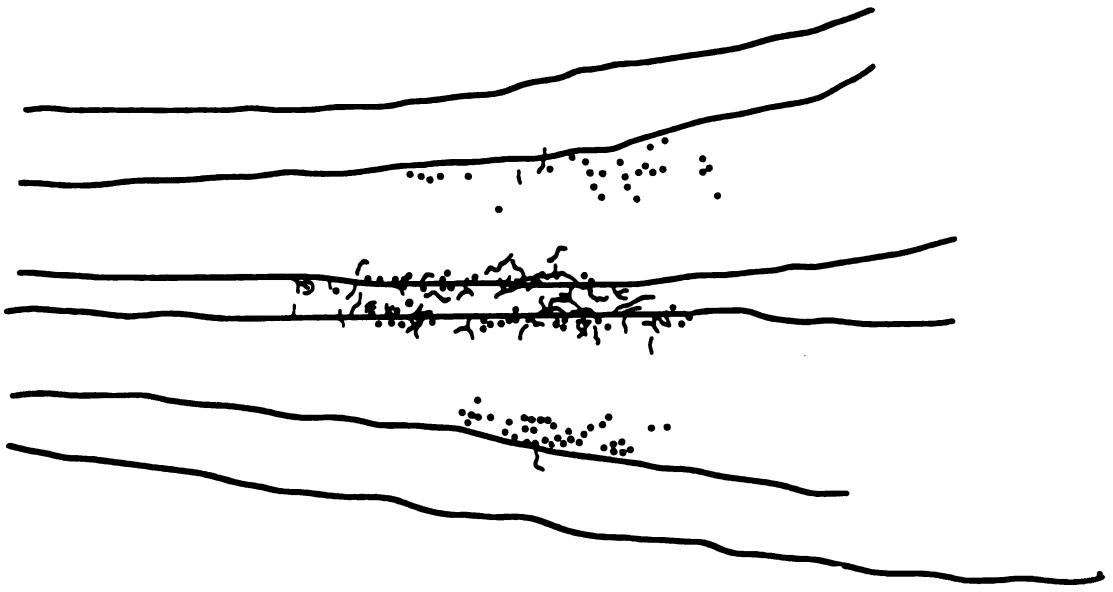

Figure 12

Figure 13. A) Neurophysin containing fibers and putative terminals (arrows) that appear to contact the soma of an SNB motoneuron. Bar = 10μm. **B)** The proximal dendrite of an SNB motoneuron that appears to be contacted by many neurophysin-containing boutons. Bar = 10μm.

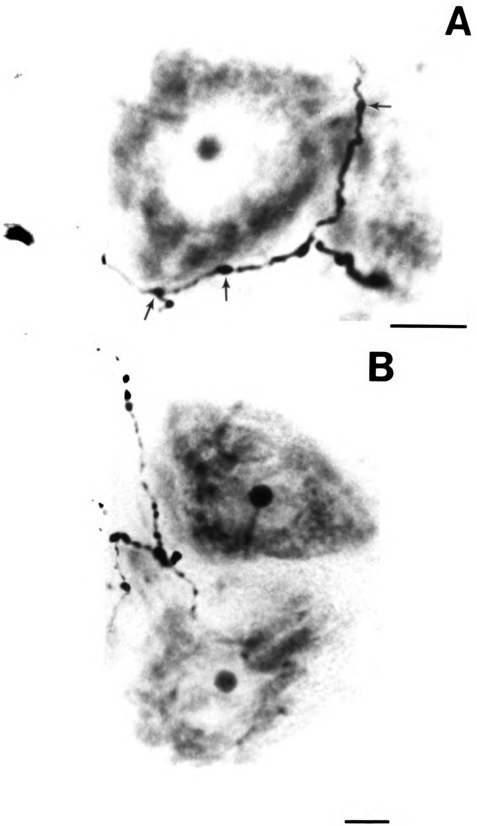

Figure 13

Figure 14. A) Neurophysin-containing fibers and putative terminals within the region of the SNB were occasionally found forming a circular "knot" configuration **B)** Neurophysin containing fibers were found adjacent to the central canal. Fibers terminated in swellings that contacted the ependymal cells of the central canal. Bar = 10μm.

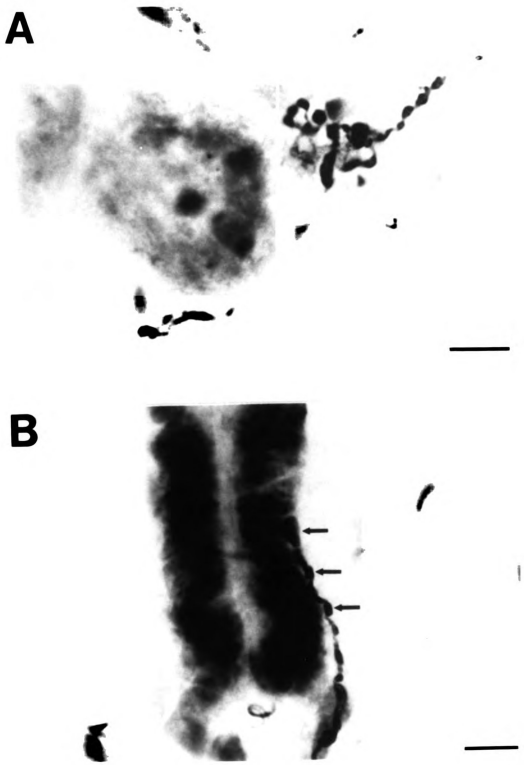

Figure 14

appeared to make *en passant* contacts and to terminate in a bouton swelling onto ependymal cells of the central canal (Figure 14).

PART 2: Neurophysin Fibers on Retrogradely Identified Motoneurons

Because the SNB motoneurons in thionin stained tissue could be either motoneurons innervating the bulbocavernosus muscle or the anal sphincter, it was not clear from the previous experiment if NP fibers specifically contacted motoneurons involved in the control of penile reflexes. Following retrograde filling of SNB motoneurons innervating the BC muscle, it was found that indeed NP fibers approached and appeared to contact the cell bodies (not shown) and the dendrites (Figure 15) of these motoneurons. NP fibers were also seen contacting motoneurons that were not labelled with Fluorogold.

In summary, fibers and terminals containing NP-immunoreactivity appeared to contact the soma and proximal dendrites of SNB motoneurons, and specifically, those SNB motoneurons innervating the BC muscle.

Figure 15. A double exposure photomicrograph using epifluorescence illumination showing a Fluorogold labelled SNB motoneuron **(BLUE)** following an injection of Fluorogold into the bulbocavernosus muscle and neurophysin-containing fibers **(RED)** indicated by the presence of rhodamine. Neurophysin-containing fibers approached and appeared to contact the dendrites of this motoneuron. Bar = 50μm.

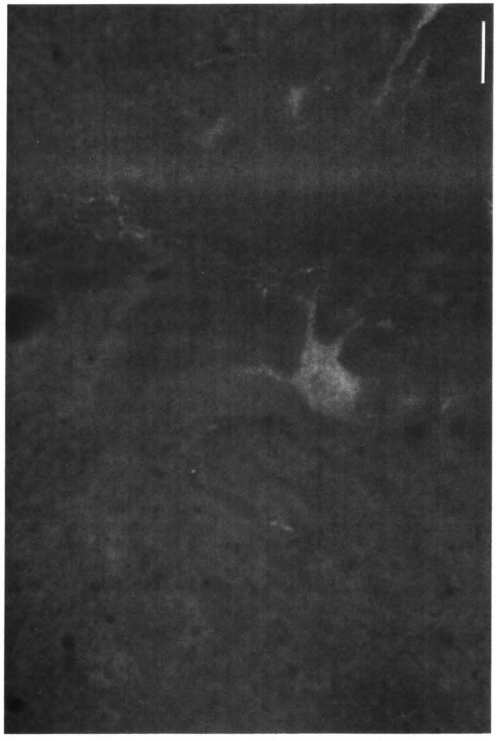

Figure 15

EXPERIMENT 3B: SEXUAL DIMORPHISM OF AND THE EFFECT OF CASTRATION ON NEUROPHYSIN FIBERS IN THE LOWER LUMBAR SPINAL CORD

Levels L5-L6 of the spinal cord of the rat contain the sexually dimorphic motor nuclei the SNB and DLN (Breedlove and Arnold, 1981), as well as the sensory input from the pudendal nerve which also exhibits a sex difference (McKenna and Nadelhaft, 1986). In order to determine if the general distribution of NP in the lower lumbar spinal cord or the NP in the region of the SNB was sexually dimorphic, Experiment 3B, Part 1 compared the location and distribution of NP fibers in the lower lumbar cord of males and females.

The synaptic input onto SNB motoneuron cell bodies and proximal dendrites is dramatically decreased following castration (Leedy et al, 1987; Matsumoto et al, 1988b) and this synaptic coverage can be restored to intact levels by exposure to testosterone for as short as 48 hours (Leedy et al, 1987). Putative terminals containing NP appeared to contact the soma and proximal dendrites of SNB motoneurons (Expt 3A). Experiment 3B, Part 2 examined whether castration affected the density of NP fibers in the region of the SNB.

METHODS

PART 1: Sexual Dimorphism

Four females and four males were over-anesthetized and perfused as in Experiment 3A. Spinal cords were removed and postfixed and cryoprotected as in Experiment 3A. Spinal cords were sectioned on a freezing microtome at 30μm in the coronal plane and processed for immunohistochemistry using antisera against NP according to the protocol in Appendix A. Following immunohistochemistry sections were mounted onto gelatin coated slides, allowed to air dry and counterstained with thionin.

The distribution of NP fibers from several levels of the lower lumbar spinal cord were documented by camera lucida drawings and compared in a qualitative manner between males and females.

PART 2: Effects of Castration

Twenty males were anesthetized and either castrated (n=10) or sham-castrated (n=10). Following a 4 week survival period, animals were perfused and spinal cords prepared for immunohistochemistry as in Experiment 3A. Spinal cords were sectioned on a freezing microtome at 30μm in the coronal plane. Alternate sections were processed for immunohistochemistry using antisera against NP using the ABC/DAB detection method (Appendix A). Following immunohistochemistry, sections were mounted onto gelatin coated slides, allowed to air dry and then counterstained with thionin.

Quantification

Under darkfield illumination, NP fibers in the region of the SNB in sections containing SNB motoneurons were drawn at 10X using camera lucida. The region of the SNB was defined as extending dorsally to the dorsal edge of the central canal, ventrally to the most dorsal limit of the ventral motor pool and bilaterally to approximately the middle of each ventral horn.

The length of the fibers was measured using a Bioquant image analysis system. To control for a differing number of sections per animal due to loss of sections during immunohistochemistry, the measurements were calculated as a mean per 30μm section. The number of fibers per 30μm section, the average length of each fiber and the total length of fibers per 30μm section were calculated. Measurement from castrates and sham-castrates were statistically compared using the Student t test ($p<0.05$).

RESULTS

PART 1: Sexual Dimorphism

The distribution of NP fibers in the lower lumbar spinal cord of the female is shown in Figure 16. In general, the distribution is very similar to that seen in the male (Figure 10). However, there did appear to be some degree of sexual dimorphism, although it was subtle. There appeared to be a greater density of fibers in the region dorsal to the central canal (lamina X) in the female compared to the male (compare Figure 16B with Figure 10C). In contrast, there seemed to a smaller number of fibers in the apex of the dorsal horn (laminas II and III) in the female

Figure 16. Camera lucida drawings showing the distribution of neurophysin-containing fibers in coronal sections through the lumbosacral spinal cord of a female rat from most caudal (**A**) to most rostral (**C**).

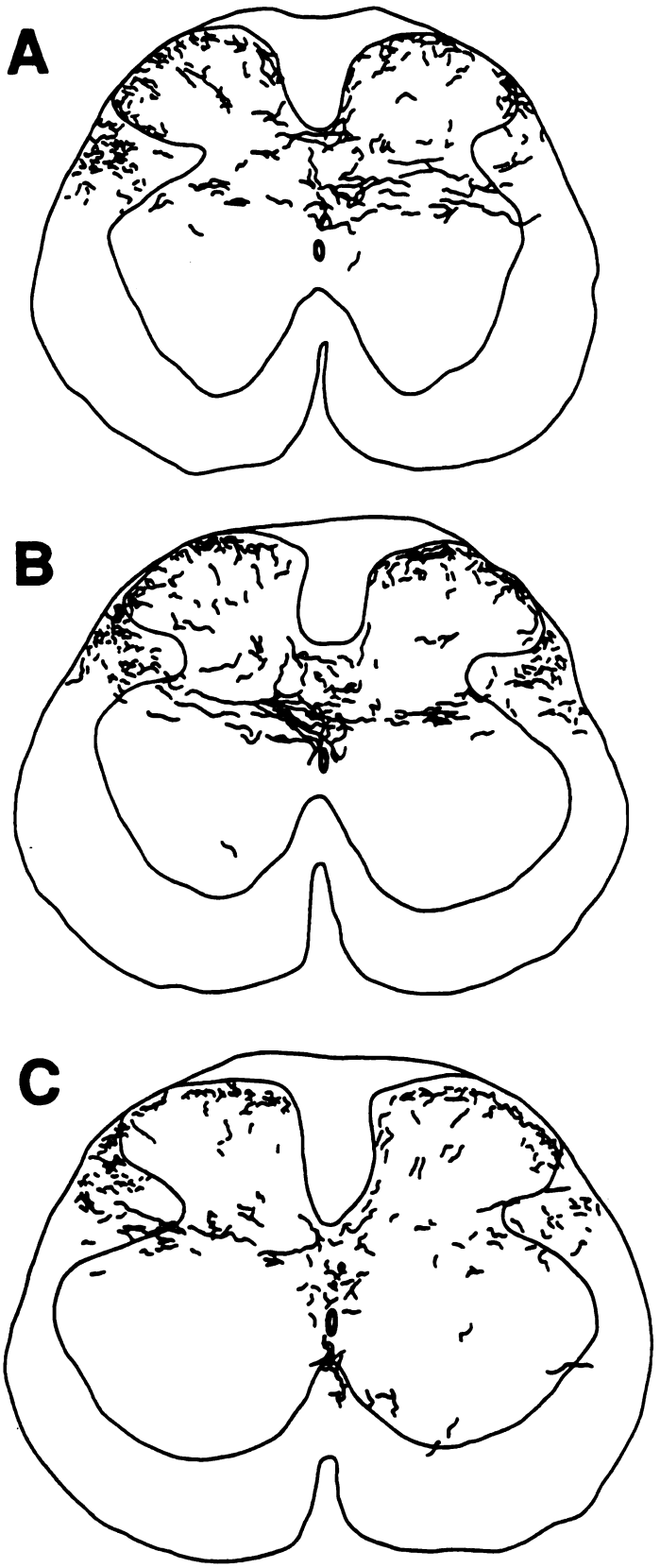

Figure 16

compared to the male (compare Figure 16B,C with Figure 10C). In the female, NP fibers were also found in the ventral horn in a similar region to that seen in the male. However, there was a slightly smaller number of fibers seen in this region in the female, the fibers were located more on the midline in the female, whereas in the male these fibers were distributed more laterally (compare Figure 16C with Figure 10C), and these fibers were in fewer and more rostral sections in the female than in the male.

PART 2: Effects of Castration

A summary of the quantification of NP fibers in the region of the SNB in castrated and sham-castrated males can be found in Table 3. The raw data from each animal is shown in Appendix B. The total length of NP fibers per 30μm section in the region of the SNB was not affected by castration in the male (Table 3). The average length of NP fibers in the region of the SNB was also not significantly altered by castration. The number of NP fibers per 30μm section in castrates and intacts was not statistically different.

DISCUSSION

Results from Experiment 3 demonstrate that NP-containing fibers and terminals are located in the region of the SNB motor nucleus. NP fibers and terminals appear to contact the cell body and proximal dendrites of motoneurons within the SNB and contact specifically those neurons innervating the BC muscle. NP fibers are also found in the lateral funiculus, in the region of the IML, in the apex

Table 3

The Mean (s.e.m.) Number of NP-Immunoreactive Fibers, Average Fiber Length and Total Fiber Length in the Region of the SNB in Castrated and Sham-Castrated Males.

	# OF FIBERS PER 30μm SECTION	AVERAGE FIBER LENGTH (μm)	TOTAL LENGTH PER 30μm SECTION (μm)
SHAM-CASTRATES	8.62 (0.9)	59.2 (1.7)	508.9 (54.4)
CASTRATES	12.24 (2.1)	63.5 (2.7)	737.7 (106.0)

of the dorsal horn (laminas II and III) and in the region dorsal to the central canal (lamina X).

There was only a subtle sexual dimorphism in the distribution of NP fibers in the lower lumbar spinal cord, with females having more fibers in lamina X, dorsal to the central canal and the fibers in the ventral horn being more medial and more rostral in females. Castration did not significantly alter the total length or the number of NP fibers in the region of the SNB of males.

The distribution of NP in the lower lumbar spinal cord of the male is similar to that of other reports describing NP in both cervical and thoracic levels of spinal cord as well as lumbar levels (Swanson and McKellar, 1979). In addition, the general location of NP fibers found in this study is consistent with the distribution of anterogradely labelled fibers following PHA-L injections into PVN (Luiten et al, 1985). The major finding of the present experiment, which expands upon previous studies, is the demonstration of direct NP input, presumably from PVN, onto spinal motoneurons. NP fibers, as well as being found in the region of the SNB, are also located in other sexually dimorphic areas, such as terminal areas for the sensory branch of the pudendal nerve (McKenna and Nadelhaft, 1986).

In contrast to the prediction in Experiment 1, very few NP fibers were found in the region of the DLN. Similarly, not many fibers were found in the region of the dendritic bundles of the SNB and DLN. Dendrites from these nuclei form elaborate and large bundles that run between the SNB and DLN (Schroder, 1980; McKenna and Nadelhaft, 1986) and it is thought that these dendritic bundles have a role in the integration of function between cells of these nuclei (Rooney et al, 1979; Rose and

Collins, 1985). Ultrastructural evidence has demonstrated that terminals are found within these bundles, each terminal contacting several dendrites (Anderson et al, 1976). Because the dendritic bundles seem like an ideal place for modulatory input and peptides are thought to act as neuromodulators, the hypothesis that NP fibers and terminals would be found within these bundles was reasonable. However, NP was found mostly on cell bodies and proximal dendrites.

It has been suggested that input onto motoneurons can be characterized by its relation to the cell body and the axon hillock (Rall et al, 1967), a result of the fact that the membrane resistance of motoneurons is spatially nonuniform (Dodge, 1979). The input of inhibitory interneurons onto motoneurons is often located on or near the cell soma (Burke et al, 1971). Because NP terminals were found on the motoneuron soma and proximal dendrites, it is possible that OXY and/or VP input onto SNB motoneurons may be inhibitory to SNB function, although no direct evidence for OXY or VP acting as inhibitory neurotransmitters exists at this time.

The finding that both putative *en passant* boutons as well as terminal boutons appear to contact the ependymal cells of the central canal is consistent with the idea that these terminals may release NP and OXY or VP into the CSF. The level of OXY in the CSF of the male rat triples following ejaculation and the source of this OXY is the PVN (Hughes et al, 1987). Another possibility is that NP/OXY/VP might alter the permeability of the CNS/CSF barrier, thereby increasing or decreasing the exposure of the spinal cord to the CSF.

The NP terminals found in swirl patterns within the region of the SNB may surround a dendrite or a small bundle of dendrites proximal to an SNB motoneuron

cell body. Alternatively, these terminals may encircle an axon terminal of other afferent input to the SNB as an additional way to modulate SNB function.

The fact that NP terminals were found directly contacting motoneurons that innervate the BC muscle, strongly suggests that OXY and/or VP play a role in the modulation of penile reflexes by directly acting on the motoneurons that innervate the muscles responsible for these reflexes. OXY and apomorphine appear to modulate penile responses at the level of the hypothalamus and the PVN seems to be an important site in this modulation (Argiolas et al, 1986; 1987a; Melis et al, 1986). If indeed these findings indicate that these neurotransmitters are altering SNB/DLN function, they may be doing so in part, through the oxytocinergic and/or vasopressinergic innervation of the SNB, presumably originating in the PVN.

NP fibers also appeared to contact SNB motoneurons that were not retrogradely labelled, which could indicate that NP fibers also innervate anal sphincter motoneurons. The PVN has been shown to project to the nucleus of Onuf in the cat, which contains the motoneurons innervating the anal sphincter in this species, (Holstege, 1987). However, even after a long survival time, Fluorogold injected into the BC muscle did not fill many SNB motoneurons. This may be because of the small diffusion of Fluorogold away from the site of injection, thereby resulting in a small injection site and not many filled motoneurons, or it may be that Fluorogold is not readily taken up by peripheral nerve terminals. In either case, the motoneurons not retrogradely filled, yet contacted by NP fibers, may be anal sphincter motoneurons or may be BC muscle motoneurons that did not fill with retrograde tracer.

It is very important to keep in mind that it is impossible to determine at the light level whether or not these putative NP terminals are indeed making chemical synapses with SNB motoneurons. Although terminal boutons and *en passant* bouton swellings are thought to be presynaptic structures, this needs to be verified at the ultrastructural level.

The sex difference that existed in the distribution of NP in the lower lumbar spinal cord was very subtle. NP fibers were slightly more dense in the area dorsal to the central canal (lamina X). The significance of these additional fibers in the female is not known but may be involved in female reproductive functions. Fibers ventral to the central canal in the female did not appear to be in regions where motoneurons are located, but rather appeared to run directly into the ventral funiculus. These fibers did not appear to innervate motoneurons of the SNB in females, which innervate the anal sphincter. This suggests that NP fibers in the region of the SNB in the male may be specific to BC motoneurons.

Measures of NP fibers in the region of the SNB were not statistically different between intact males and castrate males. Although the means of these groups were quite distinct, the variability between animals within a group was quite high, especially in the castrate group (see Appendix B). With this taken into consideration, it may be important to note that there was a trend suggesting that castrated males had more NP fibers in the SNB. This is noteworthy since the trend is in the opposite direction from what might be predicted based on the results of Matsumoto et al (1988b) and Leedy et al, (1987), demonstrating that the synaptic input onto SNB motoneurons decreases with castration. The results of the present experiment may

reflect a case where lack of statistical significance may not indicate the absence of biological significance. Tissue from intact and castrated males could readily be discerned through the microscope by an experimenter blind to the treatment group, by the higher density of fibers in the region of the SNB in many sections.

If indeed these measures are functionally relevant, an increase in fiber number and fiber length as detected with NP-immunohistochemistry, could indicate several things. An increase in NP fibers could result from a lack of release by the terminals of these fibers. NP is released along with OXY and VP in the pituitary (Johnston et al, 1975). If cell activity is low, release may decrease, but axonal transport may continue at the same rate. This might result in a "back-up" of NP within the axon, resulting in a greater fiber length in the region. This is unlikely in the present experiment, however, since the average length of NP fibers is almost identical in intact males and castrated males. Any increase in total fiber length appears to be the result of an increase in the number of fibers. This phenomenon could also result in an increase in the number of fibers detected if release occurred at the same rate as axonal transport in the intact male making some NP fibers empty and therefore undetectable. A second possibility is that the rate of synthesis has changed. Steroids are known to alter the levels of OXY mRNA in the hypothalamus (Caldwell et al, 1989). The third possibility is that increased NP fiber number and length reflects a hyperinnervation of the SNB in the absence of testosterone. Although this possibility is intriguing, much more study would be required to conclude anything about the regulation of NP in the spinal cord by testosterone.

If castration does indeed result in a functionally significant change in NP input to the SNB, this would again be consistent with the idea of OXY/VP being inhibitory in the SNB. Castration abolishes penile reflexes, the functional output of the SNB motor nucleus (Hart, 1967; 1973; Rodgers and Alheid, 1972; Davidson et al, 1978; Bradshaw et al, 1981). This idea is also consistent with Experiment 3A in which NP fibers were found almost exclusively on cell bodies of SNB motoneurons. Although there are discrepancies in the literature regarding the effects of OXY on the parameters of male reproductive behavior, some studies demonstrate that discrete electrolytic lesions to the lateral and posterior PVN abolished the ejaculation-associated increase in CSF OXY as well as reduced the postejaculatory interval (Hughes et al, 1987), which is presumably an inhibition of copulatory behavior and possibly penile function.

The presence of NP in the region of the SNB most likely reflects the presence of OXY. There are many fewer VP fibers than OXY fibers in spinal cord as detected by immunohistochemistry (Buijs, 1978; Sofroniew, 1983) and VP levels are lower than OXY levels in the spinal cord, as detected by radioimmunoassay (Lang et al, 1983; Hawthorn et al, 1985; Valiquette et al, 1985). Furthermore, there are very few VP-immunoreactive fibers in the region of the sexually dimorphic regions of the lumbar cord (Bruce Newton, personal communication). Although OXY is a good candidate for the neurotransmitter innervating the SNB, the idea that NP itself may be a biologically active peptide should not be immediately dismissed.

It has been demonstrated that NP levels are affected by gonadal steroids. NP levels in the plasma of female rhesus monkeys were elevated about 10 hours

following estrogen treatment and a prolonged elevation was found around the mid menstrual cycle, when estrogen levels were high (Robinson et al, 1976). Levels of NP in the CSF were also elevated following estrogen treatment (Perlow et al, 1982). Although neurophysins are known to be released into the general circulation along with OXY and VP (Johnston et al, 1975) it has been assumed in the past that the neurophysins were inert carrier proteins. However, it has recently been demonstrated that NP-II (VP-associated) can act independently to alter prolactin release in estrogen-primed male rats (Shin et al, 1989). In the case of the SNB, a preliminary study failed to identify OXY binding sites in this region (Thomas Insel, personal communication). Technical problems cannot be ruled out, but binding studies using radiolabelled NP as a ligand should be attempted.

EXPERIMENT 4: EFFECTS OF ELECTROLYTIC LESIONS OF PVN ON NEUROPHYSIN-IMMUNOREACTIVITY IN THE LOWER LUMBAR SPINAL CORD

It is well documented that PVN projects to spinal cord (Kuypers and Maisky, 1975; Conrad and Pfaff, 1976; Hancock, 1976; Saper et al, 1976; Swanson, 1977; Ono et al, 1978; Armstrong et al, 1980; Hosoya, 1980; Swanson and Kuypers, 1980; Schwanzel-Fukuda et al, 1984) and that NP/OXY fibers are found in all levels of spinal cord (Swanson and McKellar, 1979). It is assumed that the source of the NP in the spinal cord is the PVN. This idea has been accepted through the findings of several studies. First, in retrograde tract tracing studies of projections from brain to spinal cord, the PVN was the only nucleus found to project to spinal cord that is NP producing, (Experiment 1 & 2; Cechetto and Saper, 1988; Shen et al, 1990). Furthermore, lesions of the PVN result in a dramatic decrease in OXY levels in all levels of the spinal cord (Lang et al, 1983; Hawthorn et al, 1985). This again, is consistent with the idea that the PVN is the major source of NP/OXY in the spinal cord.

In order to verify that the source of the NP fibers found in the region of the SNB motor nucleus in Experiment 3 is the PVN, electrolytic lesions of the PVN were made and the density of NP-immunoreactive fibers in the lumbosacral spinal cord, particularly in the SNB region, was examined.

METHODS

PVN Lesions

Thirtyfour males were anesthetized and placed in a stereotaxic apparatus. An incision was made through the skin and muscle of the head to expose the skull. Coordinates from bregma for PVN, determined according to the atlas of Paxinos and Watson, were as follows: A-P: -2.1 (n=11) or -2.0 (n=6) or -1.9 (n=6); M-L: $\pm$0.6; D-V: -7.7. Holes were drilled through the skull and bilateral electrolytic lesions were made by passing current (1mA for 20 seconds) through a tungsten electrode, insulated with polyurethane with a 1mm uninsulated tip. The left side was always done first and the electrode was checked between left and right sides by verifying the passage of current through the tip into saline solution. Six males received sham lesions in which the electrode was lowered but no current was passed. Holes in the skull were filled with bone wax, the incision was closed with wound clips and the animals were allowed to recover.

Tissue Preparation and Histology

Following a survival time of 1 week, anesthetized animals were perfused with 0.87% saline at 4°C followed by 10% formaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. Brains and spinal cords were removed, postfixed overnight and cryoprotected overnight in 20% sucrose in 0.1M phosphate buffer (pH 7.4) at 4°C.

The PVN was sectioned at 30μm in the coronal plane on a freezing microtome and collected in 0.87% saline in 0.1M tris buffer (pH 7.4) (TBS) at 4°C. Alternate sections were mounted onto gelatin coated slides, allowed to air dry and then counterstained with thionin. Using these sections the presence or absence of

damage to the PVN was confirmed. Six animals with significant damage to PVN, 3 "misses" which served as controls and 6 sham-lesioned males were used for the remainder of the experiment.

Immunohistochemistry

Spinal cords were sectioned at 30μm in the coronal plane on a freezing microtome and collected into TBS (4°C). Alternate spinal cord sections and the remaining PVN sections were then processed for immunohistochemistry using antisera against NP using the ABC/DAB detection method according to the protocol in Appendix A. Following immunohistochemistry, sections were mounted onto gelatin coated slides, allowed to air dry and then dehydrated in ethanol (70%, 95%, 100%; 5min each), cleared in xylene (15min) and coverslipped.

The location and size of the PVN lesions were documented by camera lucida drawings using thionin stained tissue. The distribution of NP-immunoreactive fibers in the lower lumbar spinal cord was documented by camera lucida drawings using darkfield illumination of unstained tissue.

RESULTS

Electrolytic lesions of the PVN resulted in differing degrees of damage to the nucleus in each animal. The lesion from an animal in which both sides of the PVN were completely destroyed is shown in Figure 17. The location and size of the lesions in all other animals, where partial damage occurred, are shown in Figure 18. Only those animals in which the lateral parvocellular subnucleus was significantly damaged were used in the study because, as demonstrated in Experiment 2, 70% of the

Figure 17. Camera lucida drawings of sections through the PVN of a male rat (Animal LES-25) from anterior (A) to posterior (D). Dark stippled areas represent the region of severe damage resulting from electrolytic lesions. Light stippled areas represent the region surrounding the lesion where gliosis was apparent. See List of Abbreviations.

Figure 18. Camera lucida drawings of sections through the lateral parvocellular subdivision of PVN of individual animals that had received electrolytic lesions. Dark stippled areas represent the region of severe damage. Light stippled areas represent the region of gliosis. The lesions resulted in either bilateral damage to PVN (Animals LES-2, 3 & 30), unilateral damage to PVN (Animals LES-20 & 31) or damage adjacent to PVN (Animals LES-26, 27 & 18). See List of Abbreviations.

neurons in PVN that project to the spinal cord and produce NP are located in lp. Therefore, Figure 18 shows the damage to the lp subnucleus. Some animals received bilateral, yet partial damage to PVN. Other animals, although they had received bilateral lesions, had damage only on the left side, which was always the first side lesioned.

These unilateral lesions may have been the result of a failure of the electrode to pass current on the second side. Although the electrode was examined between the first lesion (left side) and the second lesion (right side), the unilateral lesions that resulted were always on the left side, leaving the right PVN completely intact. The PVN of sham-lesioned animals was completely intact in all cases.

Figure 18 also shows lesions from 3 animals in which cell bodies in PVN subnuclei were completely intact but the lesion was adjacent to PVN, either lateral, dorsal or ventral to PVN. These animals were examined as "controls" to determine if destroyed fibers leaving PVN, while keeping cell bodies intact, was sufficient to eliminate or reduce NP fibers in the lumbosacral spinal cord.

Animals in which lesions destroyed all or part of PVN resulted in the loss of NP cell bodies in PVN as well as a reduction in the density or pattern of NP fibers leaving the PVN. Figure 19 shows the lateral parvocellular subnucleus of PVN of a sham-lesioned animal and a lesioned animal following immunohistochemistry for NP. NP cell bodies in lp are significantly reduced and the NP fibers leaving the lp laterally are disrupted.

The distribution of NP fibers in the lower lumbar spinal cord of the male with complete bilateral destruction of PVN and a sham-lesioned male are shown in Figure

Figure 19. Sections through the lateral parvocellular subdivision of PVN showing neurophysin-immunoreactivity in a sham-lesioned (**A**) or lesioned (**B**) male. 3V = third ventricle. Bar = 200μm.

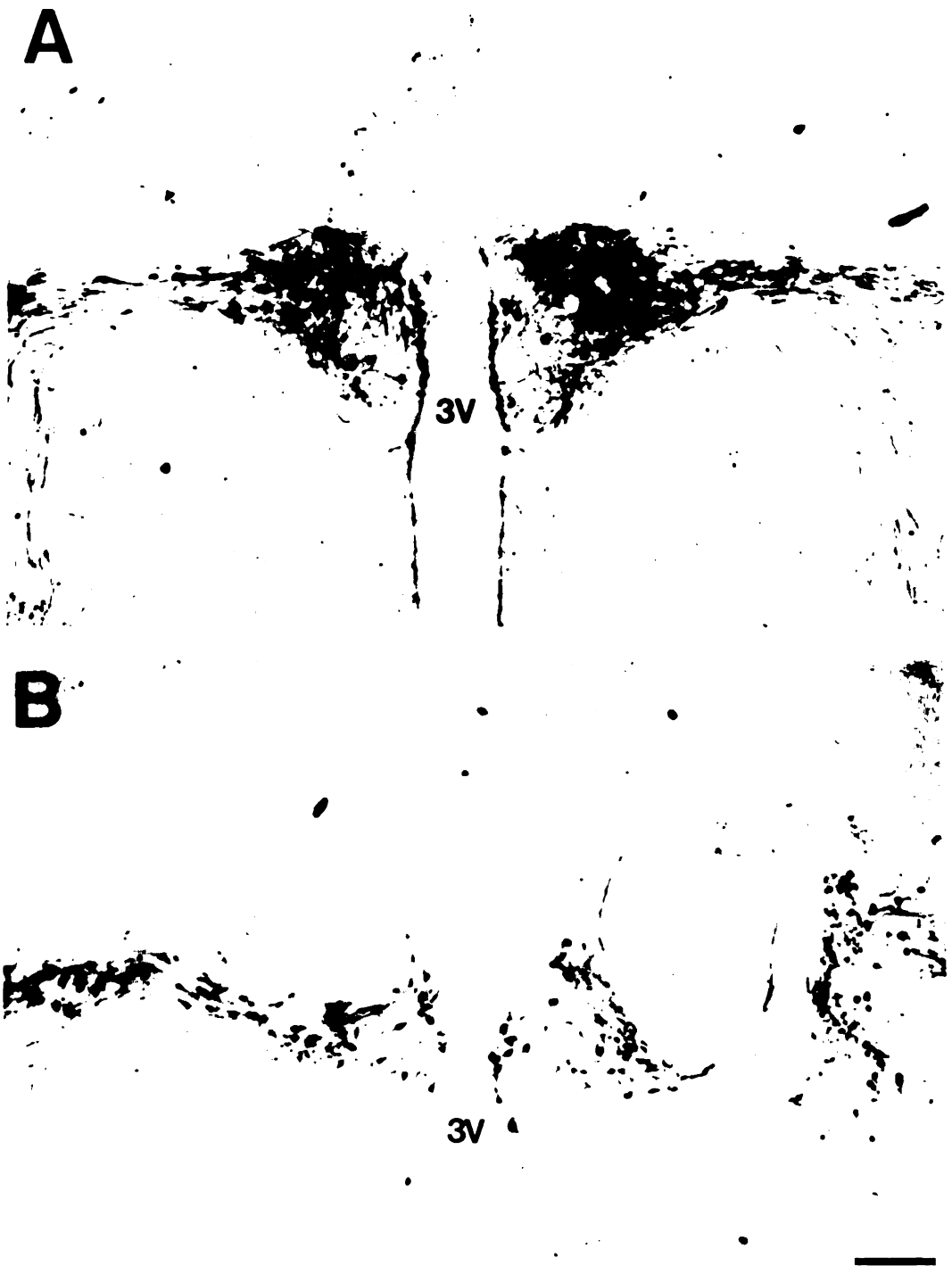

Figure 19

20. Destruction of the PVN resulted in a dramatic reduction in the density of NP innervation in this level of the cord as compared to a sham-lesioned animal. The distribution of NP fibers in a section through the level of the SNB for each of the other lesioned animals is shown in Figure 21. The effect of varying damage to the PVN on the distribution of NP in the dorsal horn and the region of the SNB is summarized in Table 4. In lesions where most of the lp subnucleus was destroyed, NP fibers in the dorsal horn as well as in the region of the SNB were significantly reduced. In the case of a unilateral lesion the NP fibers were reduced in the dorsal horn and IML on the side ipsilateral to the lesion, whereas NP fibers in the region of the SNB were only slightly reduced on the ipsilateral side and the fibers in the region dorsal to the central canal were moderately reduced on both sides. These results suggest differential ipsi- and contralateral innervation depending on the region of the cord.

When a unilateral lesion was lateral to PVN, damaging NP fibers leaving laterally, NP fibers were reduced in both the dorsal horn/IML region as well as the region dorsal to the central canal on the side ipsilateral to the lesion, whereas the contralateral side was only moderately reduced. The fibers in the region of the SNB were moderately reduced on both sides. When a unilateral lesion was dorsal to the PVN, NP fibers in the region dorsal to the central canal or in the region of the SNB remained unaltered on both ipsi- and contralateral sides. However, the NP fibers in the dorsal horn and IML were drastically reduced on the ipsilateral side. When bilateral lesions were ventral to PVN, NP fibers in the dorsal horn and IML were only moderately reduced. In contrast, fibers in the region dorsal to the central canal

Figure 20. The distribution of neurophysin-immunoreactive fibers in the lower lumbar spinal cord at the level of the SNB in **A)** a PVN lesioned male (Animal Les-25) or **B)** a sham-lesioned male. See Figure 17 for lesion site.

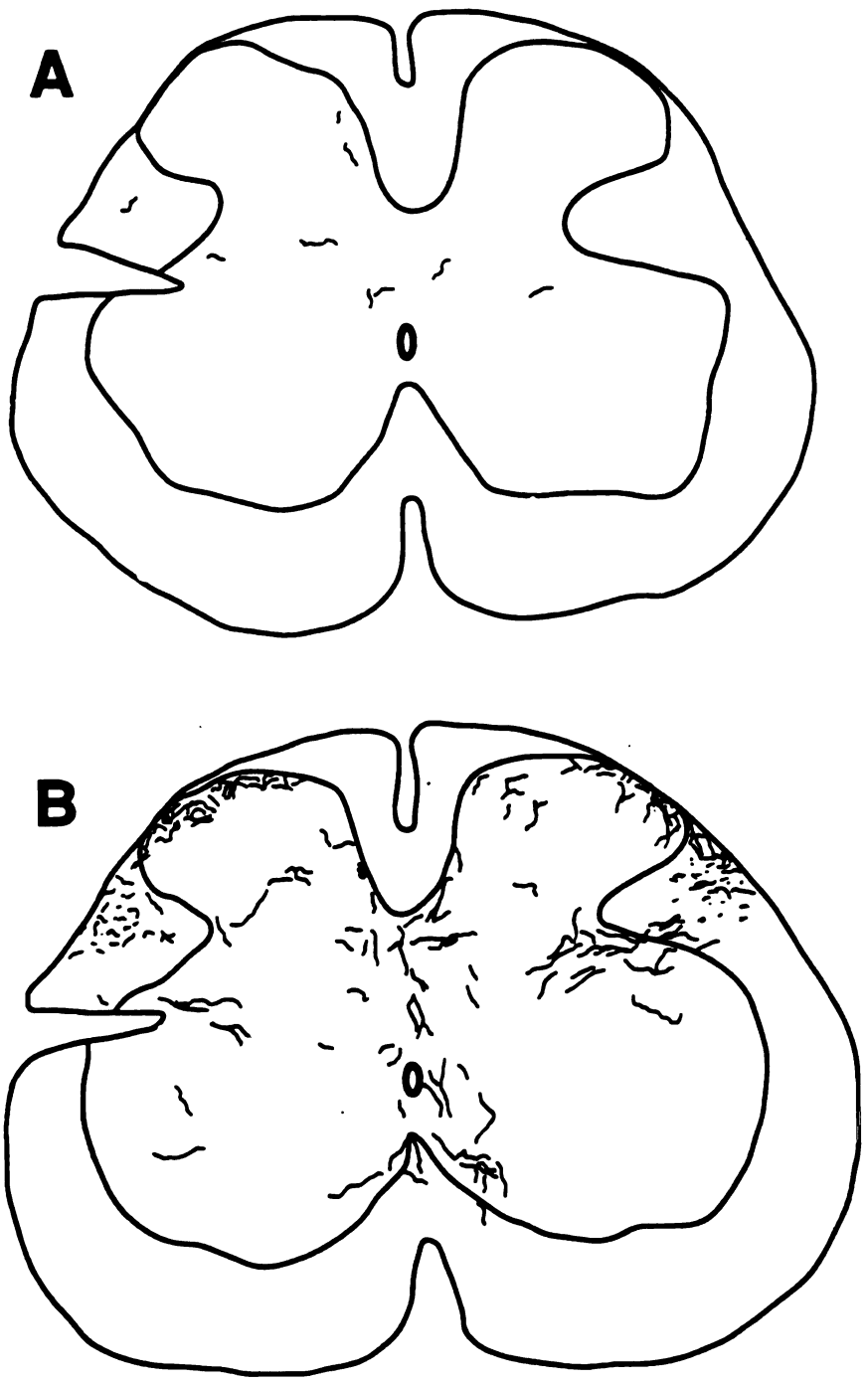

Figure 20

Figure 21. The distribution of neurophysin immunoreactive fibers in the lower lumbar spinal cord in individual animals that had received electrolytic lesions of PVN. See Figure 18 for lesion sites.

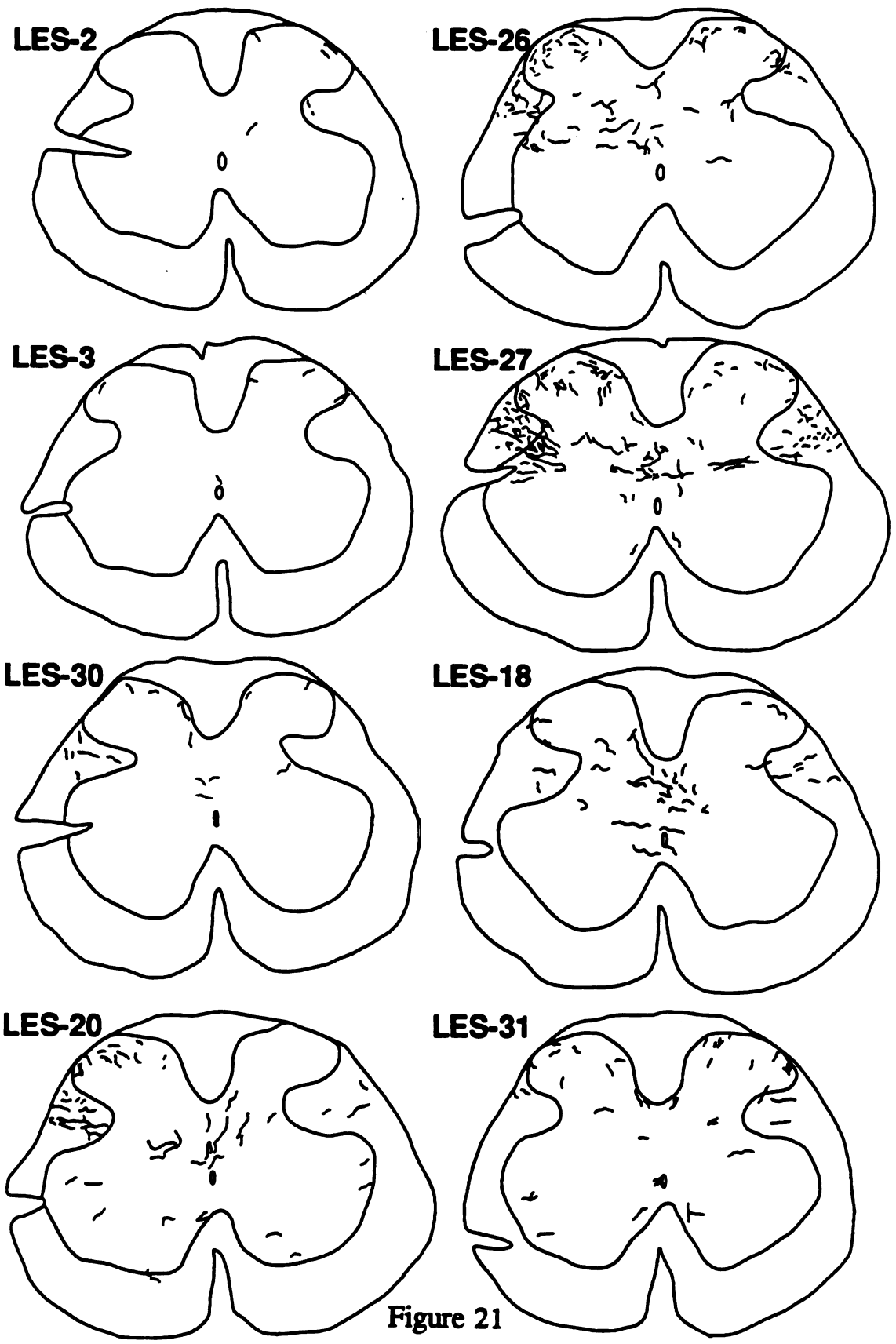

Figure 21

Table 4

Summary of the Location of PVN Lesions, Damage to the Lateral Parvocellular Subnucleus of PVN and the Distribution of NP Fibers in the Dorsal Horn of the Lower Lumbar Spinal Cord and in the Region of the SNB.

ANIMAL #	DESCRIPTION OF PVN DAMAGE	DENSITY OF NEUROPHYSIN FIBERS IN: DH & IML		REGION ABOVE cc		REGION OF THE SNB	
BILATERAL DAMAGE		R	L	R	L	R	L
LES-28	PVN destroyed R&L	ooo	ooo	ooo	ooo	ooo	ooo
LES-2	lp partially damaged on R&L	ooo	ooo	ooo	ooo	---	---
LES-3	lp destroyed on R&L	ooo	ooo	ooo	ooo	---	---
LES-30	PVN destroyed on R most destroyed on L	ooo	ooo	ooo	ooo	ooo	ooo
UNILATERAL DAMAGE							
LES-20	PVN destroyed on L PVN intact on R	+++	ooo	++	++	++	++
LES-31	PVN destroyed on L PVN intact on R	++	++	++	++	++(+)	++

Table 4 (cont'd)

ANIMAL #	DESCRIPTION OF PVN DAMAGE	DENSITY OF NEUROPHYSIN FIBERS IN: DH & IML		REGION ABOVE cc		REGION OF THE SNB	
ADJACENT DAMAGE		R	L	R	L	R	L
LES-26	Lateral lesion on L PVN intact on R&L	++	oo	++	oo	++	++
LES-27	Dorsal lesion on L PVN intact on R&L	+++	ooo	+++	+++	+++	+++
LES-18	Ventral lesion on R&L Partial damage to lp	++	++	oo	oo	oo	oo

+++ = many NP fibers (sham level)
++ = moderate NP fibers - reduced from sham levels but still substantial
oo = few NP fibers - substantially reduced from sham levels
ooo = very few NP fibers remaining
--- = no NP fibers remaining

R=right; L=left
DH=dorsal horn
IML=intermediolateral nucleus
cc=central canal
SNB=spinal nucleus of the bulbocavernosus

and the region of the SNB were markedly reduced. However, in the case of this ventral lesion, while the majority of PVN was intact on both sides, there was slight damage to the lp subnucleus. It is therefore difficult to determine if the reduction in fibers seen in the cord was due to the loss of a few cells in lp or the damage or descending fibers leaving PVN ventrally. Obviously, these possibilities are not mutually exclusive.

DISCUSSION

The results of Experiment 4 demonstrate that any type of damage to PVN, whether complete or partial, bilateral or unilateral, appears to reduce, if not abolish, NP fibers in the region of the SNB. In addition, destruction of areas lateral and ventral to PVN affect NP fibers in the SNB, whereas lesions dorsal to PVN have no effect on these fibers.

The finding that NP fibers in lumbar spinal cord are absent following complete destruction of PVN is consistent with results from studies using radioimmunoassay. PVN lesions resulted in a dramatic decrease in the levels of OXY and VP in all levels of the spinal cord (Hawthorn et al, 1985). These results, as well as the results of the present experiment, suggest that PVN is the source of NP in the region of the SNB. This idea is also consistent with findings from Experiments 1 and 2 demonstrating that PVN is the only region in hypothalamus that projects to the lower lumbar spinal cord that produces NP. All other areas known to contain NP, such as SON, SCN, POA, bed nucleus of the stria terminalis and the lateral septum, do not project to L5-L6.

Findings from unilateral lesions, showing that NP fibers are reduced in L5-L6 both ipsi- and contralaterally, but appear to be more greatly reduced on the ipsilateral side, are consistent with findings of Holstege (1987). Holstege examined the projection from PVN to the spinal cord in the cat, using radiolabelled amino acids (anterogradely) and HRP (retrogradely). Holstege found that the fiber tracts from PVN descended through the lateral funiculus throughout the length of the cord. From the lateral funiculus the fibers were distributed into the spinal cord bilaterally but with a dominance toward the ipsilateral side.

EXPERIMENT 5: IDENTIFICATION OF STEROID-CONCENTRATING NEURONS IN PVN THAT PROJECT TO LOWER LUMBAR SPINAL CORD

The SNB motor nucleus is highly steroid sensitive in adulthood and motoneurons in this nucleus concentrate radiolabelled T and DHT, but do not concentrate estradiol (Breedlove and Arnold, 1980). The hypothesis that steroid sensitive neurons are part of larger steroid sensitive neural circuits was tested in Experiment 5.

The PVN appears to be steroid sensitive in a number of ways. It has been demonstrated that neurons of the PVN concentrate androgens in the male rat (Sar and Stumpf, 1975) and estrogens in the female rat (Pfaff and Keiner, 1974; Stumpf et al, 1975; Cordimas and Morrell, 1990). The incidence of dye-coupling (an index of electrical coupling) between PVN neurons is reduced by castration in the male rat (Cobbett et al, 1987). In addition, the firing rates of oxytocinergic PVN neurons of male rats are increased following systemic injections of testosterone (Akaishi and Sakuma, 1985).

Due to the findings of more recent studies, demonstrating that there are in fact relatively few testosterone-concentrating cells in the PVN (Morrell and Lisciotto, personal communication), and that estrogen-concentrating cells of the PVN project to the medulla in female rats (Cordimas and Morrell, 1990), the present study examined the hypothesis that estrogen-concentrating neurons of the PVN project to the region of the SNB in the male rat.

METHODS

Spinal Cord Injections

Eight males were anesthetized and received injections of 0.5μl of 4% Fluorogold into the lumbosacral spinal cord, as in Experiment 1. After 12 days, males were anesthetized and castrated.

Injections of Radiolabelled Steroid

Seven days after castration, (19 days after the Fluorogold injection), males were lightly anesthetized with Metofane inhalant anesthesia and received intraperitoneal injections of ^{3}H-estradiol (70-80μCi/100g body weight), (New England Nuclear: 4-position labelled; specific activity = 106.9Ci/mmol) dissolved in approximately 0.25ml of absolute ethanol. The ^{3}H-estradiol was given in two injections of equal volume 30min apart.

Tissue Preparation and Histology

Two hours following the second injection the animals were over-anesthetized with Metofane inhalant anesthesia and perfused with 100ml 0.87% saline (4°C) followed by 300-400ml 10% formaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. The brains and spinal cords were removed and postfixed overnight in 10% formaldehyde, 25% sucrose in 0.1M phosphate buffer (pH 7.4) and then cryoprotected overnight in 25% sucrose in 0.1M phosphate buffer (pH 7.4). Following cryoprotection, the brains were cut into hypothalamic blocks, rolled in powdered dry ice until thoroughly frozen and then slowly lowered into liquid nitrogen where they were stored until sectioning.

The spinal cords were sectioned on a freezing microtome at 50μm in the longitudinal plane and alternate sections were mounted onto gelatin coated slides and allowed to air dry. Sections were either stained with thionin or left unstained and dehydrated in 100% ethanol for 1min and cleared in xylene for 2min and then coverslipped with DPX mountant. The location of the Fluorogold injection site was confirmed. Only those animals in which the center of the injection site was within the rostro-caudal extent of the SNB nucleus were used for further analysis.

Brains were removed from the liquid nitrogen and placed onto a cold cryostat chuck and secured with tissue tek. The brains were allowed to equilibrate in the cryostat overnight at -30°C. Brains were sectioned at 12μm in the coronal plane and directly thaw mounted onto photographic emulsion coated slides (NTB-3, Eastman Kodak, Inc.). Some sections were thaw mounted onto gelatin coated slides and thionin stained for use as reference sections. Emulsion coated slides were kept in desiccated, light-tight boxes at 4°C.

Following exposure times of 11-12 months, emulsion coated slides were developed in D-170 developer (Kodak), rinsed in 0.1M phosphate buffer (pH 7.4), fixed in Kodak fixer and rinsed in dH_2O. Sections were then dehydrated in 100% ethanol for 1min, cleared in xylene for 2min and coverslipped with DPX mountant.

Mapping and Quantification

The distribution of Fluorogold labelled cells and steroid-concentrating cells in representative sections through the PVN (every 36-84μm) were mapped at 20X using camera lucida. The steroid concentrating cells were defined as those cells with an accumulation of silver grains over their nucleus that was three times (3X)

background. This was determined using a Bioquant image analysis system at 100X magnification. Briefly, the video density of a cell (# pixels) was converted into grains per cell (12.88 pixels per grain) and this was compared with the number of grains per unit area of background. Background for each cell was determined by obtaining the video density of five cell-sized areas surrounding the steroid concentrating cell, calculating the number of grains per area and then taking the mean of the five areas as the background for that cell. This was done because background differed greatly from section to section as well as from area to area on a section.

RESULTS

Three of the eight males had injections within the rostro-caudal extent of the SNB nucleus (Figure 22), and these animals were used for the quantitative analysis. Fluorogold labelled cells were found in the PVN in a distribution similar to that seen in Experiments 1 and 2.

Estrogen-concentrating cells (3X background) were found throughout the PVN of the male. The majority of the estrogen concentrating cells were located in ap and lp. Many cells in lp contained Fluorogold as well has having an accumulation of silver grains over the nucleus (double labelled) (Figure 23). The location of Fluorogold labelled, estrogen concentrating and double labelled cells can be seen in Figure 24. The proportion of cells concentrating ^{3}H-E and containing Fluorogold in each subnucleus for can be found in Table 5. The majority of double labelled cells were found in lp. Few if any were found in any other subnucleus. Few ^{3}H-E concentrating cells were found in the magnocellular subnuclei. Approximately 30% of the neurons

Figure 22. Schematic drawings of longitudinal sections through the lumbosacral spinal cord showing the location of the Fluorogold injection site in the region of the SNB in individual animals (EAR 5, 7 & 9) administered ^{3}H-estradiol. The dark stippled areas represent the centers of the injection sites where the Fluorogold was most intense. The lighter stippled areas represent the spread of the Fluorogold. The black dots represent the location of SNB motoneurons.

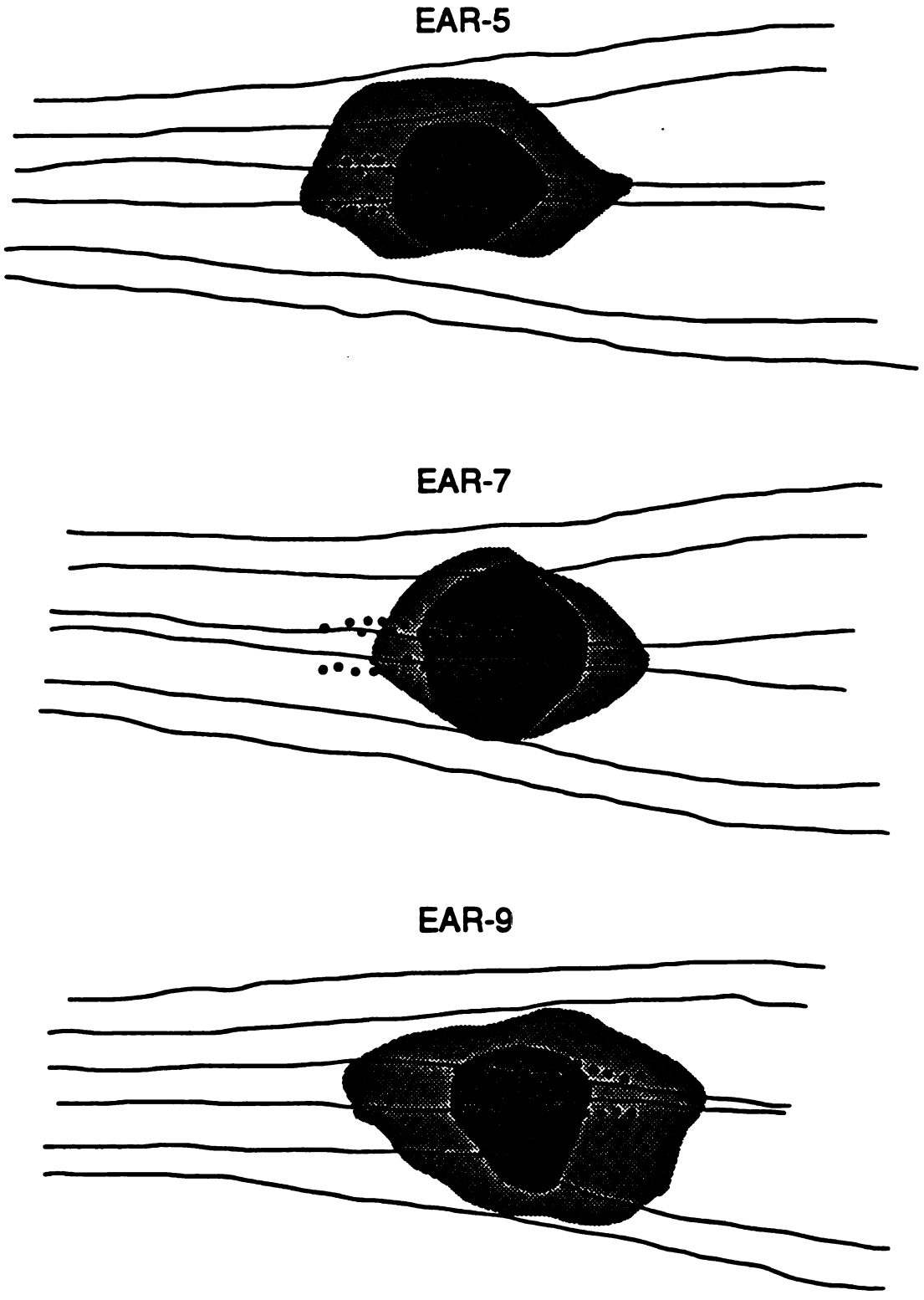

Figure 22

Figure 23. Double exposure photomicrographs using both epifluorescence and brightfield illumination showing **A)** a Fluorogold labelled cell in PVN following an injection of Fluorogold into the lower lumbar spinal cord that also has an accumulation of silver grains under its nucleus following an i.p. injection of ^{3}H-estradiol (^{3}H-E) (**large arrow**). Other cells (**small arrows**) contain Fluorogold or silver grains but not both. Bar = 20μm. **B)** A section through the lateral parvocellular subdivision of PVN showing several spinally-projecting neurons that also concentrated ^{3}H-E. Bar = 20μm.

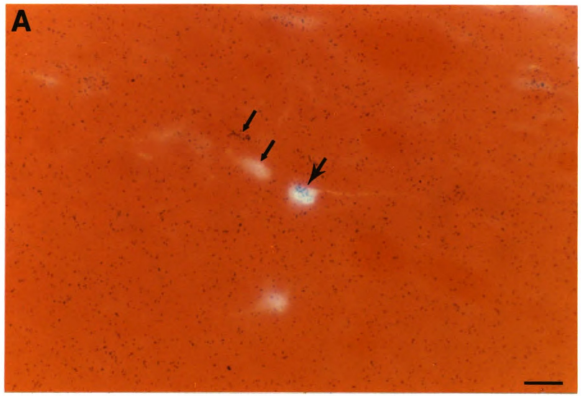

Figure 23

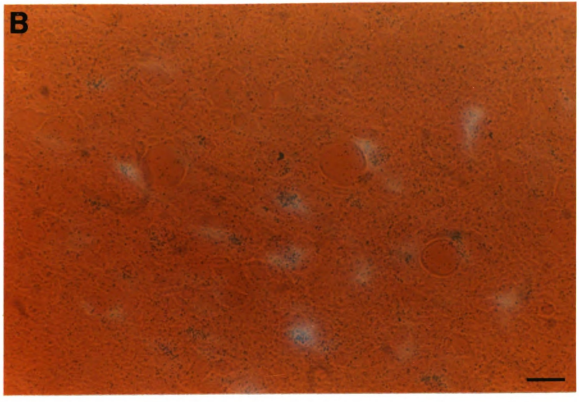

Figure 23 (cont'd)

Figure 24. Camera lucida drawings of sections through the PVN showing the location of subnuclei (boxes) and the distribution of Fluorogold labelled cells (**open circles**), ^{3}H-estradiol concentrating cells (**open squares**) or double labelled cells (**triangles**) within these subnuclei. See List of Abbreviations.

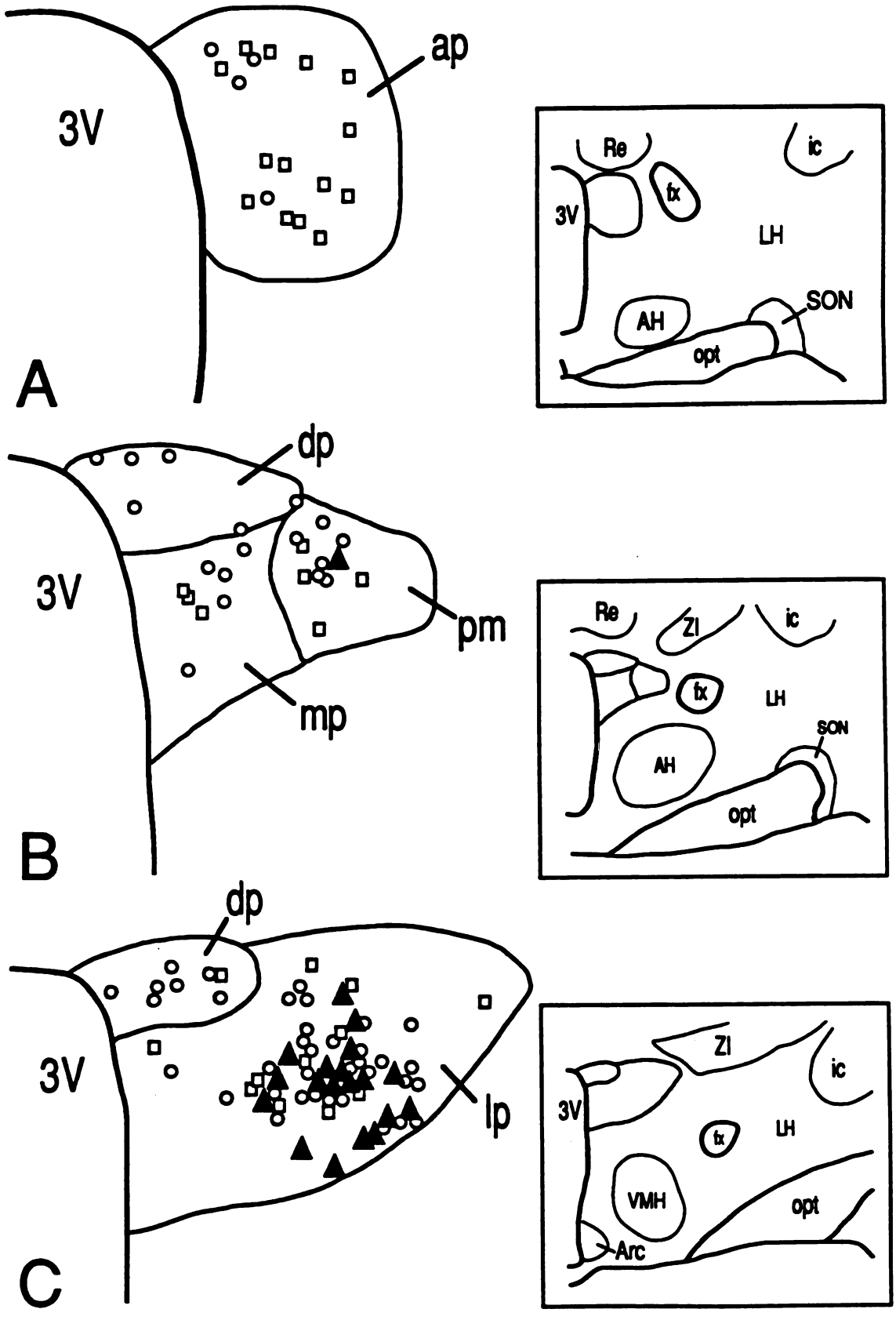
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Figure 24

Table 5

The Individual and Mean Proportion of Paraventricular Nucleus Neurons that Project to the Lower Lumbar Spinal Cord and Concentrate Radiolabelled Estradiol.

	PROPORTION OF CELLS PROJECTING TO SPINAL CORD THAT CONCENTRATE ^{3}H-ESTRADIOL*					PROPORTION OF CELLS CONCENTRATING ^{3}H-ESTRADIOL THAT PROJECT TO SPINAL CORD*				
ANIMAL #	ap	mp	pm	dp	lp	ap	mp	pm	dp	lp
EAR-5	0/11	0/9	1/20	0/23	11/112 (9.8%)	0/22	0/10	1/3	0/8	11/46 (23.9%)
EAR-7	0/11	0/16	0/12	2/33	42/148 (28.4%)	0/56	0/13	0/7	2/19	42/142 (29.6%)
EAR-9	0/15	0/7	1/14	0/40	59/186 (31.7%)	0/29	0/7	1/7	0/15	59/118 (50.0%)
MEAN (s.e.m.)	0%	0%	4% (2.1)	2% (2.0)	23.3% (6.8)	0%	0%	16% (9.7)	4% (3.5)	35% (7.9)

* ap=anterior parvocellular; mp=medial parvocellular; pm=posterior magnocellular; dp=dorsal parvocellular; lp=lateral parvocellular

in the lp that project to the lower lumbar spinal cord concentrated ^{3}H-E. Up to 50% of the estrogen-concentrating cells in lp project to the lower lumbar spinal cord.

One animal in the study (EAR-5) had substantially fewer ^{3}H-E concentrating cells than the other two animals (see Table 5). It is very likely that this is the result of poor radiolabelled ligand binding, and does not reflect a true variability among animals. Reported percentages are based on the animal with the greatest number of double labelled cells, as it is assumed that this injection site was the most successful for the purposes of the present study.

DISCUSSION

The results of Experiment 5 demonstrate that there are many ^{3}H-E concentrating neurons in the PVN of the male rat. In addition, a subpopulation of neurons in the lateral parvocellular subnucleus of PVN both concentrate ^{3}H-E and project to steroid-sensitive regions of the lumbar spinal cord.

The finding that neurons in various subnuclei of the PVN of the male rat concentrate ^{3}H-E is important because all studies previously conducted had examined ^{3}H-E concentrating cells only in the female. However, the results of the present study are consistent with findings of experiments using *in situ* hybridization for estrogen receptor (ER) mRNA (Simerly et al, 1990). ER mRNA was found in the PVN of both male and female rats, with no apparent sex difference. Cells with ER mRNA were found in ap, dp, and the anterior magnocellular subnucleus. In contrast to the present study, fewer cells containing ER mRNA were found in lp compared to other subnuclei, whereas in the present study the majority of the ^{3}H-E concentrating cells

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were found in lp. Simerly et al (1990) found few, if any cells containing ER mRNA in pm, which is consistent with findings of the present study showing few if any ^{3}H-E concentrating cells in this subdivision.

Studies using steroid autoradiography have reported varying results on the presence and distribution of ^{3}H-E concentrating cells in PVN of the female rat. Stumpf et al (1975) reported a substantial number of ^{3}H-E concentrating cells in both magno- and parvocellular subnuclei. Pfaff and Keiner (1973) reported very few PVN cells that concentrated ^{3}H-E, but state that those that were found were in the lateral caudal tip of PVN (probably lp). However, both of these autoradiography studies were done prior to the description of the PVN subnuclei (Armstrong et al, 1980; Swanson and Kuypers, 1980), therefore direct comparisons between these studies and the present experiment are difficult. Furthermore, neither study examined the concentration of ^{3}H-E by neurons of the PVN in the male rat.

The present results demonstrating that up to 50% of the ^{3}H-E concentrating cells in the lp project to lower lumbar levels of spinal cord are consistent with the findings of Cordimas and Morrell (1990). These authors found that 69.4% of the ^{3}H-E concentrating cells in PVN of the female rat project to the medulla. The majority of these cells were in the lp subnucleus. The results taken together with the present findings suggest that some of the E-sensitive neurons of the lp send collaterals to the medulla on the way to the spinal cord or that there is a sex difference in the E-sensitive projections of the PVN.

The present finding that 30% of lp neurons that project to lower lumbar spinal cord concentrate ^{3}H-E suggests that some of these steroid concentrating cells could

contain NP. Experiment 2 demonstrated that approximately 30% of the neurons in lp that project to L5-L6 contain NP. These populations may overlap to some degree, or may be mutually exclusive. However, E-concentrating neurons in the PVN of females contain neurophysin (Sar and Stumpf, 1980; Rhodes et al, 1981).

The partner experiment of the present study (results not shown) examined the concentration of ^{3}H-T by PVN neurons of the male that project to L5-L6. Due to technical difficulties only 1 animal was available for analysis. Results from this animal suggested that there were few ^{3}H-T concentrating cells in PVN. Although fewer cells resulting from technical problems cannot be ruled out, the preoptic area and the BNST regions of this animal had many ^{3}H-T concentrating cells. A low number of ^{3}H-T concentrating cells in PVN is consistent with the results of other investigators (Lisciotto and Morrell, personal communication). Few if any of these ^{3}H-T concentrating cells in PVN projected to L5-L6.

Interestingly, the present results, as well as those of Lisciotto and Morrell, demonstrating few ^{3}H-T concentrating cells in PVN are not consistent with findings of Simerly et al (1990). This paper reported many PVN cells that contained androgen receptor (AR) mRNA. The difference between the findings of this study and the present experiment may be a technical one. It is possible that *in situ* hybridization may have a higher level of sensitivity, thereby detecting cells with few receptors. In contrast, the difference could be a biological one in which the AR mRNA in many cells of the PVN may not be translated into functional AR protein. Another possibility is that the short-term castration necessary for steroid autoradiography is enough to down-regulate androgen receptor levels in PVN, even if other areas (e.g.

MPOA, BNST) are less affected. Since *in situ* hybridization does not require castration, many more PVN cells may be detected as positive.

GENERAL DISCUSSION

The present studies demonstrate that in male rats, neurons within the parvocellular subdivisions of the paraventricular nucleus (PVN) project to levels of the lower lumbar spinal cord containing sexually dimorphic motor nuclei that modulate penile reflexes. Neurophysin (NP), the coproduct of oxytocin (OXY) and vasopressin (VP), is found in these PVN neurons and in fibers and terminals that appear to contact motoneurons of the SNB, specifically those motoneurons innervating the BC muscle. These fibers and terminals were abolished following electrolytic lesions of PVN, supporting the idea that these fibers originate in the PVN. A subpopulation of neurons in the lateral parvocellular subnucleus of the PVN that project to levels of spinal cord containing the SNB, also concentrate radiolabelled estrogen.

PVN Projection to Spinal Cord and SNB Function

Based on the findings of the present experiments we suggest that the PVN innervates the motoneurons of the SNB and uses OXY and/or VP as a neurotransmitter in this pathway. Several lines of evidence are available to suggest that this pathway is related to penile response components of masculine sexual behavior. OXY is known to have effects on penile reflexes when infused into the third ventricle (Argiolas et al, 1986) and the effects of apomorphine on penile responses are thought to be via a central release of OXY (Melis et al, 1989). The site

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of action of OXY, at least in part, may be via oxytocinergic synapses onto SNB motoneurons.

In addition, however, OXY also appears to alter penile responses by acting in the PVN itself (Melis et al, 1986). The mechanism by which OXY acts in PVN is unclear, but OXY binding sites have been found within the PVN (Brinton et al, 1984) and OXY has been shown to facilitate its own release (Freund-Mercier and Richard, 1981) as well as increase the firing rate of oxytocinergic neurons (Moos et al, 1984). Furthermore, OXY-immunoreactive synapses have been reported to contact oxytocinergic neurons in the SON (Theodosis, 1985). These facts support the idea that OXY could be acting in the PVN on parvocellular oxytocinergic (and/or other peptidergic) neurons which in turn project to the spinal cord. However, the possibility exists that OXY-induced penile responses following infusion into the PVN, could result from OXY leakage into the third ventricle and consequently , act in numerous other sites along the entire ventricular system. This is especially a concern in light of several considerations: Firstly, in the study by Melis et al (1986) OXY was infused via a metal cannula, which may cause extensive damage to the area (e.g. the ventricular wall). Secondly, testing was performed for one hour following infusion, a time period sufficient for the diffusion of the OXY into the CSF and its transport to other regions of the CNS. In support of this idea are the findings from Melis et al (1986) demonstrating that OXY significantly increased penile responses when infused into the CA_1 region of the hippocampus, a periventricular area.

The anatomical evidence provided by the present experiments strongly suggests that OXY may act directly on SNB motoneurons and may alter penile

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reflexes at this level. However, there may be multiple sites of action as well as multiple mechanisms for OXY's effects on SNB function.

Pharmacological studies also support the concept that OXY from the PVN is involved in the regulation of SNB/DLN function. Evidence suggests that dopamine's effects on penile reflexes are mediated through the PVN. Apomorphine, a dopamine agonist, infused into the medial preoptic area (MPOA), facilitated penile reflexes (Pehek et al, 1989). These effects of apomorphine on penile responses, following central administration were prevented by electrolytic lesions of the PVN (Argiolas et al, 1987a). That these lesions disrupted OXY processes is strongly supported by the finding that the effects of systemically administered apomorphine were blocked by central administration of an OXY antagonist, (Argiolas et al, 1987b). The concomitant administration of apomorphine and OXY, both of which induce penile responses when given alone, did not produce an additive effect on the incidence of the responses (Melis et al, 1989). Taken together, these findings led these authors to conclude that apomorphine induces penile responses by releasing OXY within the central nervous system.

The POA is innervated by dopaminergic axons of the incertohypothalamic tract (A14) (Bjorklund et al, 1975) and the POA sends projections to the PVN (Silverman et al, 1981; Chiba and Murata, 1985). Based on the results of the present studies, demonstrating a NP-containing pathway from the PVN to the SNB, it is possible that dopamine acts on neurons in the POA that project to PVN neurons, which in turn influence penile reflexes via a direct projection to the SNB.

Neural Circuits of Male Copulatory Behavior: Role of PVN and Oxytocin

Understanding the role that PVN and OXY play in the incidence of penile reflexes, in combination with the part they have in the display of male copulatory behavior, may help to unravel how neural circuits work to coordinate all aspects of this complex behavior. Although the muscle innervated by the SNB, the bulbocavernosus, is not essential for the gross motor components of copulatory behavior in males, (Sachs, 1982), this muscle's function is undoubtedly integrated with these motor patterns to form the complete repertoire of copulatory behavior, including the initiation and the maintenance of sexual behavior, as well as the successful impregnation of females (the ultimate purpose of male sexual behavior in the rat). It is instructive, therefore, to consider what role the PVN and OXY play in other aspects of male copulatory behavior.

The neural circuits involved in male copulation are not well understood. The medial preoptic area has been the center of attention for many years because lesions of this region cause dramatic and lasting deficits in the demonstration of sex behavior by intact adult male rats (Heimer and Larsson, 1964; 1966). The behavioral deficits following POA lesions cannot be reversed, even with testosterone administration (Heimer and Larsson, 1964). However, the administration of lisuride, a non-specific monoamine receptor agonist was able to restore behavior in most POA lesioned animals. (Hansen et al, 1982).

The medial amygdala and bed nucleus of the stria terminalis, as well as the A14 dopaminergic region, have been described as relevant input to the MPOA with regard to male copulatory behavior (Chiba and Murata, 1985). Lesions of these areas

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disrupt specific aspects of male sex behavior but do not abolish it altogether as do lesions of the MPOA. Efferents of the MPOA project to the midbrain via the medial forebrain bundle (Swanson, 1976) and some areas of the midbrain that receive input from MPOA, such as the ventral tegmentum, have been implicated in male sex behavior (Hansen and Gummesson, 1982; Brackett and Edwards, 1984).

The means by which the MPOA receives or sends information to the spinal cord, to coordinate its function with sympathetic, vascular, sensory and reflexive functions, represents a major gap in our understanding of copulatory behavior. Much of this information is undoubtedly relayed through brainstem sites. For example, one brainstem site, with which the MPOA has reciprocal connections (Chiba and Murata, 1985), the paragigantocellularis nucleus of the reticular formation, has been shown to project to and exhibit an inhibitory influence on the SNB (Marson and McKenna, 1990). Based on the results of the present studies, the PVN may also be considered a site by which the MPOA influences SNB processes. In addition, however, the PVN is involved in the timing of the behavior without controlling actual behavioral output. Lesions of the lateral and posterior PVN increased the mount and intromission latencies, while reducing the post ejaculatory interval (PEI) (Hughes et al, 1989). These results suggest that PVN plays an excitatory role in the behavior itself, but may have an inhibitory role during the post-copulatory period. Thus the PVN may play a role in coordinating or timing the gross motor patterns of male sex behavior with penile reflexes.

OXY plays a significant role in mating behavior but there is confusion in the literature as to whether OXY's effects are stimulatory or inhibitory. This confusion

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may be the result of differences in methods of administration, doses or testing paradigms, or there may be, in fact, multiple mechanisms by which OXY acts to modulate behavior.

OXY levels in cerebrospinal fluid (CSF) have been shown to increase two-fold above basal levels 5 minutes after ejaculation in the male rat and reach levels 3 times those of basal levels 20 minutes following ejaculation (Hughes et al, 1987). OXY in the CSF implies that this peptide is being released by synapses within the CNS. The source of this OXY is presumably the PVN since electrolytic lesions of PVN abolished the increase in CSF OXY levels (Hughes et al, 1987). OXY, administered i.v. 5 minutes prior to testing, had little effect on behavior and only reduced the number of intromissions (Stoneham et al, 1985). However, OXY given i.v. 60 minutes prior to testing resulted in reductions of both the ejaculatory latency and post-ejaculatory latency (Arletti et al, 1985).

Central administration of OXY (1ng into lateral ventricle) resulted in stimulatory effects on behavior, shortening EL and PEI (Arletti et al, 1985). An i.c.v. injection of an OXY antagonist resulted in inhibition of behavior, increasing IL and EL (Argiolas et al, 1988), again suggesting that OXY is stimulatory. However, other studies have reported that OXY appears to inhibit mating behavior. OXY infused into the third ventricle prior to testing, increased the ML and IL. When OXY was infused into the third ventricle after testing, the result was a lengthened PEI, suggesting an inhibitory role for OXY (Stoneham et al, 1985). In comparison, VP had no effect on male sex behavior in the rat (Sodersten et al, 1983).

Because hypophysectomy has no effect on male mating behavior (Pfaff, 1973), OXY's effects on behavior must involve the central release of this peptide. Presumably, the source of this OXY is the PVN. However, it is not clear what the role of the PVN is in the modulation of mating behavior. Since the findings of the present study demonstrate that PVN projects to the SNB via a NP-containing pathway, it is possible that some portion of OXY's effects on copulatory behavior is through the projection of the PVN to levels of the spinal cord that contain the motor nuclei responsible for penile reflexes, the sensory input from the pudendal nerve, as well as nuclei that control the sympathetic functions of visceral organs that may be involved in mating. It would be interesting to examine whether the PVN also receives inputs from this level of the spinal cord, as occurs in females (Akaishi et al, 1988), in which case the PVN might act as a sensory and motor (reflexes) relay, in turn sending and receiving information to and from the MPOA. The PVN and POA are known to have reciprocal connections with one another (Silverman et al, 1981; Chiba and Murata, 1985).

PVN and Gonadal Steroid Hormones

The results of the present study demonstrate that neurons of the PVN that project to steroid-sensitive levels of the spinal cord also concentrate radiolabelled estrogen. The accumulation of silver grains over the nucleus of a neuron following i.v. injections of radiolabelled steroid, is presumed to indicate the presence of nuclear steroid receptors.

The function of nuclear steroid receptors, although poorly understood in the brain, is fairly well understood in other steroid sensitive tissues such as uterus.

Briefly, steroid hormones bind to their intracellular receptor to form steroid/receptor complexes. These complexes in turn modulate gene expression by interacting with specific regions of the DNA called steroid response elements (SRE's) (see Yamamoto, 1985 for review).

In addition to nuclear steroid receptors, there is evidence for membrane steroid receptors as well. In the absence of synaptic function and protein synthesis, the addition of 17ß-estradiol to the media of brain slices containing the medial amygdala, resulted in brief hyperpolarizations and an increased potassium conductance in neurons within seconds of its administration (Nabekura et al, 1986). Steroids can also alter the firing rate of PVN neurons. Electrophysiological studies have demonstrated that the firing rate of tonically firing (oxytocinergic) neurons of the PVN in anesthetized male rats is increased following systemic injections of T 2 days prior. Interestingly, systemic injections of estradiol had no effect in males (Akaishi and Sakuma, 1985). Because of the two day time period between injection of the steroid and electrophysiological recording, it is impossible to tell if these changes in membrane characteristics were through genomic effects (e.g. synthesis of ion channel proteins etc) or whether the steroids altered the neuronal membrane directly in some lasting way. Studies of the PVN slice in which steroids are added directly to the bath for short periods of time would address this issue.

Dye coupling of neurons in the PVN, a measure of electrical coupling, is affected by steroids with castration reducing the number of coupled cells (Cobbett et al, 1987). The mechanisms behind electrical coupling of cells via gap junctions are not well understood. It is not clear whether the synthesis of the proteins that make

up the gap junction channels are the regulating factor, or whether the channel proteins are already in the membrane and membrane contact and membrane fluidity affect the formation of functional channels (See Loewenstein, 1981 for review). It can be easily seen how steroids, in either case, might alter gap junction formation in PVN, either via genomic mechanisms or through membrane effects.

The function of PVN neurons that concentrate estrogen and project to lower lumbar levels of the spinal cord is unknown at this time. It is possible that estrogen acts in PVN neurons to regulate the synaptic coverage of SNB motoneurons from afferents originating in PVN. The density and number of synapses on SNB motoneuron soma and proximal dendrites is altered by castration (Leedy et al, 1987; Matsumoto et al, 1988b) and it is not known if these effects are through direct actions of steroids on receptors within SNB motoneurons or whether these effects are on steroid sensitive afferents. The results of the present experiments suggest that it is possible that steroids act on SNB afferents directly.

In addition, both the development and maintenance of the dendritic arbor of SNB motoneurons are dependent on the levels of endogenous steroids (Kurz et al, 1986; Goldstein et al, 1990). The plasticity of the SNB dendrites may result from changes in the density or functional strength of synaptic input from steroid sensitive afferents, such as the PVN.

Steroids may act on spinally-projecting neurons of the PVN to regulate the synthesis of neurotransmitters and/or neuropeptides used in this pathway. It has been shown that OXY mRNA levels in PVN are altered by ovarian steroids in female rats (Miller et al, 1989). If two or more transmitters are used in a single spinally-

projecting PVN neuron, as is the case in many other systems, estrogen may act to regulate the ratio of levels of these two substances. This may be important in the case of neuropeptides that may act to modulate the function of other neurotransmitters.

The function of the SNB/BC neuromuscular system (penile reflexes) is highly dependent on gonadal steroids (Hart, 1967; 1973; Rodgers and Alheid, 1972; Davidson et al, 1978; Bradshaw et al, 1981). Castration abolishes penile reflexes and T administration restores reflexes to intact levels. While administration of the reduced T metabolite, DHT, was able to restore penile reflexes in castrate males (Gray et al, 1980), administration of the aromatized metabolite, E, failed to facilitate reflexes in the ex copula situation (Hart, 1979; Gray et al, 1980; Meisel et al, (1984). However, E treatment was sufficient to restore copulatory behavior in castrated males and these males exhibited penile reflexes during mating at the same rate as intact males (O'Hanlon et al, 1981; Meisel et al, 1984).

While the elicitation of penile reflexes in the ex copula situation may involve only lumbosacral spinal circuits, the display of these reflexes during copulation may require higher levels of neural integration that involve hypothalamic circuits as well. Therefore, estrogen's ability to restore penile reflexes during copulation may involve estrogen sensitive PVN neurons that project to the lumbosacral spinal cord. This may be seen as further support for the concept that the PVN interfaces the gross motor patterns of male copulatory behavior with penile reflexes.

Steroid Sensitive Neural Circuits

The location of steroid concentrating neurons in the brain is well documented (Pfaff and Keiner, 1973; Sar and Stumpf, 1975; Stumpf et al, 1975). The functions of the nuclei containing these neurons are known in some cases, but surprisingly little is known about the projections of these steroid concentrating cells. Motoneurons of the SNB and DLN are androgen-concentrating (Breedlove and Arnold, 1983c) and their innervation of the BC and IC muscles is well documented (Schroder, 1980; McKenna and Nadelhaft, 1986). Steroid concentrating neurons in the BNST, PVN, central amygdala and the central gray of the female rat send projections to the medulla (Cordimas and Morrell, 1990), but it is not known if these projections are onto other steroid sensitive cells.

The findings of the present experiment suggest that some neurons of the PVN that project to levels of spinal cord that contain the androgen-concentrating cells of the SNB also concentrate estrogens. These findings provide the basis for the description of a neural circuit that is steroid sensitive. The idea that steroid sensitive neural pathways exist, in which some neurons of all nuclei within a polysynaptic pathway are sensitive to steroids, has been proposed for other systems as well. In the male Syrian hamster, the steroid-concentrating neurons of the posterior medial amygdala project to the steroid concentrating neurons of the medial BNST, the medial MPOA and the shell of the VMH. In contrast, the rostral medial amygdala, which contains very few steroid concentrating neurons, projects to the lateral BNST, the lateral MPOA and the core of VMH, which also lack steroid concentrating cells (Gomez, 1990). In addition, regions of the forebrain that project to the medial

preoptic nucleus in the rat, correspond closely with regions known to contain steroid-concentrating cells (Simerly and Swanson, 1986).

These findings and the results of the present study are consistent with the idea that neural circuits, thought to be involved in steroid-dependent behaviors and functions, are made up of nuclei all of which contain some neurons that are steroid sensitive. It has been suggested previously that these steroid-sensitive neural circuits could act to amplify hormonal effects via a cascade mechanism (Simerly and Swanson, 1987). In addition, the findings of the present studies suggest that the neurons in different locations within a circuit may be sensitive to different steroids, as in the case of the SNB, which is androgen sensitive yet may receive input from the PVN, which concentrates estrogen. This is consistent with the idea that steroid sensitive neural circuits may contain a level of differential sensitivity that would accommodate the complexity of the effects of hormones on behavior.

In the case of the SNB neuromuscular system, it can be proposed that a steroid-sensitive neural circuit exists in which androgen-concentrating SNB motoneurons innervate the BC muscle, which also contains androgen and estrogen receptors (Krieg et al, 1974; Dube et al, 1976; Dionne et al, 1979). Motoneurons of the SNB may be innervated by estrogen-concentrating neurons of the PVN, which , in turn, may receive input from MPOA neurons that concentrate either androgen and/or estrogen.

In conclusion, it is suggested that this circuit may provide the pathway for the coordination between aspects of copulatory behavior that are regulated by the

MPOA and penile reflexes that are regulated by the SNB/BC neuromuscular system.

Future Directions

The findings of the present studies, while providing many answers, also generate many questions. Several experiments will need to be performed in order to better understand the role of PVN's projections to lower lumbar spinal cord.

As mentioned above, the presence of NP in fibers and putative terminals contacting SNB motoneurons suggests the presence of OXY and/or VP. However, immunohistochemistry using antibodies directed specifically against OXY or VP will need to be performed in order to determine if indeed OXY is the neurotransmitter involved in this projection. Secondly, autoradiographic binding studies using a specific radiolabelled ligand for the OXY receptor would determine if indeed there are OXY binding sites on SNB motoneurons. This, in conjunction with the ultrastructural verification of oxytocinergic synapses onto SNB motoneurons, would strongly confirm the hypothesis that PVN modulates penile reflexes via a oxytocinergic synaptic input directly onto SNB motoneurons.

While the above experiments are necessary to confirm the hypothesis, several other experiments would be informative as well. The idea that NP may act as a neurotransmitter could be tested by performing autoradiographic binding studies using radiolabelled NP. Although this may seem controversial, the suggestion that oxytocinergic binding sites may be lacking in this region, in addition to the fact that vasopressinergic fibers are scarce, warrants further investigation.

Because only 20% of neurons in the PVN that project to lower lumbar spinal cord contain NP, the identification of other neurotransmitters or neuropeptides in

this projection would be important. In addition, the colocalization of other peptides with OXY in this projection would help to clarify the mechanisms behind this projection. Substance P and met-enkephalin would be good candidates since they are both found in parvocellular neurons of the PVN (Ljungdahl et al, 1978; Cechetto and Saper, 1988) and in fibers in the region of the SNB (Micevych et al, 1986).

The finding that neurons of the PVN in the male rat concentrate estrogen leads to questions about the role of these steroid receptors. Surprisingly, preliminary studies have suggested that OXY mRNA in neurons of the lateral parvocellular subnucleus is not altered by castration (Kashon and Clemens, unpublished observations), the region in which many of the estrogen concentrating, spinally projecting neurons are found. The identification of specific lp neuron populations, such as those neurons projecting to lumbar cord or those that contain steroid receptor protein, may be necessary to detect a specific effect of steroids on OXY mRNA in the PVN of the male rat.

Synaptic input onto SNB motoneurons is known to be regulated by gonadal steroids (Leedy et al, 1987; Matsumoto et al, 1988b). The regulation of PVN input onto SNB motoneurons by estrogen would not only be an important question to the understanding of PVN's role in SNB function but would also provide information as to whether synaptic input is regulated at the pre- or postsynaptic level. Because the SNB concentrates T and DHT but not E and spinally projecting neurons of the PVN appear to concentrate E but not T, any effects of E on PVN input onto SNB motoneurons would have to occur at the level of the presynaptic neurons.

Summary and Conclusions

The present studies demonstrate that neurons within the parvocellular subdivisions of the PVN project to sexually dimorphic levels of the spinal cord that contain the motor nuclei responsible for penile reflexes. These neurons contain NP, the coproduct of OXY and VP, and NP is found in fibers and terminals that appear to contact motoneurons of the SNB. These fibers and terminals are abolished following electrolytic lesions of PVN suggesting that these fibers originate in the PVN. A subpopulation of neurons in the lateral parvocellular subnucleus of the PVN that project to levels of spinal cord that contain the SNB, also concentrate radiolabelled estrogen, suggesting that there may be estrogen sensitive input onto androgen sensitive motoneurons.

In this regard, the PVN must be part of the neural circuit that controls male copulatory behavior. It is suggested that the PVN may act as a relay center within the hypothalamus in which it serves to integrate the gross motor patterns of male sexual behavior that are regulated by areas such as the MPOA, with penile reflexes that are controlled by the SNB/BC neuromuscular system.

The tracing of neural circuits that involve steroid sensitive neurons and the identification of the transmitters used in these pathways, are important steps in understanding the mechanisms by which steroids act on the brain to alter behavior.

APPENDICES

APPENDIX A

APPENDIX A

Protocol for Neurophysin Immunohistochemistry using the Rhodamine-tagged Avidin Detection Method.

1. Rinse with 0.87% saline in 0.05M tris buffer (pH 7.4) with 0.2% Triton X100 (TBS/TX) 3 X 5min at R.T.

2. Incubate in 3% Normal goat serum in TBS/TX for 30min at R.T.

3. Rinse 3 X 5min with TBS/TX.

4. Incubate in 1:5000 rabbit anti-neurophysin serum in TBS/TX for 48hr at 4°C.

5. Rinse 3 X 5min at R.T. with TBS/TX.

6. Incubate in goat anti-rabbit serum (1drop) in 10ml TBS/TX for 60min at R.T.

7. Rinse 3 X 5min at R.T. with TBS/TX.

8. Incubate with rhodamine-tagged avidin (2μm/ml TBS/TX) for 60min at R.T. in the dark.

9. Rinse 4 X 5min with TBS/TX.

Protocol for Neurophysin Immunohistochemistry using the ABC/DAB Detection Method.

1. Rinse with 0.87% saline in 0.05M tris buffer (pH 7.4) with 0.2% Triton X100 (TBS/TX) 3 X 5min at R.T.

2. Incubate in 3% Normal goat serum in TBS/TX for 30min at R.T.

3. Rinse 3 X 5min with TBS/TX.

4. Incubate in 1:5000 rabbit anti-neurophysin serum in TBS/TX for 48hr at 4°C.

5. Rinse 3 X 5min at R.T. with TBS/TX.

6. Incubate in goat anti-rabbit serum (1drop) in 10ml TBS/TX for 60min at R.T.

7. Rinse 3 X 5min at R.T. with TBS/TX.

8. Incubate in a solution of the avidin-biotinylated HRP complex (ABC) (2drops A: 2drops B in 10ml TBS/TX) for 90min at R.T.

9. Rinse 4 X 5min at R.T. with TBS.TX.

10. React with Diaminobenzidene solution (DAB) containing glucose oxidase (1ml to 10ml DAB).

11. Rinse 4 X 5min at R.T. with TBS/TX.

APPENDIX B

Appendix B

Table A

The Proportion and Percentage for Individual Animals of Paraventricular Nucleus Neurons That Project to Lower Lumbar Spinal Cord and Contain Neurophysin.

		TOTAL # FG	TOTAL # DOUBLE LABELLED	% DOUBLE LABELLED	% DOUBLE LABELLED THAT ARE IN EACH SUBNUCLEUS *					% IN EACH SUBNUCLEUS THAT ARE DOUBLE LABELLED *				
					ap	dp	mp	pm	lp	ap	dp	mp	pm	lp
NPFG-5	#	949	163	—	0/163	21/163	6/163	18/163	118/163	0/53	21/238	6/127	18/102	118/429
	%	—	—	17.1	0	12.9	3.7	11.0	72.4	0	8.8	4.7	17.6	27.5
NPFG-6	#	816	97	—	3/97	14/97	9/97	11/97	60/97	3/36	14/207	9/106	11/110	60/357
	%	—	—	11.9	3.1	14.4	9.3	11.3	61.9	8.3	6.8	8.5	10.0	16.8
NPFG-16	#	579	141	—	1/141	28/141	10/141	6/141	96/141	1/16	28/180	10/105	6/30	96/248
	%	—	—	24.4	0.7	19.9	7.1	4.3	68.1	6.3	15.6	9.5	20.0	38.7
MEAN (sem)	%	781.3 (108.2)	133.7 (19.4)	17.8 (3.6)	1.3 (0.9)	15.7 (2.1)	6.7 (1.6)	8.9 (2.3)	67.5 (3.0)	4.9 (2.5)	10.4 (2.7)	7.6 (1.5)	15.9 (3.0)	27.7 (6.3)

* ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; pm=posterior magnocellular; lp=lateral parvocellular

Appendix B

Table B

Raw Data for the Quantification of Neurophysin Fibers in the Region of the SNB in Sham-Castrated and Castrated Males.

ANIMAL #	# OF FIBERS PER 30µm SECTION	AVERAGE FIBER LENGTH (µm)	TOTAL LENGTH PER 30µm SECTION (µm)
SHAM-CASTRATES			
NP-15	13.2	61.92	814.6
NP-16	11.7	54.78	638.3
NP-17	7.0	55.61	391.3
NP-18	4.1	69.93	286.0
NP-28	7.6	56.09	428.3
NP-29	8.3	55.44	461.4
NP-30	10.5	59.49	625.6
NP-31	10.1	66.31	672.9
NP-32	8.4	55.40	465.7
NP-33	5.3	57.19	305.0
MEAN	**8.62**	**59.2**	**508.9**
(s.e.m.)	**(0.9)**	**(1.7)**	**(54.4)**
CASTRATES			
NP-11	12.1	55.38	668.5
NP-12	11.4	65.67	749.2
NP-13	17.1	58.00	994.2
NP-14	18.5	58.08	1073.5
NP-36	9.0	59.70	535.0
NP-37	5.4	78.65	421.3
NP-38	5.9	63.87	375.6
NP-39	11.9	70.02	833.0
NP-40	4.8	73.66	356.5
NP-41	26.3	52.09	1370.5
MEAN	**12.24**	**63.5**	**737.7**
(s.e.m.)	**(2.1)**	**(2.7)**	**(106.0)**

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