DISTRIBUTION AND OVERLAP OF FOS AND ANDROGEN RECEPTOR PROTEIN IN THE BRAINSTEM OF MALE RATS: IMPLICATIONS FOR REPRODUCTIVE BEHAVIOR

By

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ABSTRACT

Appetitive and consumatory sexual behaviors are critically dependant upon gonadal steroids, the medial preoptic area, and functional androgen receptors (AR). Utilizing the expression of the protein product of the immediate-early gene c-fos as a marker of behavior-dependent neural activation, anatomical sites involved in reproductive behavior have previously been colocalized with AR in regions of the forebrain, midbrain, and lumbar spinal cord.

Because brainstem pontomedullary afferents express AR mRNA and preferentially synapse onto sex-activated cells, it is probable that areas in the medulla and pons are involved in the display of reproductive behavior, possibly orchestrated via androgen receptor dependant activation. To more fully assess brainstem contributions to reproductive behaviour, AR receptor and Fos immunoreactivity (AR-ir and Fos-ir, respectively) were compared in the pons and medulla of copulating (cop), non-copulating (non-cop), and socially isolated (si) male rats, allowing the identification of potential targets of androgenic influence on copulation.

Copulation-selective Fos-ir was detected in auditory processing areas of the dorsal cochlear nucleus in the medulla, and the nucleus of the trapezoid body in the pons. In the medullary linear nucleus, the cops and non-cops did not differ in the amount of Fos, however, both contained more Fos when compared to the si animals. Equivocal patterns of Fos expression were detected in the intermediate reticular nuclei, and the posterodorsal and reticulo-tegmental areas.

Contrary to studies localizing AR mRNA by in situ hybridization, AR-ir was not detected in auditory or somatosensory areas, and except for the prepositus nucleus, areas carrying vestibular information were AR negative, as were sites containing cranial nerve motoneurons. In contrast, nuclei of the solitary tract displayed dense AR-ir, as did several sites in the tegmental and central gray areas.

Consistent with previous reports implicating inhibitory supraspinal ventral medullary nuclei in sexual behavior, the raphe pallidus, the lateral paragigantocellular and the ventral gigantocellular reticular nuclei contained both AR-ir and copulation-induced Fos-

ir. However, AR-ir and Fos-ir did not overlap in the trapezoid body or the dorsal cochlear nucleus following sexual behavior.

Overall, these data indicate that androgens may influence neuronal functioning in brainstem areas in the medulla that potentially regulate male rat sexual behavior.

DEDICATION

To my Mother for constantly inspiring me.

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INTRODUCTION

Rat Sexual Behavior

The display of masculine sexual behavior in the rat requires the integration of a set of reflexes in a precise and stereotyped temporal sequence (Meisel and Sachs, 1994). The initiation of copulation is dependant upon soliciting behaviors produced by the female. These behaviors include: approach and avoidance, where the female will attempt to entice the male to pursue her, and ear wiggling, hopping, and darting, which are indicators of arousal and signals her receptivity and interest to mate (Nelson, 2000; Erskine, 1989). The display of lordosis by the female, a dorsoflexion of the spine, is a critical behavior that allows copulation to take place; the female arches her back, deflects her tail to one side, and remains in a stationary position to allow the male to mount her. Ear wiggling, hop-darting, and approach-avoidance behaviors, as well as the facilitation of lordosis (elicited by the male) are dependant upon the internal endocrine state of the female; female rats will display maximal lordosis and sexual solicitation behavoirs when serum levels of estrogens and progestins are high, a time that coincides with the release of ova. In natural settings, female rats will also pace the bout of reproduction as a specific amount of vaginal stimulation is needed to achieve a successful pregnancy; multiple intromissions promote sperm transport, as well as prolonging corpora luteal functioning, allowing the uterine wall to develop and prepare for implantation of the fertilised ovum (Nelson, 2000). Insufficient vaginal stimulation, or none at all, will not maintain luteal function and the female will continue to display a natural estrous cycle. Pacing behavior is evident in testing situations in the lab when the female is able to escape, in between mounts, via a tunnel that occludes the male from entering, or when a bi-level chamber is used (see Mendelson and Pfaus, 1989).

In response to the solicitation behaviors of female rats in estrous, experienced and inexperienced male rats will pursue females and once in contact, display several sociosexual behaviors, such as anogenital investigation, ultrasonic vocalization, and

mounting with pelvic thrusting. With mounting behavior, sexually experienced males palpate the flanks of the female with their forepaws to elicit the lordosis response. Once displayed, the male may gain entrance to the female's vagina, termed intromission (or intromission pattern), after a number of mounting and thrusting attempts. With enough intromissions, a male will achieve an ejaculation, but the bout of coitus is not a continuous event. Male rats typically display a number of mounts and intromissions over several minutes, with periods of inactivity, anogenital investigation, grooming, or environmental exploration, before a single ejaculation is achieved (Meisel and Sachs, 1994).

Males display a characteristic set of behaviors after ejaculation, encompassing the refractory or post ejaculatory period. A 22 kHz ultrasonic vocalization has been detected emanating from the male, but the function of this behavior is currently unknown (Meisel and Sachs, 1994). In addition to 'singing', males engage in genital grooming, as well as dragging their pelvic region on the bottom of the testing cage, possibly to mark their scent. In general though, they are uninterested in the female, which repeatedly attempts to initiate copulation by displaying solicitation behavior. The termination of the post ejaculatory interval is followed with repeated mounting and intromitting behavior, and a male rat will typically ejaculate several times, often until exhaustion, in a single day of copulating. Full recovery from sexual exhaustion, which usually lasts four to five days, is coincidental with the female's estrous cycle which is similar in length (Nelson, 2000). Interestingly, the postejaculatory period can be shortened by activation of the sympathetic nervous system by administering a tail pinch (Wang and Hull, 1980), or infusion of the GABA antagonist, bicuculline, into the medial preoptic-hypothalamic area (Fernandez-Guasti, Larsson, Vega-Sanabria, 1986).

Observations of male and female rat sexual behavior in the lab retain some ecological validity, as the display of mating in wild rats has been reported to be behaviourally similar to that described above (Pfaus, Mendelson, Phillips, 1990).

However, despite the display of these stereotyped reflexes in response to a receptive female, it has been reported that some male rats tested in the laboratory will not initiate mating in the presence of an estrous female, and if these males do, only do

so sporadically and inefficiently (Pfaus and Wilkins, 1995). One theory to account for this phenomenon suggests that mating can be disrupted if sexually naive males are placed into a sex testing arena with a short acclimatisation period but without previous familiarity of the new surroundings. It has been observed that the animal will not copulate as efficiently and will not achieve a stable pattern of sexual behaviour suggesting the nervous system is somehow affected by the stress of a novel environment before the attainment of stable reproductive behaviors (Pfaus and Wilkins, 1995). In the wild, though, a male rat tends to copulate in familiar environments and generally will not do so in unfamiliar territory (Pfaus and Wilkins, 1995), but this does not explain why a minority of rats do not display sexual behavior in the lab under any circumstances (Crowley, Popolow, Ward, 1973; but see Agmo, 1999 for a review).

Theoretical Considerations of Sexual Behavior

There have been several different attempts to describe male rat sexual behavior in terms of a conceptual framework. Frank Beach conceptualized the behavior of copulation as having two distinct parts; sexual arousal, or precopulatory behaviors, and copulatory performance (Everitt, 1990). In the first distinction, precopulatory behaviors are under the control of the sexual arousal mechanism (SAM) which determines the entire set of behaviors displayed by the male before mounting and intromitting are performed after exposure to an estrous female. In the male, the SAM is important for determining the amount of sexual receptivity of the estrous female by an evaluation of her behavioral displays as well as her scent, and also is important for integrating these signals and summating the male's 'urges' to a threshold where the display of mounting and intromitting are more likely to take place. Once the male has reached a threshold for responding, a second and separate mechanism takes over. This intromission and ejaculatory mechanism (IEM) integrates the sensory stimuli and initiates the execution of mounting and intromitting until ejaculation is achieved. This dichotomy between initiation and performance has remained to this day; however, a more refined description has been put forth by Sachs (1978) based on a factor analysis. The initiation factor, which is

conceptually similar to Beach's sexual arousal mechanism, encompasses the latencies to mount and intromit after presentation of an estrous female. A sexually experienced male will be considered sexually aroused if he initiates copulation quickly when in close contact with a receptive female when compared to a male that does not initiate copulation, or takes a long period of time to do so. Beach's intromission and ejaculatory mechanism is defined by Sachs (1978) as containing three different, but interactive, processes. The copulatory rate factor is measured by the 1) interintromission interval (III), 2) ejaculation latency, and 3) postejaculatory refractory period. Highly aroused and sexually experienced male rats will display a decreased III and a subsequently decreased latency to ejaculation, and as well, may initiate copulation faster after ejaculation, decreasing the postejaculatory interval to maximize reproductive fitness with a female in estrous. Finally, the intromission count factor describes the number of intromissions needed to achieve a single ejaculation and is meant to reflect the amount of stimulation needed to achieve an ejaculation; the fewer the intromissions needed suggests a decreased threshold for responding. Admittedly, the theoretical concepts outlined above are not mutually exclusive and both the SAM and the IEM of Beach, and the initiation factor, copulatory rate factor, and the intromission count factor of Sachs each rely on each other for the successful completion of copulation and impregnation of the female. As one researcher has stated, 'a male rat with no legs will have endlessly long mount and intromission latencies, but in no way can be said to be deficient in sexual motivation and arousal' (Everitt, 1990).

The concepts represented above have been used in the past to describe components of sexual behavior. In a more simplistic manner, the concepts of appetitive and consumatory drives have been adopted from ethology to conceptualize sexual behavior more recently. Those behaviors which bring the male into close contact with the female and make copulation more likely are termed appetitive behaviors (i.e., anogenital investigation and pursuit behaviors), and those behaviors which encompass the act of sexual reproduction (i.e., mounts, intromissions, and ejaculation) are termed consumatory (see Nelson, 2000). It should be noted, though, that the appetitive and

consumatory distinctions have been employed to describe a number of different behaviors, not just those related to reproduction.

Anatomical Localisation of Areas Regulating Sexual Behavior

Early Research on the Neural Control of Reproductive Behavior

Anatomical evidence indicates several areas within the mammalian nervous system are needed to co-ordinate the behavioural activities involved in sexual reproduction in both males and females. Elucidation of these areas comes from studies using excitotoxic or electrolytic lesioning techniques, as well as stimulation studies. Based upon previous findings in female guinea pigs that bilaterally placed lesions between the optic chiasm and the infundibulum disrupt mating behavior independent of normal pituitary functioning, Brookhart and Dey (1941) reported some of the earliest research on the nervous control of sexual behavior. In these initial studies, lesions of the ventral hypothalamic region reduced mounting and intromitting components of sexual behavior, and this in turn reduced the number of successful pregnancies by these males, but the lesions had no effect upon androgen secretion, sperm production, or seminal plug formation and thus suggested disruptions in the nervous system somehow affected the normal display of male reproductive behavior. However, the lesions were substantial and destroyed tissue throughout the extent of the hypothalamus and thalamus, and thus, a determination if a single collection of cells was important for mating was not possible (Heimer and Larsson 1964). Given this, Heimer and Larsson in the 1960's presented evidence from a more refined lesioning study that a specific locus within the diencephalon was important for sexual behavior. First, they demonstrated that a complete lesion that isolated the forebrain from the rest of the caudal nervous system augmented mating in male rats; lesioned animals became hypersexual- the latency to ejaculate was substantially reduced due to a decrease in the number of intromissions required, as well, initiation of mating after ejaculation was increased as the post ejaculatory interval was significantly shortened (Heimer and Larsson 1964). However,

more restrictively placed lesions revealed that the medial preoptic area (MPOA) was important for the normal display of male sexual behavior; extensive destruction of the entire MPOA completely abolished mating permanently, whereas lesions in discrete portions temporarily abolished mating in rats (Heimer and Larsson, 1966/67). In addition to this, bilateral implantation of electrodes just lateral to the third ventricle in the hypothalamus, and into the medial division of the medial preoptic area (mMPOA), increased the display of a number of sexual behaviors, decreased the number of mounts and intromissions needed for ejaculation, and decreased the post-ejaculatory refractory period (Merari and Ginton, 1975; Malsbury, 1971; Stephan, Valenstein, Zucker, 1971), suggesting this area is the critical area within the hypothalamus for the display of sexual behavior. Bilateral destruction of the mMPOA results in a complete loss of sexual behavior (Hansen, Kohler, Goldstein, and Steinbusch, 1982; Klaric and Hendricks, 1986; Maillard-Guteknust and Edwards, 1994; Twiggs, Popolow, Gerall, 1978), as does severing the inputs and outputs of the medial-lateral or dorsal fibres (Szechtman, Caggiula, and Wulkan, 1978).

Further, evidence indicates there is a distinct sexual dimorphism within the central portion of the mMPOA; Male rats and hamsters have been reported to display an increased volume (Gorski, Gordon, Shryne, Southam, 1978) and larger dendritic field compared to female rats (Greenough, Carter, Steerman, and DeVoogd, 1977). This area has subsequently been labelled the sexually dimorphic nucleus of the preoptic area (SDN-POA; Simerly and Swanson, 1986) and contains a dense collection of fibers which contain serotonin, however, even though its development is androgen dependant, its exact role in the regulation of reproductive behaviour, if any, has not been proven (Gorski et al, 1978).

The MPOA is reciprocally connected with several areas from the olfactory bulbs, cortex, midbrain, brainstem, and spinal cord and is implicated in a number of behaviors such as temperature homeostasis, thirst, regulation of hormone secretion, and maternal behavior (Simerly and Swanson, 1986). Major areas within the hypothalamus send inputs to the MPOA, as well, areas such as the lateral hypothalamus and lateral preoptic area send efferents, via the medial forebrain bundle, to the MPOA. Several areas in the

amygdala and other limbic regions, the encapsulated part of the bed nucleus of the stria terminalis, raphe nuclei, as well as areas within the brainstem such as the ventral tegmental area, periaqueductal gray, central tegmental fields, locus coeruleus, and the pedunculopontine region all terminate within regions of the MPOA. Further, monamines, gut peptides, hypothalamic releasing factors, pituitary hormones, enkephalins, and calcitonin gene related peptides are expressed within cells of the MPOA or are contained within afferents synapsing into discrete regions within the nucleus (Simerly, Gorski, and Swanson, 1986).

The Medial Preoptic Area and Its Role in Copulation

Because of the complex array of inputs and outputs to several regions within the central nervous system, the MPOA is in a position to integrate a substantial amount of sensory information derived from visual, auditory, tactile and olfactory sources (Simerly and Swanson, 1986; Coolen, Peters, and Veening, 1998). As stated above, destruction of the MPOA results in cessation of consumatory sexual behaviors, but despite this, it is unknown if it is the motivation to seek sexual contact that is affected or if it is the actual performance of the act that it compromised.

Evidence suggests that the MPOA may be organized to activate certain precopulatory behaviors such as anogenital investigation, and pursuit and mounting. Destruction of this structure may not affect the actual performance of copulation (i.e. the ability to intromit and ejaculate), *per se,* but instead destruction results in a decreased 'desire' to approach and initiate copulation with a receptive female. Support for this suggestion has come from studies showing that MPOA lesioned males lose the preference for an estrous female, when given the choice between her and a male, or an anestrous female (Paredes, Tzschentke, Nakach, 1998). This study suggests the MPOA processes sex relevant stimuli from estrous females and 'activates' investigatory, leading to subsequent consumatory behaviors through afferents from olfactory and amygdala sources.

However, evidence which seems to contradict the assertion that the MPOA is important for activation of appetitive or arousal behaviors is derived from a study of lesioned males where indicators of desire where the dependant variable; lesioned males will bar press for access to an estrous female (Everitt and Stacey, 1987), and will endure painful shock to reach an estrous female residing on the opposite end of an electrified grid (Nelson, 2001), suggesting pathways or nuclei subserving arousal mechanisms are left intact, and thus, this suggests also that the MPOA is important for performance.

In addition to this evidence, MPOA lesioned monkeys will also not initiate sexual behaviors when presented with a receptive female, but they will still masturbate to ejaculation, again suggesting that mechanisms regulating appetitive behaviors are still fully functional in these males. This evidence has also been taken to suggest, though, that regions subserving performance duties are left intact- such as stimulation of erection- and this has led to the suggestion that MPOA lesioned animals display a failure to transition from arousal to copulation (Meisel and Sachs, 1994). In other words, they fail to move from appetitive to consumatory behavior.

To further elucidate the MPOA's role in reproduction, researchers have looked at other physiological indicators of sexual arousal beyond the ones described above. Perhaps one of the most unambiguous displays of sexual arousal is the display of non-contact erections (NCE; Liu, Salamone, Sachs, 1997a, b). Sexually experienced males display NCE's when presented with the scent of an estrous as opposed to an anestrous female (Sachs, Akasofu, Citron, Daniels, Natoli, 1994). Males with MPOA lesions display a deficit in copulatory behaviors without affecting the display and number of non-contact erections. In contrast, the incidence of NCE's was severely decreased, with only minor effects on copulatory performance, in animals with lesions to an area reciprocally connected to the MPOA, the bed nucleus of the stria terminalis.

Finally, only one group has proposed that the MPOA is organized for both appetitive and consumatory behaviors, a conclusion that is based upon electrophysiological data in freely moving and copulating male rats (Shimura, Yamamoto, Shimokochi, 1994). Cellular activity in the MPOA is elevated above baseline precopulatory levels with both pursuit and after mounting and pelvic thrusting; the return to baseline cellular activity

was correlated with the postejaculatory refractory period, concomitant with sexual satiety. However, because of a lack of consensus and compelling evidence, the current view is that the MPOA regulates consumatory sexual behavior.

The importance of the MPOA to sexual behavior has been extended to include other species such as cats (Hart, Haugen, and Peterson, 1973), dogs (Hart 1974), goats (Hart 1986), and monkeys (Slimp, Hart, and Goy, 1978), however, several areas within the olfactory bulb, cortex, diencephalon, mesencephalon, and metencephalon, as well as the spinal cord are bidirectionally connected to the MPOA, suggesting several areas outside of the hypothalamus are also involved in the regulation of sexual behavior (see Simerly and Swanson, 1986).

The Spinal Cord and Projection Sites in the Brainstem

In the lumbar spinal cord (L4/L3 region), an area within lamina 10 that contains newly described lumbar spinothalamic (LSt) cells that project to the thalamic parvocellular subparafasicular nucleus (SPFp), is preferentially activated with ejaculation (Truitt and Coolen, 2002). Destruction of these cells via excitotoxic lesions prevent ejaculation but do not affect other aspects of appetitive and consumatory sexual behavior in male mice (Truitt and Coolen, 2002). It is thought that this area is the site for the generation of ejaculation in males, but may only be involved in the relay of somatosensory information from the penis to the MPOA of the hypothalamus by way of the SPFp in the thalamus. Regulation of ejaculation may be generated via oxytocinergic fibres originating in the paraventricular nucleus (PVN) that synapse on to cells within the intermediolateral (IML) column of the lumbar spinal cord (L6), and regulate seminal emission through inputs to the internal and external genitalia (Ackerman, Lange, and Clemens, 1997). The PVN has been implicated in modulation of sympathetic and parasympathetic transmission to the lumbar spinal cord, as cytotoxic lesions of the PVN with NMDA prevent seminal emission (by decreasing oxytocin neurotransmission) without affecting any other aspects of mating (Ackerman, Lange, and Clemens, 1997).

In addition, the periaqueductal gray (PAG) in the tegmentum, and the lateral paragigantocellular reticular nucleus (LPGi), in the caudal ventral medulla, have been implicated in the regulation of copulation. Discrete columns of cells within the PAG, that project to the MPOA, are activated with copulation (Murphy and Hoffman, 2001). In addition, the PAG is intimately connected with the LPGi (Murphy and Hoffman, 2001); This area is thought to be the primary descending inhibitory input to the motoneurons regulating penile reflexes, as severing the axons and subsequent stimulation of the LPGi decreases the latency to achieve erection, causes the striated perineal muscles to contract, and culminates in ejaculation in *ex copula* tests (Marson and McKenna, 1990), and decreases the latency for ejaculation through a reduction of the number of intromissions during *in copula* tests (Lui and Sachs, 1999).

The LPGi of the ventral medulla is considered a hub of sensory integration by a number or researchers (Hermann, Holmes, Rogers, Beattie, Bresnahan, 2003). This area receives direct input from all divisions of the cochlear nuclei, areas involved in somatosensory processing such as the gracile and cuneate nuclei, as well this area sends afferents to respiratory groups in the medullary reticular formation. Through its reciprocal connections with the PAG, the LPGi is regulated by hypothalamic, preoptic, and limbic sources leading to the suggestion that this area may regulate a number of behavioral responses in addition to sexual reproduction, such as pain perception, acoustic startle, breathing and heart rate, aggression, as well as tactile information processing (Hermann et al., 2003). That the MPOA is linked to the pelvic region through the PAG and LPGi suggests that information received from the penis and other motor areas of the spinal cord produces a functional circuit that is also important for postural changes associated with the behavoirs elicited during reproduction (i.e., mounting and intromitting) (Murphy and Hoffman, 2001).

The sexually dimorphic spinal nucleus of the bulbocavernosus (SNB; Breedlove and Arnold, 1980), that receives serotonergic axonal input from the LPGi, and raphe magnus and pallidus (Marson and McKenna, 1990, 1996), regulates measures of penile functioning by directly affecting the activity of the bulbocavernosus (BC) and ischiocavernosus (IC) muscles of the perineal cavity connected to the penis (Meisel and

Sachs, 1994). During copulation, these muscles are involved in the generation of penile flips and cups needed for successful insemination of the female (Hart and Melese d'Hospital, 1983). Stimulation of the SNB by electrodes elicits activation of cells within several areas in the brainstem such as the gigantocellularis and lateral gigantocellularis nuclei (Gi-LPGi continuum; Meisel and Sachs, 1994). However, the SNB is also innervated by other areas within the brainstem such as the locus coeruleus and subcoeruleus, A5 adrenergic cells, reticular cells within the medulla and pons that control micturition and other eliminative functions (Marson and McKenna, 1990; Shen, Arnold, Micevych, 1990).

Other Areas in the Nervous System Implicated in Male Sexual Behavior

Perturbations in sexual behavior can also be produced with the destruction of regions which are reciprocally connected to the MPOA. For instance, not only does the BNST regulate non-contact erections, but it is important for the normal display of ejaculation; electrolytic lesions in this region produce males that displayed an increase in the number of intromissions, and for those that do ejaculate, the post ejaculatory refractory period is increased (Valcourt and Sachs, 1979; Claro, Segovia, Guilamon, Del Abril, 1995). An opposite effect on copulation is observed with amygdala lesions.

Destruction of the amygdala produces animals that are hypersexual, and which indiscriminately mount animate and inanimate objects (see Meisel and Sachs, 1994). Paradoxically, however, discrete amygdala lesions produce animals with sexual performance deficits (De Jonge, Oldenburger, Louwerse, van de Poll, 1992); destruction of the corticomedial division of the amygdala produces animals with an increased latency to achieve a single ejaculation, while decreasing the number of ejaculation displayed in a copulatory bout, without affecting mounting or intromitting, whereas destruction of the basolateral amygdala produces no observable deficits. In addition, the deficits observed in the corticomedial lesioned animals is only evident if the female rat is

improperly primed- that is, females treated with estrogen only (and hence do not display the full pattern of sexual behaviors). Males performed normally if the female rats were injected with both estrogen and progesterone, suggesting that the deficits observed in the corticomedial lesioned animals were context specific and behaviourally sensitive.

Areas processing olfactory stimuli have also been implicated in the induction of copulation in rats, gerbils, and hamsters (Singer, 1991; Clancy, Macrides, Singer, Agosta, 1984). Removal of the vomeronasal organ that projects to the accessory olfactory bulb and subsequently to the MPOA and BNST via the amygdala, prevented copulation after presentation of a pheromone discharged from the female vagina known to induce mount, intromission, and ejaculation in hamsters and gerbils (Clancy, Macrides, Singer, Agosta, 1984; Murphy and Schneider, 1970). As well, bulbectomized rats display longer intromission latencies and only a few will mount and intromit to ejaculation, even when administered gonadotropins or gonadal steroids (reviewed in Meisel and Sachs, 1994).

Maillard-Guteknust and Edwards (1994) have provided evidence that connections to the MPOA from the rostral brainstem are important for the display of male sexual behavior. In this study, unilateral destruction of connections to the MPOA combined with contralateral destruction of areas in the caudal brainstem produced males that mated infrequently in 30 minute tests, and those that did mate to ejaculation, took longer than control males. However, the lesions were quite extensive throughout several regions of the brainstem, some encompassing the retrorubral fields and pedunculopontine tegmental nuclei, as well as parts of the periaqueductal gray and central gray regions making interpretation difficult.

The dorsolateral tegmental area (DLTg) in the pons has also been implicated in sexual behavior as studies have shown it may mediate some aspects of motivation to seek copulation (Edwards and Einhorn, 1986). Axons through the medial forebrain connect the DLTg to the MPOA, and destruction of the DLTg significantly impairs or abolishes copulation possibly by eliminating the rewarding effects of social and genital contact in sexually experienced males (Edwards and Einhorn, 1986).

Lesioning studies, however, are limited in interpretation as it is often unknown if destruction of the particular region of interest is regulating appetitive and/or consumatory behaviors, or if it is the axons passing through that particular region which are regulating aspects of copulation. Modern techniques use markers of cellular activation in an attempt to isolate and implicate structures which may be important for the display of male sexual behavior. Some of the earliest markers included the use of 2deoxy-glucose, a metabolic energy source which is taken up by activated cells, but more modern markers include the use of immediate early genes and their subsequently translated protein products, which are expressed after behavioral manipulations (discussed below).

Copulation Induced Fos Immunoreactivity is Expressed in Discrete Regions within the Nervous System

Initial reports indicated that the expression of the immediate early gene (IEG), *c-fos*, could be rapidly induced within fifteen minutes after exposure of PC12 cells to growth factors (Greenberg, Greene, and Ziff, 1985). Subsequently, growth factor stimulated *c-fos* mRNA expression was shown to be substantially enhanced in the presence of agents that affected the electrical activity of the cell (Curran and Morgan, 1985), and this led to the suggestion that cellular activity could be coupled to the genetic transcription machinery and modulate long-term changes in cellular physiology and morphology perhaps by increasing the number of boutons and evoked junctional currents at synapses (Sanyal, Sandstrom, Hoeffer, Ramaswami, 2002). In addition, because these IEG's are rapidly stimulated above baseline, they are suitable markers of induced cellular activity, and can be used to map the neuroanatomical location of areas in the nervous system that become activated after behavioral manipulations.

In mammals, the distribution of Fos immunoreactivity has been observed in several areas in the forebrain and spinal cord after sexual behavior. For example, the MPOA, BNST, medial amygdala (MeA), central tegmental fields (CTF), parvocellular subparafasicular nucleus (SPFp), nucleus accumbens (NAc) and spinal LSt cells, as well

as several other areas, are activated after various aspects of sexual behavior in rats (Greco, Edwards, Zumpe, Michael, Clancy, 1998; Greco, Edwards, Michael, Zumpe, Clancy, 1999; Greco, Edwards, Zumpe, Clancy, 1998; Truitt and Coolen, 2002; Truitt, Shipley, Veening, Coolen, 2003; Coolen, Peters, Veening, 1997; Kollack-Walker and Newman, 1995; Struthers, 2001; Robertson, Pfaus, Atkinson, Matsumura, Phillips, Fibiger, 1991; Baum and Everitt, 1992; Wersinger, Baum, Erskine, 1993).

However, mapping cellular activation in response to differing amounts of copulation may also be used to dissociate the contributions of certain brain regions to appetitive and consumatory behaviors. For instance, Fos immunoreactivity was detected in the brains of rats that received only olfactory input versus rats that received both olfactory input and genital stimulation from copulation (Wersinger, Baum, Erskine, 1993). In this study, levels of Fos immunoreactivity were significantly increased in the MPOA, CTF, MeA, and BNST in males allowed to mate to copulation (i.e., they displayed both appetitive and consumatory behaviors), but the display of Fos was eliminated in the SPFp after transection of the pelvic nerve (Wersinger, Baum, Erskine, 1993). These data suggest that the SPFp is important for the transmission of genital/ visceral information as this area appears to only express Fos after ejaculation. This is further supported by data showing that unilateral destruction of the SPFp eliminates Fos in the BNST and MPOA following ejaculation (Baum and Everitt, 1992), and that the SPFp sends efferents to these forebrain areas, but these connections are not reciprocated (Heeb and Yahr, 2001).

The distribution of copulation induced activation of cells in the spinal cord has been described in different areas, termed laminae, of the spinal cord. Lamina X cells (interneurons located around the spinal canal) in lumbosacral regions also express Fos immunoreactivity after copulation (Greco et al., 1998). This group of cells also resides in the same level of the cord as the motoneurons of the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB), which sends afferents to the perineal muscles, bulbocavernosus and ischiocavernosus (Breedlove and Arnold, 1980). However, unlike the lamina X cells, SNB motoneurons may use Jun as their primary IEG, as these levels are elevated above basal intensity preferentially after consumatory elements of sexual

behavior; nuclear Fos immunoreactivity is not detected in this region after consumatory sexual behavior (Greco et al, 1998; Monks and Watson, unpublished observations).

Confirming stimulation and lesion studies implicating limbic and hypothalamic sites in the regulation of sexual behavior (as mentioned above), Fos immunoreactivity can be induced in the BNST and MeA in males that mate to ejaculation, but this pattern is not observed if males simply mount and intromit (Coolen, Peters, Veening, 1997). In addition, the periaqueductal gray (PAG) in the midbrain displays an increase in Fos immunoreactivity after mating in male rats (Greco, Edwards, Michael, Clancy, 1996).

Further, recent work suggests that the nucleus gigantocellularis (Gi) is also involved in regulating penile reflexes (Holmes, Hermann, Rogers, Bresnahan, Beattie, 2002); lesioning of the Gi-LPGi reduced the latency to first erection in *ex copula* penile reflex tests, while subsequently increasing the frequency in display of erections when compared to sham operated controls.

While it has been shown that areas within the olfactory bulbs, forebrain, and midbrain, as well as the lumbosacral spinal cord, are important for the regulation and expression of sexual behavior, studies regarding the contribution of sites within the brainstem are not definitive. It has been reported that copulation induced Fos expressing cells within the MPOA are colocalized with efferents from areas within the pontine region of the upper brainstem (Struthers 2001), and that pudendal motoneurons are connected to brainstem sites such as the medial and spinal vestibular nuclei, the Gi-LPGi, pontine reticular nucleus, and regions in the parvocellular reticular area, suggesting these areas may be involved in copulation. However, descriptions of the distribution of Fos immunoreactivity within the medulla and pons after sexual behavior are lacking.

Thus, a major aim of the current study is to quantify cells in the pons and medulla that express Fos immunoreactivity in males that have displayed appetitive and consumatory behaviors, and compare this to non-copulating and socially isolated animals.

Gonadal Steroids and Reproductive Behavior in the Male Rat

Gonadal steroids such as testosterone and estrogen exert permanent changes upon the nervous system developmentally via regulation of the process of apoptosis (organizational effects), and further increase the likelihood of the display of certain behaviors in adulthood such as copulation and aggression (activational effects).

Steroid hormone receptors are a family of transcription factors that exist in the cell membrane, cytosol, and sometimes unbound within the nucleus (Evans, 1988). The classic view of steroid receptor functioning proposes that genetic transcription and eventual protein synthesis is activated after 1-2 hours of ligand binding; these are the so called 'genomic' effects of activated steroid receptors. Genomic effects of gonadal steroids are well documented in peripheral and nervous tissues. For example, neuronal cadherins (N-Cadherins) and calcium gene-related peptide are both directly regulated by androgens by a cell autonomous manner in spinal motoneurons (Monks, Getsios, MacCalman, Watson, 2001; Monks, Vanston, Watson, 1999). As well, N-Cadherins are regulated by androgens in the hippocampus of rats (Monks, Getsios, MacCalman, Watson, 2001). Developmentally, gonadal steroids are important for the creation of sexually dimorphic structures in the brain and spinal cord by regulation of the perinatal cell death process (Breedlove and Arnold, 1980; Arnold and Gorski, 1984). Further, androgens also have important neurotrophic-like and neuroprotective effects upon facial motoneurons after injury (Jones, Brown, Damaser, 2001); the recovery of function after facial nerve crush is substantially augmented in hamsters after exogenous androgen or estrogen treatment, perhaps by attenuating the stress response cells display after trauma, and by up-regulating the expression and synthesis of scaffolding proteins such as beta-tubulin (Jones et al, 2001).

However, several reports of rapid effects of steroids on reproductive and nervous tissues have been reported; these rapid effects are termed 'non-genomic' or 'non-classical' steroid effects (Revelli, Massobrio, Tesarik, 1998). These can be elicited: 1) in cells such as spermatozoa that do not accomplish mRNA production; 2) in the presence of transcription or protein synthesis inhibitors; and 3) by cell impermeable steroids

complexed to bovine serum albumin (see Revelli et al., 1998; Benten, Lieberherr, Sekeris, Wunderlich, 1997). In splenic T cells bathed in the androgen receptor blocker, cyproterone, bovine serum albumin-conjugated testosterone induces an increase in intracellular calcium within seconds (Benten et al., 1997), suggesting androgens can induce rapid non-genomic effects, possibly mediated by a recently described cell surface androgen receptor (Lieberherr and Brosse, 1994). Behaviourally, exogenous estrogen treatment of gonadecotmized male rats stimulates female chemoinvestigation within 35 minutes (Cross and Roselli, 1999), suggesting a non-genomic action on male sexual behavior.

The specificity of hormone action on cellular physiology is maintained through the restricted expression of steroid receptors in subpopulations of nervous and peripheral tissues. Early reports using radiolabelled testosterone or its major metabolite, dihydrotestosterone (DHT), as well as radiolabelled estrogen, reported that distinct populations of cells in the nervous system accumulated these steroids (Pfaff and Keiner, 1973; Pfaff, 1968; Sar and Stumpf, 1977a, b). Moreover, the distribution of mRNA for estrogen and androgen receptors as demonstrated by *in situ* hybridization mirrored the radiolabelled accumulation data (Simerly, Chang, Muramatsu, Swanson, 1990). Specifically, several areas within the hypothalamus, such as the MPOA, and telencephalic areas such as the septal nucleus, displayed the highest levels of mRNA expression. mRNA was also detected in areas of the cortex, olfactory bulbs, the hippocampus, amygdala, basal ganglia, and thalamus.

In several mammalian species tested to date, gonadal steroids have an important role in activating sexual behavior. Castration of male rats produces gradual deficits in consumatory sexual behavior where ejaculation is lost first, then mounts, followed by mounting behavior, leading to eventual cessation of reproduction after long-term deprivation of gonadal steroids (Meisel and Sachs, 1994). Exogenous treatment with testosterone reverses the effects of castration, where the restoration of copulation begins first with mounts, followed by intromission, and eventually ejaculations (Meisel and Sachs, 1994).

Subsequent studies have shown that the major testosterone metabolites both participate in the activation and maintenance of sexual reproduction (Baum and Vreeberg, 1973). These findings are further supported by studies showing androstanediols, the metabolically reduced derivatives of the androgen, DHT, also have facilitative effects upon chronically castrated male rats; 3β diol and DHT treatment restores mounts, intromissions, and ejaculations to near precopulatory performance and in a similar manner as estrogen plus DHT, whereas 3α diol plus DHT, or 3α diol alone, has no effect (Morali, Oropeza, Lemus, Perez-Palacios, 1994). 3β diol binds with high affinity to estrogen receptors and mimics the effects of estrogen in a number of different behaviors, whereas 3α diol does not interact with nuclear estrogen receptors (Morali et al., 1994). Moreover, the restoration of penile reflexes in chronically castrated male rats is under the control of the androgen receptor; testosterone and dihydrotestosterone both facilitate penile erection, whereas estrogen treatment is ineffective (Gray, Smith, Davidson, 1980). Taken together, these data support the conclusion that activation of centrally distributed estrogen receptors, as well as peripherally residing androgen receptors are both necessary for the display of masculine sexual behavior (Morali et al., 1994).

Because of the high concentration of androgen receptors in the MPOA and the dependence of sexual behavior on circulating gonadal steroids, as well as numerous physiological and anatomical studies, this collection of cells has been suggested to be the critically important region for the display of masculine sexual behavior. However, a recent study has suggested that androgens can also regulate the display of copulation in areas outside of the MPOA (Harding and McGinnis, 2003). Castrated male rats implanted with testosterone directly into the ventromedial nucleus (VMN) of the hypothalamus displayed a subsequent restoration of sexual performance. Importantly, the diffusion of testosterone was limited to VMN neurons only, as only these cells contained nuclear immunostaining and no effect upon weights of reproductive tissues was observed, suggesting this region was sufficient to activate sexual behavior via androgen receptor dependant mechanisms.

Given that the brainstem is intimately connected with areas in the brain rostrally, and areas in the spinal cord caudally, it is also possible that other steroid accumulating, androgen receptor expressing cells in the brainstem could participate in the regulation of sexual behavior. To test this assumption, we quantified the distribution of androgen receptor (AR) immunoreactive cells in areas of the brainstem where Fos expression was associated with copulation, as this has previously not been done.

METHOD

Animals

24 adult male Long Evans rats 63-90 days old were obtained from Charles River Laboratories (Saint-Constant, Quebec) and housed three animals per cage with access to tap water and lab chow *ad libitum*. 12 female Sprague Dawley rats 90 days of age were obtained from the University of British Columbia breeding colony or from local stock at Simon Fraser University's Animal Care Facility. Female rats were singly housed and had access to tap water and lab chow *ad libitum*. All animals were subject to a 12/12hr light/dark cycle, with lights off at 1000hrs. All animals were treated according to the Canadian Council on Animal Care guidelines and all protocols were approved by the University Animal Care Committee at SFU.

Surgery

Bilateral ovariectomies (OVX) were performed under Fluothane anaesthesia. Briefly, an incision was made through the skin and muscle in between the rib cage and the flanks, exposing the ovaries internally. After removal of the ovaries, the muscle wall was sutured with cat gut and the skin was stapled with wound clips. Females were allowed to recover for two weeks before use in the training and testing phases (details below).

Apparatus

Ten gallon (50x25x30 cm) fish tanks obtained from Hagen (Richmond, BC) were used as testing arenas. Four arenas in total were used, but two of the cages were slightly modified; the glass was removed from one end of these cages and each was replaced with wire mesh. During the testing and training phases, the arenas contained

fresh bedding and the tanks were cleaned between each session with diluted Qautricide, and the bedding was replaced.

Steroid Treatments

OVX females were primed with 10ug of Estradiol Benzoate (Steraloids) and 500ug of Progesterone (Steraloids) 48 and 4 hours, respectively, before training and testing was to begin. The steroids were dissolved in corn oil and a 0.1 ml bolus was injected subcutaneously

Training

Each male was placed in the testing apparatus five minutes before the training phase was to begin to acclimate to the surroundings. After this period, OVX females were then placed into the cages. Males were allowed to copulate for 1 hour, after which time, they were placed back into their home cages. This procedure was repeated for six days, with a different female paired with a different male on successive training days. Observations were made to ensure males were displaying appropriate sexual behaviors and were becoming more proficient after each testing session. Females were only used every four days, and training took place at the same time each day (1200hrs) in the colony room under red light illumination. Females were replaced during the training sessions as needed to stimulate sexual behaviors in the males. To be included in the study, males had to achieve a single ejaculation within 15 minutes the day before the 'test phase' to ensure an acceptable level of copulatory efficiency; three males did not achieve this level of performance and were not included in the study.

Test

Males were randomly divided into three different groups the day of testing: copulators (Cop;n=7), non-copulators (NC; n=7), and social isolates (SI; n=7). Each male was placed into separate testing arenas five minutes prior to testing, and at this

point, a wire mesh lid was placed over top of all three cages. For the SI male, a plexiglass lid was placed over top of the cage prior to testing (the wire mesh screen was placed over top of this). Vaseline was placed on the top edges to ensure a tight seal and that pheromonal cues emanating from the primed females were prevented from stimulating relevant brain areas in this control group. Once the five minutes had elapsed and the SI male was sealed into his cage, the NC female was placed into her cage adjacent to the NC male's cage. A primed female was then added to the Cop male's cage and the timer was started. The male was allowed to copulate until a single ejaculation, and was left in the cage for one hour more with the stimulus female. The non-copulator male and female, as well as the social isolate, were also all left in their respective testing arenas. After this time, the experimental animals were euthanised and then the females were returned to their home cages.

Thus, one test session consisted of 1) a male that was allowed to mount, intromitt, and ejaculate (the Cop males), 2) a male that was separated from a primed female via two wire mesh screens and was able to smell, see and hear the female, but was prevented from copulating (the NC males), and finally 3) a male that was sealed in a testing arena and only allowed to see and possibly hear the female, but prevented from physical and pheromonal contact (the SI males). On each test day, a single cohort of males (but sometimes two cohorts) comprising of a copulator, a non-copulator, and a social isolate was tested. After each testing session, the males were euthanised within approximately twenty minutes of each other (see below).

Copulatory Measures Collected

For this study, the frequency of mounts, intromissions, and ejaculations were collected; A mount was scored when the male raised up on his hind legs, and placed his forepaws on the back of the female and palpated her flanks, began to thrust his pelvis (but not always), but did not insert his penis. An intromission was counted if the male displayed mounting behavior, thrusted his pelvis rapidly, and achieved penile insertion-penile insertion was assumed when the male rapidly 'jumped back' (i.e., dismounted) off

of the female and groomed his penis. An ejaculation was counted when males displayed a number of mounts and intromissions, but, in the final intromission before and ejaculation, did not dismount rapidly, and instead, displayed a conspicuous deep thrust, followed by an extended 'clutching' of the female's flanks, and a subsequent falling on his side as the female leaped forward. In addition, 1) the latency to mount; the time from the addition of the primed female to the first mount, 2) the latency to intromit; the time from the introduction of the stimulus female to the first intromission, and 3) the ejaculation latency; the time from the introduction of the female to the first ejaculation were all also quantified (Tohei, Watanabe, Taya, 1998). The interintromission interval and the hit rate were also calculated as further measures of male sexual performance. The interintromission interval was calculated by dividing the ejaculation latency by the total number of intromissions and was used as an indicator of the male's level of arousal, and the hit rate was calculated by dividing the total number of intromissions by the total number of mounts plus intromissions. Observations of genital grooming (licking and grooming of the pelvic region) and non-contact erections (the display of an erection not associated with mounting, pelvic thrusting, or intromitting- males typically display NCE's when they can not display sexual behavior in response to proceptive behaviors from the female) were also made for the NC group, as well as the location within the cage these males spent most of the time during testing. The SI males were scored on general behavior (locomotor activity, defensive burying activity, inactivity, and exploration) and for the display of non-contact erections or genital/penile grooming. The females were observed for the display of ear wiggling and hop-darting to ensure proper priming of sexual behavior via steroid administration.

Tissue Preparation

Animals were killed with a blended mixture of O2 and CO2, and the chest cavity was opened to expose the heart. Transcardial perfusions were done with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS, which was used to adequately fix the tissue. The brain and spinal cord were dissected out and post-fixed

in 4% PFA for two hours. The tissue was placed in a 20% PBS-sucrose solution overnight for cryoprotection. Once the brain and spinal cord had sunk in the PBS-sucrose solution, both were removed, blocked in the coronal plane and placed on a freezing microtome (American Optical). The brainstem was cut into 50μm coronal sections beginning at the pyramidal decussation at the top of the cervical spinal cord (approximately –14.00mm from bregma; according to Paxinos, Carrive, Wang, and Wang, 1999) to the caudal ventral periaqueductal gray (approximately –8.0mm from bregma), and alternate sections from every animal were divided into thirds and placed into separate aliquots containing DeOlmos solution for protection and placed in the freezer (-20C) until processed for immunocytochemistry (ICC; Watson, Weigand, Clough, Hoffman, 1986). The spinal cord was also cut at 50μm coronally through the lumbar spinal levels, placed in DeOlmos and then in the freezer (-20 C) until processed for ICC (Watson, et al., 1986).

Immunocytochemistry

Fos was detected in alternate sections using immunocytochemistry. Free floating brainstem and spinal cord sections of the copulators, non-copulators, and social isolates processed in the same dish were washed six times in PBS containing Triton-X 100 (PBSx). Sections were incubated in 3% hydrogen peroxide and PBSx for 15 minutes to eliminate endogenous peroxidase activity, and then washed in PBSx three more times. A 90 minute incubation in normal goat serum (NGS; Bio/Can Scientific, Toronto, Ontario) in PBSx was used to block non-specific binding, and then the tissue was incubated in a rabbit anti-Fos primary antibody in PBSx and 1% NGS for 48 hours (Oncogene c-*fos*, Ab-5; used at a concentration of 1:80,000). This antibody recognizes amino acid residues 4-17 of the human Fos protein, and does not react with the 39 kDa Jun protein. The tissue was then washed in PBSx three times and incubated in a goat anti-rabbit secondary antibody (Vector *Elite* kit) at a dilution of 1:400 to recognise the primary antibody. After three more washes in PBSx, the tissue was incubated for one hour in an avidin-biotin horseradish peroxidase complex prepared according to manufacturer's

instructions (Vector Laboratories). The tissue was developed using diaminobenzidine hydrochloride (DAB) in Tris buffer with 3% H_2O_2 and 8% nickel chloride (NiCl) added for intensification; this solution precipitated a dark blue-black reaction product. Sections were mounted on gelatin-coated slides, dehydrated in graded alcohol solutions, and coverslipped with Permount (Fisher) for evaluation. Elimination of the primary antibody was used as a negative control.

For androgen receptor (AR) ICC (primary antibody, PG-21, was obtained from Dr. G. Prins at the University of Chicago, Illinois and used at a concentration of 1:3000), free floating brainstem and spinal cord sections from all three groups (copulators, non-copulators, and social isolates; 2 separate runs were done) were processed in the same dish and treated exactly the same way as above with slight modifications; PBSx contained 0.05% gelatin (PBS-GT) and the protocol also contained an extra avidin/biotin step to block non-specific staining. This step was added before incubation of the secondary antibody. Briefly, sections were first incubated in unconjugated avidin (Vector) diluted in PBS-GT and containing 0.015% normal goat serum for 20 minutes, and then incubated in a biotin/PBS-GT/normal goat serum solution for a similar amount of time. Again, the primary antibody was visualized via a DAB-hydrogen peroxide reaction with NiCl intensification; however, the incubation times were slightly modified on two different ICC runs (reacted for 5 minutes the first time, and then subsequently reacted for 10 minutes a second time as the first run produced ambiguous staining in some areas). Sections were mounted on gelatin-coated slides, dehydrated in graded alcohol solutions, cleared in Xylene, and coverslipped with Permount (Fisher) for visualisation. As a negative control, the primary antibody was omitted for some of the slices. To test the specificity of the AR antibody, the spinal nucleus of the bulbocavernosus, the dorsolateral nucleus, and lamina V-X interneurons in the lumbar spinal cord were used as a positive control as these areas have previously been reported to stain intensely for this antigen (Breedlove and Arnold, 1980). In addition, the sexually monomorphic retrodorsolateral nucleus was used as a negative control as AR-ir is not substantial in this nucleus. A subset of the AR slides was counter-stained with Neutral Red to verify the placement of several nuclei. Briefly, coverslips were soaked off

by placing them in Xylenes overnight. The sections were rehydrated through a graded solution of alcohols and deionized water, subjected to the counter-stain, and then placed back into the graded alcohol solutions, cleared in Xylenes and then recoverslipped with Permount for visualisation.

Microscopy

An observer blind to experimental conditions counted nuclear profiles of Fos and AR on coded slides. All mounted sections containing Fos or AR immunoreactivity were analysed under a standard light microscope (Nikon Eclipse E600), and putative areas of interest were first compared and verified with the architectonic atlases of Paxinos and Watson (1997), and Paxinos et al. (1999) using the 4X objective. Once the area of interest had been identified, cellular profiles were identified using the 10X objective. Subsequently, positive labelling was considered 'nuclear' if cells displayed consistent staining throughout the entire nuclear envelope of the cell and lack of immunoreactivity in the nucleolus under the 60x objective. The number of cells displaying Fos immunoreactivity was quantified for each of the copulator, non-copulator, and social isolates, using a 20X objective and an overall average number of labelled cells per section was calculated for each of these three groups. For androgen receptor staining, labelled cells were quantified on a mixed group of copulator, non-copulator, and social isolates, and are also represented as an average number of labelled cells per section.

For subjective ratings of the staining in the areas which contained Fos and AR immunoreactivity, a slide representative from each of the copulators, non-copulators, and social isolates was analyzed. First, the area(s) with the most intense labelling was identified and used as the measure of 'darkest' stain for that animal. Each area was subsequently compared to this and assigned a comparable rating (i.e., no staining, light, moderate, or dark), and this procedure was done in each of the animals. To arrive at an overall rating for each anatomical structure of interest, the subjective ratings obtained from each animal were compared to each other.

Statistical Analysis

All statistical analyses were carried out using Statistical Package for the Social Sciences (version 11.0 for Windows). Multiple one-way ANOVA's were used to test the overall differences in Fos immunoreactivity in areas analyzed, and planned comparisons were carried out using independent samples 2-way T-tests in areas where the ANOVA reached statistical significance. No correction for family wise error rate was made, as each T-test performed consisted of a family of three. For both the ANOVA's and T-test's, a rejection criterion was set at 0.05.

RESULTS

Sexual Performance and Behavioral Observations

On measures of sexual performance, Cop's displayed an average of 8 (±2.36 SEM) mounts and 10 (±1.1 SEM) intromissions. The average latencies to mount, intromit, and ejaculate were 70 seconds (± 21.74 SEM), 91 seconds (±32.85 SEM), and 617 seconds (±123.94 SEM), respectively. These values agree with previous reports of copulatory performance in rats of the same strain (Greco, Edward, Zumpe, Michael, Clancy, 1998). The average interintromission interval was 57.59 seconds (± 10.28 SEM), with an average hit rate of 62% successful intromissions with each mounting attempt. In the non-copulator group, 86% (6/7) of the male rats were observed to display genital grooming behaviors, and 50% (3/6) of those were observed to display non-contact erections and penile grooming. All males spent the majority of their time during the testing session near the wire mesh screen. They were observed digging in front of, and climbing, the wire mesh fence, and these behavoirs were displayed as soon as the estrous female was placed in the cage, but not during the acclimatization period just prior to her introduction. The females of the non-copulator group were observed to display typical proceptive behaviors such as hopping, darting, and ear wiggling; as well these animals were observed climbing on the wire mesh screen that separated them from the males suggesting the hormone manipulations were successful. Finally, in the social isolate group, males displayed such behaviors as pacing back and forth in the cage and sometimes digging and defensive burying behaviors. These males were never observed to groom their genitals, or to display non-contact erections or penile grooming.

Distribution of Fos Immunoreactivity

Previous results indicate that lamina X neurons in the lumbar spinal cord preferentially express more Fos immunoreactivity in copulators when compared to both

non-copulators and social isolates, where Fos expression was comparable in these two control groups (Greco et al., 1999; and Monks and Watson, unpublished observations). As can be seen in figure 1, there was more Fos in lamina X of copulators compared to non-copulators ($t=2.99$, $p=0.03$) and social isolates ($t=4.12$, $p=0.004$), but these two groups had similar amounts of Fos expression (0.648, $p=0.545$, n.s.), replicating previous results and confirming our behavioral manipulations.

Ratings of relative levels of Fos and AR expression are presented in Table 1. As can be seen, several areas within the medulla and pons expressed Fos immunoreactivity, as well as androgen receptor immunoreactivity. The darkest amounts of Fos immunoreactivity were expressed in the subtrigeminal/ lateral reticular nucleus (LRtS5), the nucleus ambiguus (Amb) that controls the pharynx and larynx, the nucleus gigantocellularis (nGi), and dorsomedial divisions of the dorsal cochlear nucleus. Similarly, the trapezoid body (Tz) contained immunoreactivity in the most medial divisions only. In the A5/dorsal periolivary region (DPO), and the pontine nucleus (Pn), Fos appeared dark in the nuclei, and distributed throughout.

Strong staining was observed in areas processing tactile sensory information, such as the external cuneate, cuneate, and nucleus X in the medulla. In addition, staining in the lateral paragigantocellular and gigantocellular reticular nuclei, the linear nucleus (Li), as well as the lateral and intermediate reticular nuclei all contained strong staining observed in cells distributed throughout the pons and medulla. In the pons, strong labelling was also observed in cells of auditory relay centres such as the supra paraolivary nucleus (SPO). The alpha, dorsal, and ventral divisions of the subcoeruleus (SubC), and areas of the reticular core (Simerly et al., 1990) such as the reticular tegmental area (reticular, ventral, and dorsal divisions) also contained strong staining. The dorsal raphe (caudal and lateral divisions) and the central gray (alpha, beta, and gamma divisions) in the pons contained strong staining. In an adjacent area to the periaqueductal gray region, the locus coeruleus (LC) and the medial micturition centre, barrington's nucleus (Bar), both contained cells displaying moderate to strong staining, as did the dorsal and ventral nuclei or the lateral lemniscus. Little Fos expression was observed in medullary vestibular and visceral processing areas; there was no Fos

expression in the superior vestibular nucleus or several divisions of the solitary tract, however, weak staining was observed within the medial division of the vestibular nucleus (MVe) and more caudal divisions of the ventral lateral solitary nucleus (SolVL), as well as in the prepositus (Pr). In addition, Fos immunoreactivity was not observed in the pontine trigeminal, facial, or the hypoglossal motor nuclei. In all areas that displayed Fos immunoreactivity in the non-copulator and social isolates, the staining appeared to be comparable to that of the Copulators in intensity; however, more subtle differences in staining perhaps could not be detected with the subjective rating scale used in this study. Fos immunoreactivity was eliminated in sections where the primary antibody was omitted.

Group Differences in Fos Expression

Overall ANOVA's revealed statistically significant differences in the: intermediate reticular nucleus ($F(2, 18)=8.1$, $p=0.003$), raphe pallidus ($F(2, 18)=4.11$, $p= 0.034$), linear nucleus ($F(2, 18)=3.57$, $p=0.049$), nucleus gigantocellularis ($F(2, 18)=5.99$, $p=0.01$), lateral paragigantocellular reticular nucleus ($F (2, 18)=6.8$, $p=0.006$), and dorsal cochlear nucleus ($F (2, 18)=14.51$, $p.<0.0001$) in the medulla; the nucleus of the trapezoid bodies ($F (2, 18)=18.56$, $p<0.0001$), posterodorsal tegmental area ($F (2, 18)=3.95$, $p=0.039$), and the reticulotegmental area ($F (2, 18)=4.94$, $p=0.02$) in the pons.

Tests for differences in means revealed that the LPGi ($t=2.442$, sig.=0.031; figure 2), the DC ($t=3.73$, sig.=0.003; figure 3), RPa ($t=2.507$, sig.=0.028; figure 4), Tz ($t=4.15$, sig.=0.001; figure 5), nGi ($t=2.398$, sig.=0.05; figure 7 and figure 11) of the Cop group contained significantly more Fos immunoreactivity when compared to the NC group. In addition, the RPa ($t=2.6$, $p=0.023$), nGi ($t=2.6$, $p=0.039$), LPGi ($t=2.9$, $p=0.024$), Tz ($t=5.4$, $p=0.002$), and the DC ($t=3.94$, $p=0.002$) of the Cop's also displayed more Fos immunoreactivity when compared to the SI animals. These areas did not differ significantly between the NC and SI groups.

The linear nucleus contained a unique pattern of results in that the Cop's and NC's did not differ in the amount of Fos expression, but the Cop ($t=2.67$, $p=0.02$) and the NC ($t=2.535$, $p=0.037$) displayed increased expression when compared to the SI's (figure 6).

In the medulla, the intermediate reticular nucleus (IRt) in the Cops displayed more Fos expression than in the SI's ($t=4.301$, $p=0.003$), however, the Cop's did not differ from the NC's ($t=1.874$, $p=0.085$), as well, the Nc's and SI's did not differ statistically in Fos expression ($t=2.101$, $p=0.057$). In the pons, the posterodorsal (PDTg) and reticulo-tegmental (RtTg) contained a similar pattern of results: The Cop's had more Fos immunoreactivity when compared to the SI's (for the PDTg- $t=2.87$, $p=0.027$; for the RtTg- $t=2.62$, $p=0.038$); the Cop's did not differ significantly from the NC's (for the PDTg- $t=1.424$, $p=0.18$; for the RtTg- $t=2.162$, $p=0.065$), and the non-copulators and social isolates had comparable amounts of Fos expression (for the PDTg- $t=1.85$, $p=0.108$; for the RtTg- $t=0.92$, $p=0.39$). These means are depicted in figure 8.

Androgen Receptor Distribution in Relation to Copulation Induced Fos Immunoreactivity

Immunostaining for the androgen receptor overlapped with Fos in regions of the caudal ventral medulla, which heavily innervate motoneurons controlling penile reflexes; Nuclear androgen receptor immunoreactivity was quantified in the lateral paragigantocellular reticular nucleus (figure 10, B and F), the nucleus gigantocellularis (figure 10, C and G), and the raphe pallidus (figure 10, D and H), as these areas also contained copulation induced Fos immunoreactivity. The lateral paragigantocellular nucleus contained an average of 67.5 cells per section (±6.7 SEM), the raphe pallidus contained an average of 26.54 cells per section (±3.6 SEM), and the nucleus gigantocellularis displayed a mean of 10.19 cells per section (±1.1 SEM). The lateral paragigantocellular reticular nucleus and nucleus gigantocellularis, as well as the raphe pallidus, all contained staining that was light to moderate. Other areas that were associated with copulation induced Fos staining, such as the dorsal cochlear nucleus,

linear nucleus, and the nucleus of the trapezoid bodies, all did not display androgen receptor immunoreactivity.

Androgen Receptor Immunoreactivity in Brainstem Regions Not Associated with Copulation Induced Fos Expression

Areas processing vestibular information did not contain androgen receptor immunoreactivity, with the noted exceptions of moderate staining in the dorsal medial spinal trigeminal and very light staining in the prepositus nucleus. Areas processing visceral information such as the medial and lateral nucleus of the solitary tract, all of the divisions of the parabrachial nucleus, and the area postrema contained dense AR-IR. Except for the nucleus ambiguus (figure 10, A and E), cranial motor neurons in the jaw (mo5), tongue (n12), face (n7; figure 11), and spinal trigeminal (SP5) all were AR negative. It appeared that the mesencephalic 5th nucleus (Me5) in the pons contained immunoreactivity, but the cells were small (probably interneurons or glia) and interspersed amongst the Me5 nuclei. The darkest and most abundant AR immunoreactivity was detected in several areas within pontoraphe regions such as the dorsal raphe, paramedian and median raphe nuclei, and the raphe obscurus and pallidus. Moderate levels of AR immunoreactivity were observed in the posterodorsal and ventral tegmental nuclei, but was absent in the reticulotegmental nucleus. Dark staining was observed in all areas of the central gray, the ventral periaqueductal gray, locus coeruleus, and barrington's nucleus in the pons. However, light staining was observed in the divisions of the subcoeruleus. No staining was observed in the medial/lateral ventral paraolivary complex or the inferior olivary.

Finally, AR immunoreactivity was observed in the spinal nucleus of the bulbocavernosus, dorsolateral nucleus, interneurons of lamina V-X in the dorsal horns, and the ventral periaqueductal gray, but was absent in the retrodorsal lateral nucleus as has been described previously (Breedlove and Arnold, 1980; see figure 9). In addition, nuclear AR immunoreactivity was eliminated in sections where the primary antibody was omitted.

Overlap of AR and Copulation-Independent Fos

There were several sites within the pons and medulla that contained Fos immunoreactivity that did not appear to be selectively activated with appetitive or consumatory sexual behaviors and that overlapped with several sites containing AR immunoreactivity (See Table 1). The activation of Fos may have been induced by handling prior to being placed in the testing arena, or by being introduced into the testing environment, or it may represent constitutively activated Fos that is not associated with behavior. For example, dark immunoreactivity for Fos was observed in the parvocellular reticular nucleus (PCRt) with light AR immunoreactivity in the same region. Both AR and Fos immunoreactivity were observed to be very dark within the paramedian reticular nucleus (PMn), and this was similar for the subtrigeminal lateral reticular nucleus (LRtS5) and nucleus ambiguus (Amb). Moderate Fos immunoreactivity was observed in the raphe obscurus (ROb), with only light AR staining in the same region. In the pons, both the dorsal raphe and central gray (alpha, beta, and gamma divisions) contained moderate staining for Fos, but contained dark AR immunoreactivity. Finally, both the locus coeruleus (LC) and barrington's (Bar) contained light Fos immunoreactivity, but contained dark immunoreactivity for AR.

The table below represents the areas expressing Fos and AR immunoreactivity. See the 'Microscopy' section for a description of how the data were collected. The data represent the combined ratings of a representative animal from the Cop, NC, and SI groups.

Table 1:

Subjective Density Ratings of Fos and Androgen Receptor Immunoreactivity

Medulla Area	Fos Rating	AR Rating	Pons Area	Fos Rating	AR Rating
AP	+++	+++	Pr	+	+
MdD	+	none	SP5O	++	none
Ecu	+++	none	Tz(m)	+++	none
Cu	+++	none	MVPO	+++	none
PMn	+++	+++	LSO	none	none
Ro	+++	none	SGe	++	none
12n	none	none	DPO(A5)	+++	none
SP5	none	none	SubC	++	+
PCRt	+++	+	Pn	+++	none
LRtS5	+++	+++	ml	+++	none
LRt	+++	none	LC	+	+++
IRt	+++	++	Bar	+	+++
AmbC	+++	+++	DRC, DRI	++	+++
Gi	+	none	MPB	+	none
nGi	+++	+++	MPBv	+	none
LPGi	+++	+	PnR	++	++
Sol	none	+++	CGalpha	++	+++
SolVL	+	+++	CGbeta	++	+++
Li	++	none	RtTg	+	none
RMg	++	+	VLL	+	none
RPa	+	+	DLL	++	none
IOc	+	none	PDTg	+	none
IO	none	none	MO5	none	none
SPVe	none	none	SPO	++	none
MVe	+	none	VLTg	+	++
Nucleus X	+++	none			
ROb	++	+			
DC	+++	none			

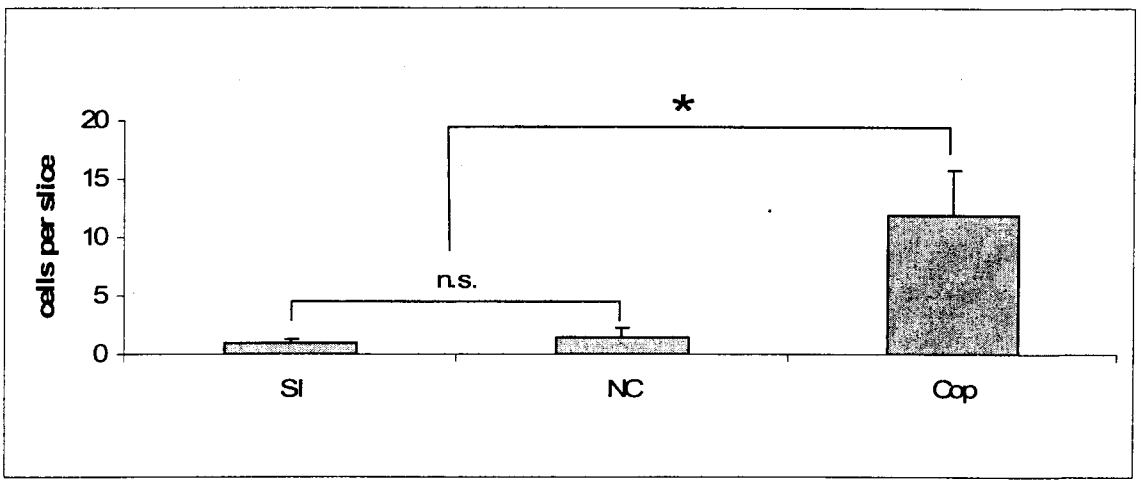
7n	none	none
ml	+++	none

Intensity Ratings: + light, ++ moderate, +++ dark

Figure 1 depicts the average number of cells per slice in Lamina X of copulators (Cop; n=7), non-copulators (NC; n=7), and social isolates (SI; n=7). Note, n.s. = non-significant at the 0.05 level; asterisk = significant at the 0.05 level.

Figure 1:

Fos Immunoreactivity in Lamina X of the Lumbar Spinal Cord

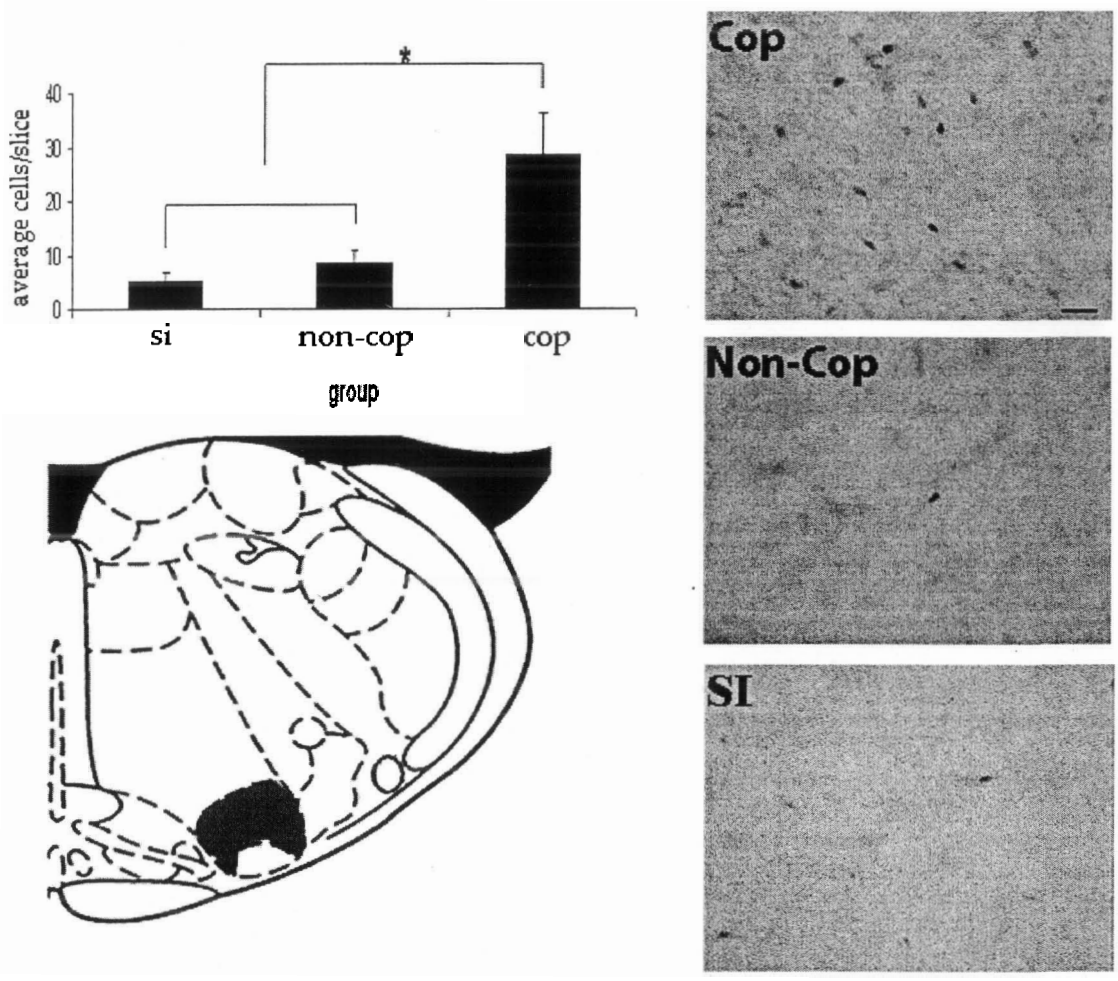


Note: n.s.= not significant; *= significant at the 0.05 level; error bars indicate standard error of the mean.

Figure 2 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the LPGi. The schematic diagram represents the location within the medulla that the LPGi is located; the light red depicts that this area is dark for Fos immunoreactivity. Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 2:

Fos Immunoreactivity in the LPGi



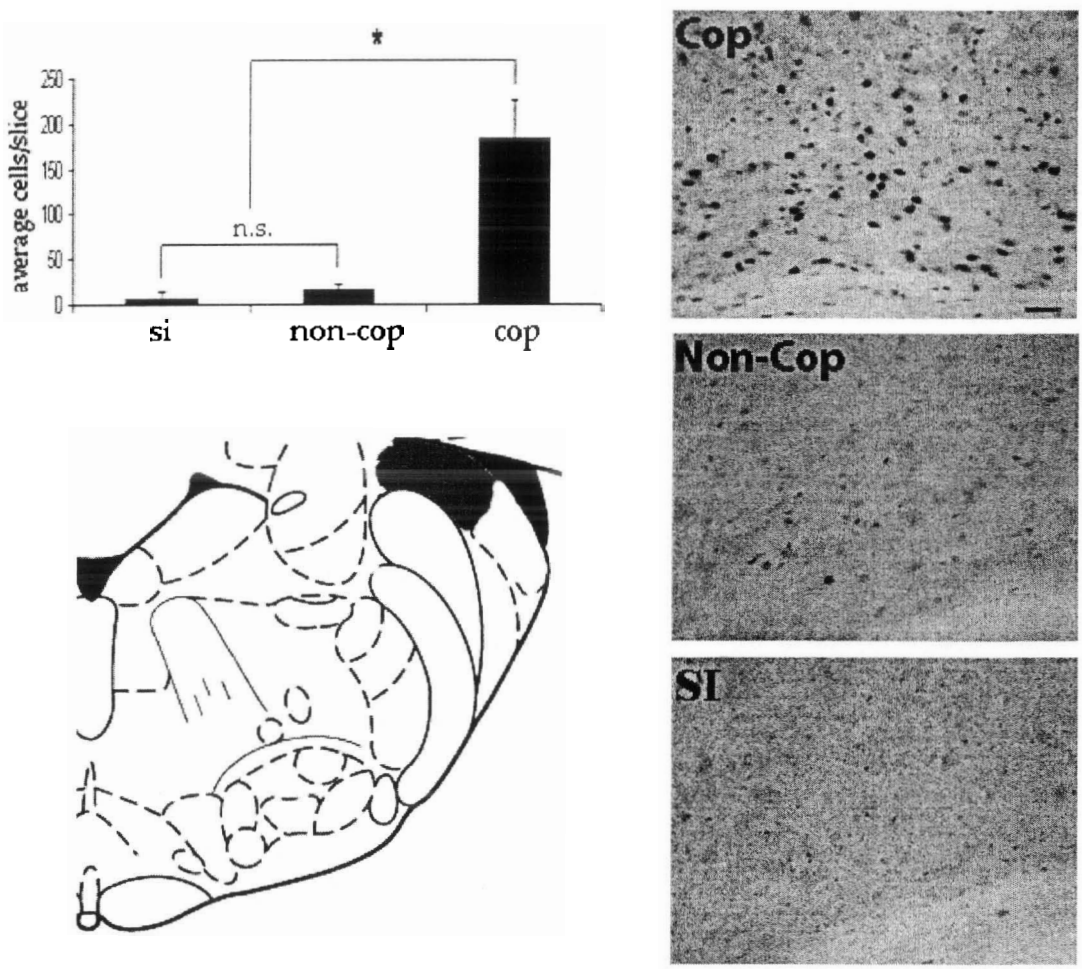
Note: n.s.= not significant; *= significant at the 0.05 level. scale bar is 50μm; error bars indicate standard error of the mean.

Figure 3 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the DC. The schematic diagram represents the location within the medulla that the DC is located; the red depicts that this area is dark for Fos immunoreactivity. Note that immunoreactivity is localized in the dorsal regions only.

Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 3:

Fos Activation in the Dorsal Cochlear Nucleus



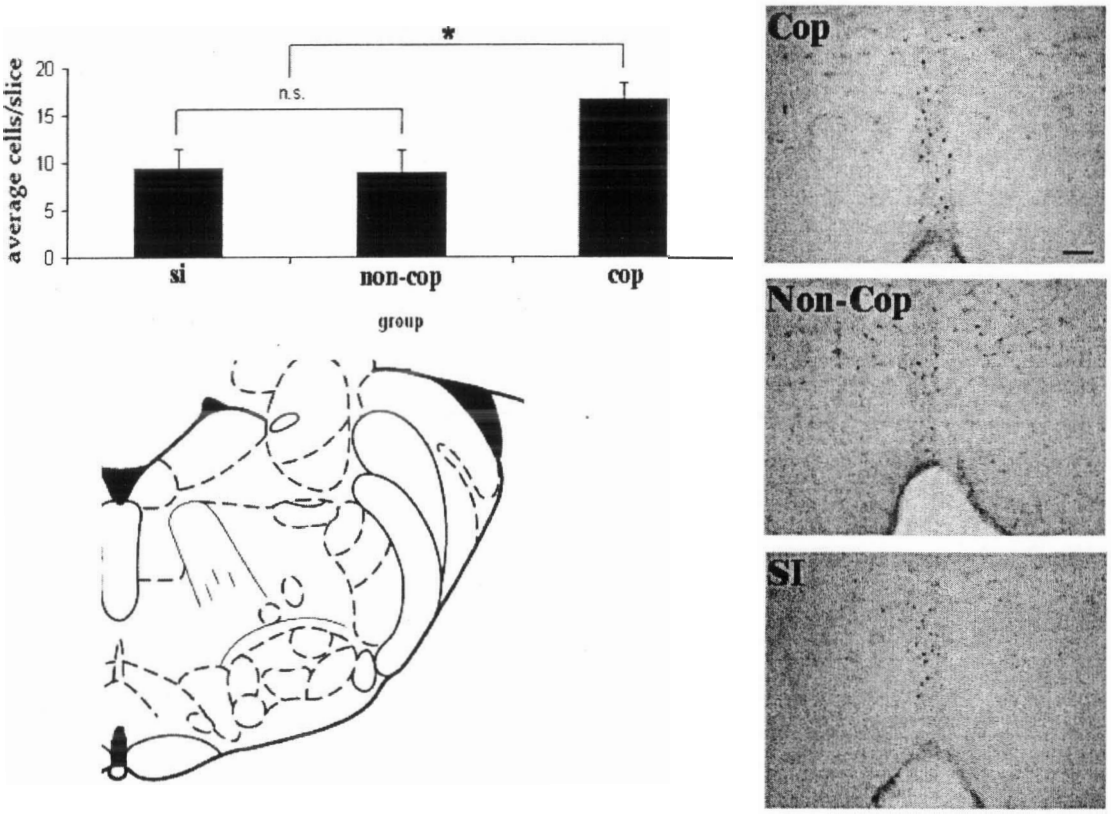
Note: n.s.= not significant; *= significant at the 0.05 level, scale bar is 50μm; error bars indicate standard error of the mean.

Figure 4 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the RPa. The schematic diagram represents the location within the medulla that the RPa is located; the red depicts that this area is dark for Fos

immunoreactivity. Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 4:

Fos Activation in the Raphe Pallidus



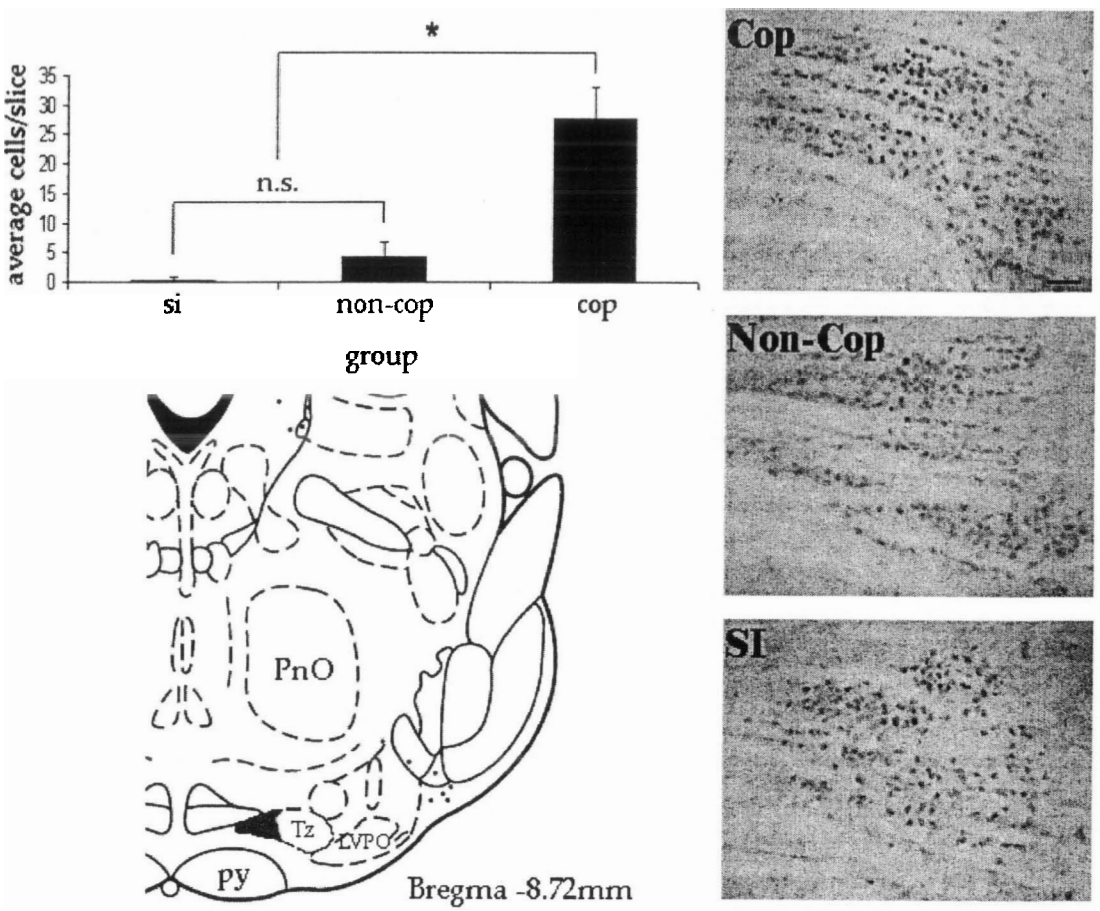
Note: n.s.= not significant; *= significant at the 0.05 level, scale bar is 50μm; error bars indicate standard error of the mean.

Figure 5 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the Tz. The schematic diagram represents the location within the pons that the Tz is located; the red depicts that this area is dark for Fos immunoreactivity. Note that immunoreactivity is localized in the medial divisions only.

Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 5:

Fos Activation in the Trapezoid Body



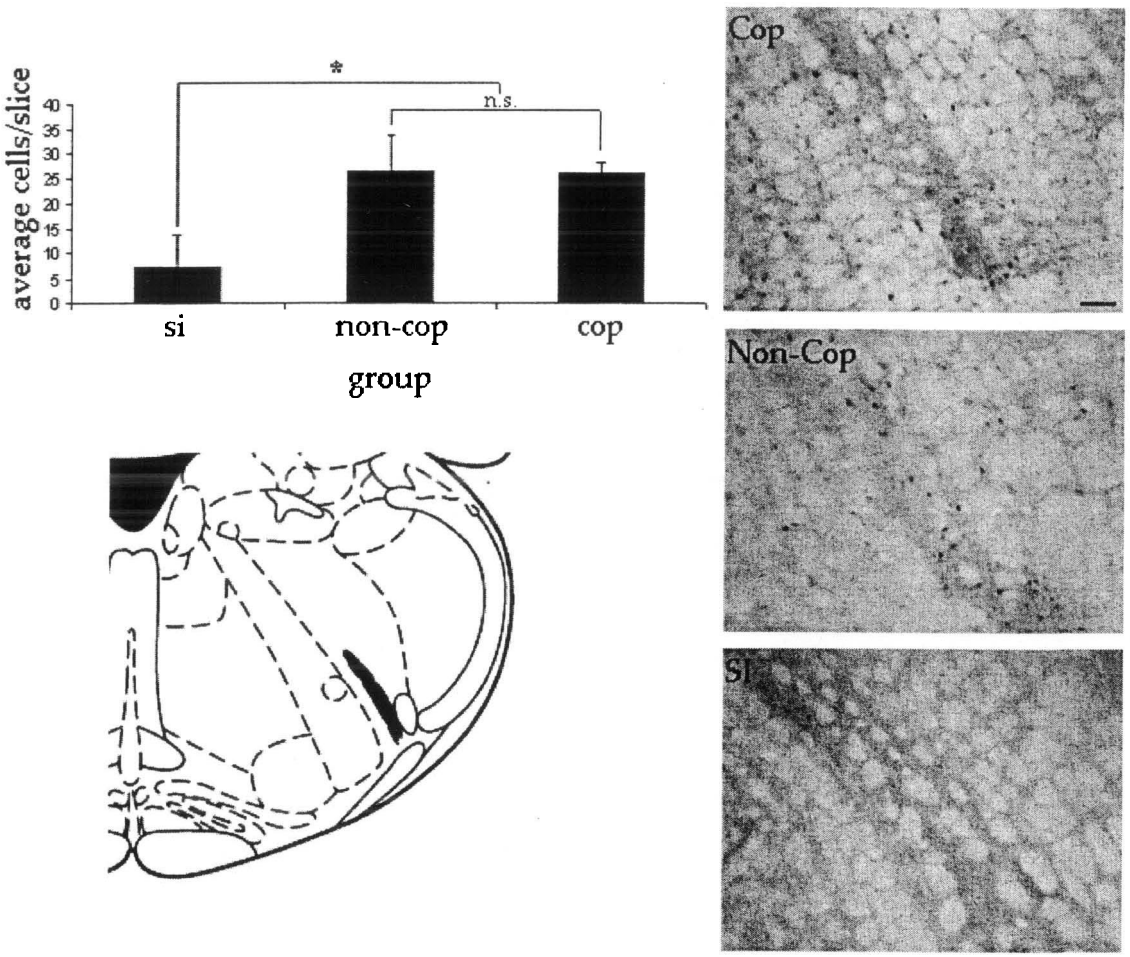
Note: n.s.= not significant; *= significant at the 0.05 level, scale bar is 50μm; the error bars indicate the standard error of the mean.

Figure 6 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the linear nucleus. The schematic diagram represents the location within the medulla that the Li is located; the blue indicates that this area is moderate for

Fos immunoreactivity. Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 6:

Fos Activation in the Linear Nucleus



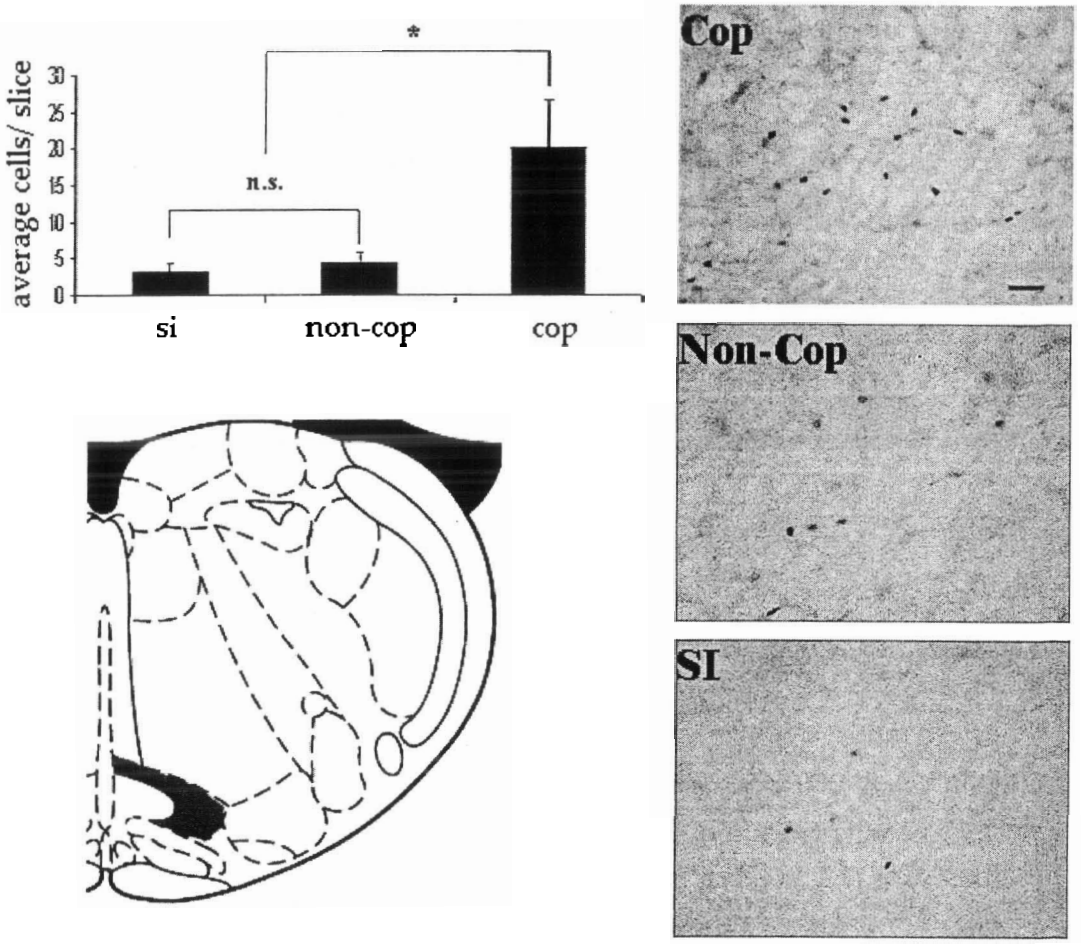
Note: n.s.= not significant; *= significant at the 0.05 level, scale bar is 50μm; error bars represent the standard error of the mean.

Figure 7 represents the mean cells per slice of the social isolates, non-copulators, and the copulators in the nGi. The schematic diagram represents the location within the medulla that the nGi is located; the red indicates that this area is dark for Fos

immunoreactivity. Representative photomicrographs of Fos in the copulators, non-copulators, and social isolates.

Figure 7:

Fos Distribution in the nucleus Gigantocellularis



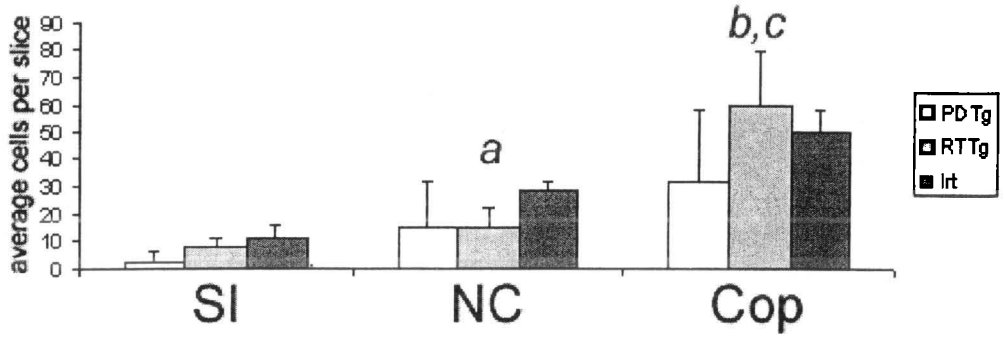
Note: n.s.= not significant; *= significant at the 0.05 level, scale bar is 50μm; error bars represent the standard error of the mean.

Figure 8 represents the average number of cells per section of copulators (Cop; n=7), non-copulators (NC; n=7), and social isolates (SI; n= 7) in the intermediate reticular nucleus (IRt), posterodorsal tegmental nucleus (PDTg), and reticulotegmental

nucleus (RtTg). Note: a= non-statistically significant comparison between the NC and SI; b= statistically significant comparison between the Cop and SI; b= non-statistically significant comparison between the Cop and NC. Error bars represent standard error of the mean.

Figure 8:

Fos Distribution in the IRt, PDTg, and RtTg



Note: n.s.= not significant; *= significant at the 0.05 level

Figure 9 represents androgen receptor immunopostive cells of lamina X, the spinal nucleus of the bulbocavernosus, and the dorsolateral nucleus in the lumbar spinal cord (L5-L6). In addition, note that the staining for AR is not substantial in the retrodorsal lateral nucleus.

Figure 9:

Androgen Immunoreactivity in the Lumbar Spinal Cord

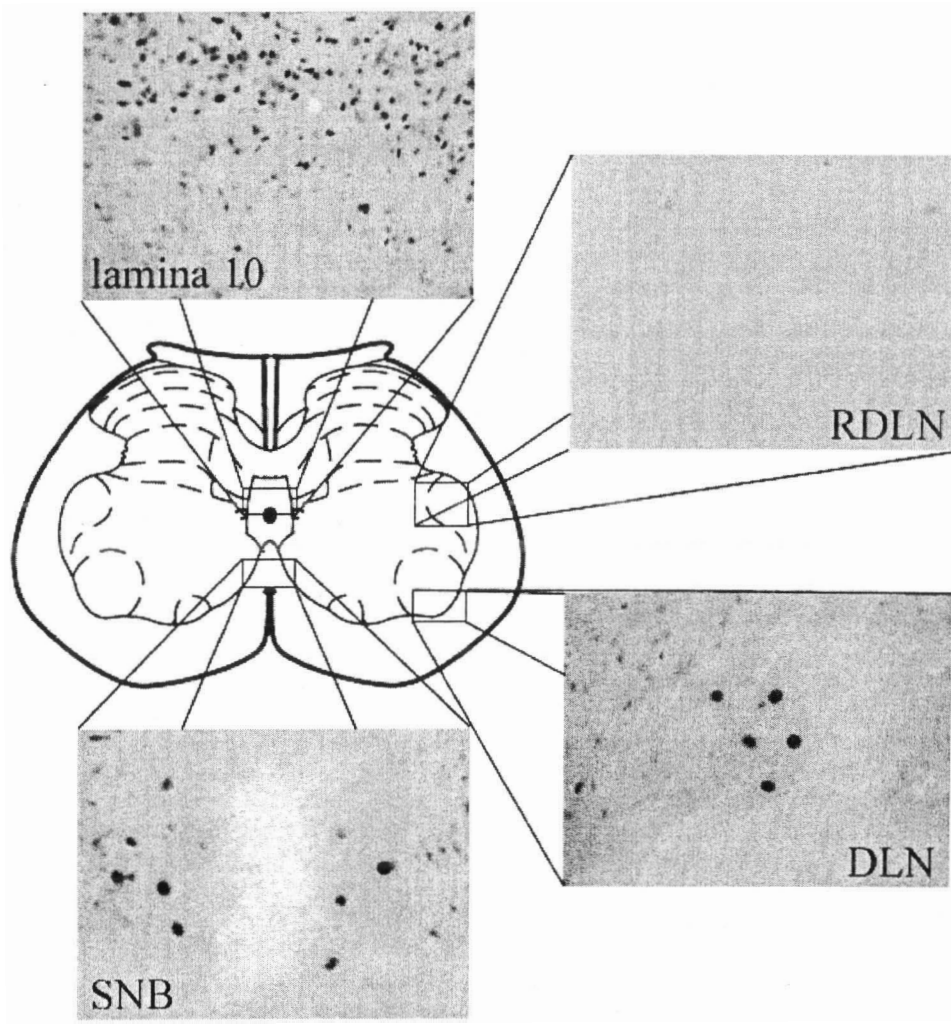
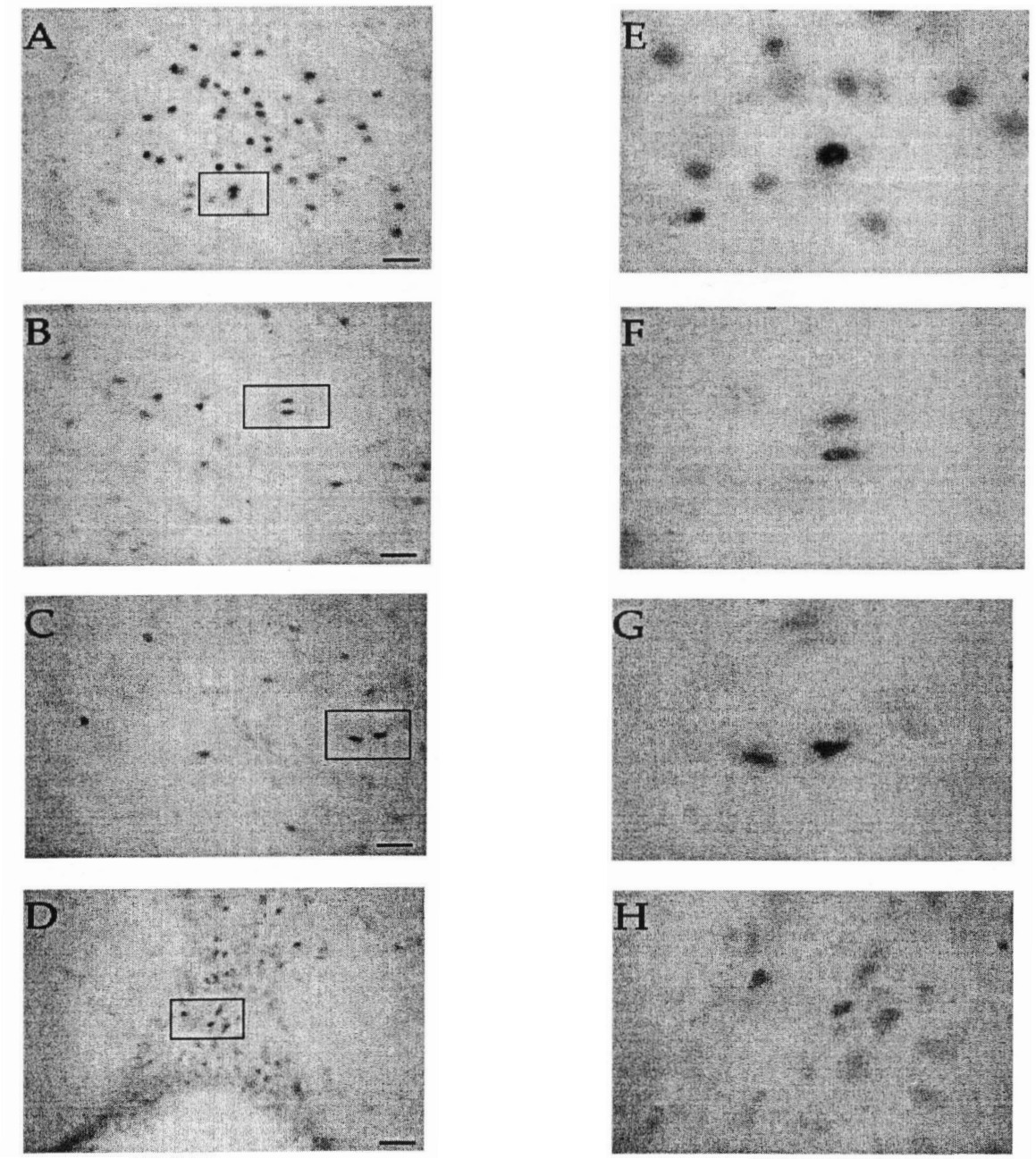


Figure 10 depicts representative photomicrographs of androgen receptor immunoreactivity in cells of the nucleus ambiguus (10X for A and 60X for E), the lateral paragigantocellular nucleus (10X for B and 60X for F), the nucleus gigantocellularis (10X for C and 60X for G), and the raphe pallidus (10X for D and 60X for H). A & E, B & C, and F & G represent dark immunostaining; D and H are examples of light staining.

Figure 10:

Androgen Receptor Immunoreactivity in Copulation Induced Fos Expressing Areas

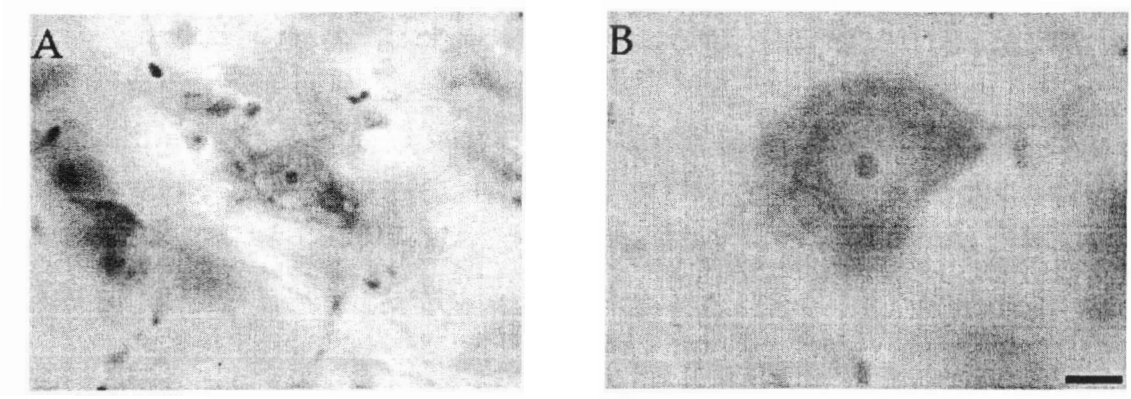


Note :Scale bar represents 50μm.

Figure 11 depicts representative photomicrographs (100X objective) of cells in the nucleus gigantocellularis (A) and the facial nucleus (B). Note that in A, the nucleus is immunopositive for the androgen receptor (light brown staining), and that the nucleus in B is immunonegative.

Figure 11:

Comparison of the nGi and Facial Nucleus



Note: Scale bar represents 50μm

DISCUSSION

One of the primary aims of the present study was to identify sites within the brainstem that expressed Fos immunoreactivity after appetitive and consumatory sexual behavior and compare this to non-copulating and socially isolated controls. In addition, a secondary aim was to describe their overlap with the distribution of androgen receptors in these areas.

Fos immunoreactivity in the nucleus of the trapezoid body (Tz) was preferentially augmented in copulating male rats when compared to the non-copulators and social isolates. The distribution of Fos immunoreactivity was not uniformly distributed in the copulators, though, as it appeared preferentially in medial divisions of the nucleus only. In the non-copulators, however, Fos was expressed in only a few cells, and seemed extremely rare in the social isolates.

This nucleus has been described in rats as being a collection of globular cell bodies of about 16.5 μm in diameter and located medially to the lateral superior olive, and just superior to the pyramidal tract in the pons (Webster, 1995). The most salient feature of this collection of cells is the prominent bouton ending, called the chalice of Held, synapsing preferentially onto contralateral Tz dendrites (Webster, 1995). These cells stain densely for glycine and serotonin, and project laterally to the base of the lateral superior olive, which is selectively sensitive to high frequency cues, suggesting the Tz may process auditory information (Webster, 1995). That the Tz processes auditory stimuli based upon studies using Fos immunoreactivity as a marker of activation after presentation of different tonal values (Friauf, 1992).

Further, a recent study using anterograde and retrograde axonal tracing techniques has revealed that some fibres originating in the medial subparafasicular (SPF) thalamic nucleus terminate on the nucleus of the trapezoid bodies as well as other areas of the auditory brainstem (Yasui, Nakano, Mizuno, 1992). Auditory information is transmitted to the SPF, as is sensory information carried via the ascending spinothalamic tract (LeDoux, Ruggiero, Forest, Stornetta, Reis, 1987) and this has led to the suggestion that the SPF may integrate acoustic and somaesthetic information, and transmit this to the

amygdala and areas of the hypothalamus such as the medial preoptic area (Yasui et al., 1992; LeDoux et al., 1987; LeDoux, Ruggerio, Reiss, 1985).

A similar pattern of Fos activation was observed in the dorsal cochlear nucleus (DCN); copulators expressed dark immunoreactivity, preferentially in the dorsal medial regions of the nucleus, whereas the non-copulators expressed very little, with Fos immunoreactive cells being extremely rare in the social isolates. The DCN may be considered a secondary auditory relay centre as afferents from the auditory nerve heavily innervate this nucleus in both the rat and the cat (Webster, 1995). Moreover, the anatomical distribution of different cell types suggests that a tonotopic map from the primary auditory apparatus is conserved in the DCN (Webster, 1995). This conclusion was based on studies using 2-deoxy glucose (2DG) and Fos immunoreactivity as markers of cellular activation in rats and mice (Ryan, Furlow, Woolf, Keithley, 1988; Friauf, 1992; Ehret and Fischer, 1991; Sato, Houtani, Ueyama, Ikeda, Yamashita, Kumazawa, Sugimoto, 1992).; As each tone that was presented was increased in frequency, there was a characteristic pattern of 2DG uptake with the most dorsal regions being activated with 16-60 KHz tones, and the most ventral regions being activated with1-8 KHz tones. A similar distribution of Fos was obtained after auditory stimulation.

Thus, given the above information, one interpretation of the distribution of Fos in the present study suggests that the pattern of immunoreactivity in copulators is a reflection of cells activated with tones roughly ranging from 20 to 60 KHz, in both the dorsal cochlear nucleus and the nucleus of the trapezoid bodies. This pattern of activation may correspond to 1) the 50 KHz vocalization emitted by males when they come in contact with conspecifics (Brudzynski and Pniak, 2002), 2) the ultrasonic vocalizations males produce just prior to ejaculation (White and Barfield, 1990), 3) the 22 KHz vocalization emitted after ejaculation in the refractory period, or 4) the call that is often emitted when male rats anticipate an estrous female in the laboratory setting (Bialy, Rydz, Kaczmarek, 2000). These data also suggest that the tonotopic map, as revealed by Fos immunoreactivity, may be conserved not only in the dorsal cochlear nucleus, but perhaps also in the nucleus of the trapezoid bodies.

The lateral paragigantocellular reticular nucleus (LPGi) is located in the rostral ventral medullary region. The LPGi is bordered medially by the inferior olivary complex, and laterally by the intermediate reticular nucleus and trigeminal sensory nuclei in more caudal medullary regions (termed the 'retrofacial LPGi'); and is located just medial to the facial nerve motoneurons (termed the 'juxtafacial LPGi') in more rostral slices (Aston-Jones, Shipley, Grzanna, 1995). The principal afferents of the locus coeruleus (LC) in the pons arise primarily from the LPGi, as numerous densely labelled cells were observed in the LC after injections of the anterograde tract tracer, wheat germ agglutinin conjugated to horse radish peroxidase, into the LPGi (Aston-Jones, Shipley, Grzanna, 1995).

Also located in the caudal ventral medulla are the cells of the ventral gigantocellular reticular field (GiV), located in caudal regions, and the cells of the alpha division of the gigantocellular reticular filed, located more rostrally (GiA; Jones 1995). The GiV and GiA have been considered as a single collection of cells by some anatomists and termed the magnocellular reticular field in the cat (FTM; in Jones, 1995). The GiV is superior to the inferior olivary complex and the cells are contiguous with those of the raphe pallidus and obscurus, whereas the GiA adjoins the cells of the raphe magnus medially; both are also continuous with the medial spinoreticular cells of the LPGi (Jones 1995). In the present study, nucleus gigantocellularis (nGi) will replace GiA and GiV (as adopted from Marson, 1997).

Electrophysiological data has suggested that the nGi and LPGi are part of a common circuit regulating penile reflexes (Hermann et al., 2003); activation of the dorsal penile nerve elicits responses in the nGi-LPGi complex, whereas mechanically evoked erectile reflexes are blocked via electrical stimulation of this complex (Hubscher and Johnson, 1996; Johnson and Hubscher, 1998).

Consistent with previous reports implicating supraspinal ventral medullary neurons in sexual behavior, in the present study, the lateral paragigantocellular reticular nucleus and the nucleus gigantocellularis contained more Fos immunoreactivity in the copulators when compared to both the non-copulators and the social isolates. The non-copulators and the social isolates did not differ in the amount of Fos staining, suggesting the

augmentation of Fos above baseline in the copulators is associated with appetitive and consumatory behaviors.

Previous studies have shown that LPGi neurons terminate within lumbar and sacral cell groups (Murphy and Hoffman, 2001), and that destruction of the LPGi releases the motoneurons innervating the penis from tonic inhibition as indicated by shorter latencies to achieve erection in *ex copula* tests (Marson and McKenna, 1990).

Another afferent of the LPGi is the periaqueductal gray (PAG), a structure that spans the rostral pons-diencephalic border, and also sends inputs to the medial preoptic area in the hypothalamus (Murphy, Rizvi, Ennis, Shipley, 1999; Rizvi, Murphy, Ennis, Behbehani, Shipley, 1996). Given this anatomical connectivity, it has been suggested that activation of the MPOA-PAG-LPGi circuit by cues from an estrous female may stimulate penile reflexes and reproductive behaviors by removing tonic inhibition on motoneurons innervating the perineal musculature (Murphy and Hoffman, 2001).

Thus, one interpretation of Fos immunoreactivity within the LPGi and nGi in the present study is that it is a reflection of the 'activation' of inhibitory circuits on penile reflexes that normally accompanies ejaculation (i.e., the post-ejaculatory refractory period). However, staining may also represent sensory information associated with the release of seminal fluids (Hermann et al., 2003); though the design of the study does not allow for the temporal dissociation of Fos and thus Fos activation may also arise from other appetitive and consumatory behaviors such as anogenital investigation, mounting, thrusting, or intromitting; these regions receive projections from areas in the brain and also from somatic motoneurons residing in several different levels of the spinal cord (Murphy and Hoffman, 2001; Hermann, et al., 2003).

Another ventral medullary nucleus, the raphe pallidus (RPa), also displayed more Fos immunoreactivity in the copulators when compared to both the non-copulators and socially isolated controls. In addition, the levels of Fos did not differ between these two control groups, but it appeared that a number of cells in this nucleus were activated. These neurons are located in the extreme ventral portion of the medulla, and can be easily distinguished as they occupy the central area between the pyramidal tracts in more caudal divisions of the medulla.

Several studies indicate that raphe neurons project to several regions in the spinal cord (i.e., sacral, lumbar, and thoracic regions; see Holstege and Kuypers, 1987a, b; Marson, 1997). Serotonergic raphe pallidus neurons have been reported to project to several areas in the lumbar spinal cord that regulate movement (Fung and Barnes, 1989), urinary continence and elimination (Marson, 1997), and erectile reflexes (Bancila, Giuliano, Rampin, Mailly, Brisorgueil, Calas, Verge, 2002), as well, they directly project to cells of the MPO area (Leanza, Pellitteri, Russo, Stanzani, 1991). In addition, they receive inputs from areas in the rostral pons such as the micturition centre, Barrington's nucleus (Bancila et al, 2002), and the RPa cells appear to be excitatory in nature (Fung and Barnes, 1989; Marson, 1997). Thus, it is likely that the raphe pallidus is primarily involved in urogential reflexes, but also, given that it receives inputs from regions of the limbic system such as the amygdala, sensory input and movement may be regulated by emotional processing areas (Holstege and Kuypers, 1987).

In the present study, Fos immunoreactivity in the RPa is potentially attributed to somatomotor information arising from hind-limb movement (Fung and Barnes, 1989) as males in all three experimental groups engaged in some type of motor behavior, but could also potentially arise from expulsion of bladder contents (i.e., urination). However, previous studies have shown that activation of this region (that is, raphe nuclei such as the pallidus and obscurus, and surrounding inferior olivary nuclei) with application of the serotonin receptor agonist, DOI, produces an inhibition of sexual behavior when injected directly to the ventral medulla (Watson and Gorzalka, 1992). Thus, Fos activation in this region may reflect the inhibition of copulation in a similar manner described above for the LPGi and nGi. Again, though, the design of the study does not allow for the temporal dissociation of the stimulation of Fos in cells of this region. Moreover, because of the location of the DOI injections in the study mentioned above, localization of action is not possible as the drug may have affected other ventral medullary raphe nuclei.

In the present study, we also observed AR immunoreactivity in the lateral paragigantocellular reticular nucleus, raphe pallidus, and nucleus gigantocellularis supporting a role for androgenic influence on cell groups regulating penile reflexes in the ventral medulla, as these cell groups contained copulation induced Fos

immunoreactivity. The exact nature of the influence of androgens in the ventral medulla is not known as it may involve classical genomic effects upon regulating cellular morphology, may involve non-genomic effects in regulating cellular physiology, or both. However, not all regions that appeared to contain reproductively induced Fos contained immunoreactivity for androgen receptors, as the nucleus of the trapezoid bodies and the dorsal cochlear nucleus were AR negative. These areas also do not appear to concentrate gonadal steroids such as testosterone or estrogen and do not contain either AR or ER (alpha and beta) mRNA, and thus it appears that gonadal steroids do not directly influence these structures in any type of hormonally related behavior such as copulation (Shughrue, Lane, Merchenthaler, 1997; Simerly et al, 1990).

A unique pattern of Fos staining was observed within the linear nucleus (Li) in the medulla. This nucleus displayed equivalent amounts of Fos immunoreactivity in copulators and non-copulators, however, both groups displayed more Fos when compared to the social isolates. The Li was observed in slices that contained the parvocellular reticular nucleus (PCRt), and was bordered laterally by the spinal trigeminal tract and medially by the intermediate reticular nucleus (IRt). The Li stretched dorsoventrally from the level of the ambiguus nucleus in the IRt, to approximately the level of the rubrospinal tract, however, in more rostral slices, the Li appeared to overlap with cells in the IRt that bordered the PCRt (for example, see panel 67 in Paxinos and Watson, 1997).

Given that Fos was stimulated above that of the social isolates in approximately equal amounts in both the copulators and non-copulators, and that the copulators displayed anogenital investigation, mounting, intromitting, and ejaculation, with no increase in Fos above that of the non-copulators, the distribution of activated cells suggests that this area may be part of a l mechanism associated with precopulatory behaviors.

The distribution of Fos was equivocal in the intermediate reticular nucleus of the medulla, and both the posterodorsal and reticulotegmental regions in the pons; the copulators displayed more Fos immunoreactivity when compared to the social isolates, however, the copulators and non-copulators did not differ in staining, nor did the non-

copulators and social isolates, making it difficult to state any firm conclusions about the role these nuclei may play in appetitive and consumatory sexual behaviors.

It appeared that there was a non-significant trend in the augmentation of Fos in the intermediate reticular nucleus between the three groups and perhaps if the number of animals was increased, the number of cells displaying Fos may have been enough to reach statistical significance. The IRt may have a non-specific and non-essential role in the regulation of consumatory and appetitive behaviors, however, as it is part of a larger and more diffuse circuit regulating several different physiological and behavioral functions (Jones, 1995). This also applies to the posterodorsal tegmental nucleus, as it has been described as the pontine extension of the medullary reticular nucleus (Jones, 1995), and contained a similar pattern of Fos immunoreactivity between the groups.

In the reticulotegmental nucleus, Fos activation appeared to be stimulated in cells above baseline in both the copulators and non-copulators, however, the amount of variability in the copulators appeared quite high and this was the reason that it just missed statistical significance. It is perhaps likely that the degree of variability in cells expressing Fos that was observed in this region of the copulators is correlated to a certain degree to the difference in copulatory performance; visuomotor centres in the brain send a substantial amount of input to the RtTg, and this region also projects heavily to areas in the cerebellum involved in visual orientation, movement, and balance (Ruigrok and Cella, 1995; Berretta, Bosco, Smecca, Perciavalle, 1991; Newman and Ginsberg, 1992). However, the differences in copulatory performance may simply be natural variation associated with animals that have the amount of sexual experience, such as the ones in the current study.

The distribution of androgen (AR) and estrogen receptor protein and mRNA has been quantified in several brain and spinal cord regions of the male and female rat (Simerly et al., 1990; Shughrue, Lane, Merchenthaler, 1997;Papka, Storey-Workley, Shughrue, Merchenthaler, Collins, Usip, Saunders, Shupnik, 2001). The distribution indicates that, within the brainstem, areas subserving sensory and motor function contain AR mRNA, as do pre- and post-cerebellar nuclei, reticular core neurons (with the exception of the pedunculopontine nucleus), and midline raphe neurons, with the highest

expression of AR mRNA being localized in areas processing visceral sensory information such as the nucleus of the solitary tract (Simerly et al., 1990). Other areas to note which contained moderate amounts of AR mRNA (as determined by the amount of silver grains, above background, associated with the cells) include the trigeminal motor nucleus, facial nucleus, nucleus ambiguus, periaqueductal gray, and inferior olive.

In agreement with the in situ hybridization data, radiolabelled dihydrotestosterone appears to accumulate in these regions (with some discrepancies discussed below), which are catecholaminergic in nature, supporting a role for the interaction of steroid and catecholamine systems in the regulation of a number of physiological and behavioral functions, including copulation (Heritage, Stumpf, Sar, Grant, 1980).

The protein distribution of the androgen receptor has been described for only a few nuclei in the brainstem. Reports indicate that AR immunoreactivity is observed in cranial nerve motoneurons of the nucleus ambiguus, hypoglossal nucleus, facial, and trigeminal nuclei (Yu and McGinnis, 2001; Clancy, Bonsall, Michael, 1992), and this distribution agrees well with the AR in situ hybridization data (Simerly et al, 1990) and radiolabelled steroid accumulation data (Heritage et al., 1980). Paradoxically, however, with the exception of the nucleus ambiguus, androgen receptor immunoreactivity was not detected in cranial nerve motoneurons or throughout all levels of spinal trigeminal cells of the pons and medulla, in the present study. In addition, discordant with the mRNA data for AR (Simerly et al, 1990), immunoreactivity was not detected in the inferior olivary complex, medial vestibular nucleus, spinal vestibular nucleus, or cuneate nuclei.

Previous reports have localised estrogen concentrating cells in cuneate nuclei via autoradiography (Heritage, Grant, Stumpf, 1977), but there appear to be no estrogen receptors in these regions as mRNA for the alpha and beta isoforms was not detected in one study (Shughrue et al., 1997), and it is likely that the novel plasma membrane-associated estrogen receptor X is also not expressed in this region, as described by another study (Toran-Allerand, Guan, MacLusky, Horvath, Diano, Sing, Connolly, Nethrapalli, Tinnikov, 2002).

Moreover, data concerning the distribution of cells which accumulate radiolabelled androgens such as DHT where described in A2 noradrenergic cells of the solitary nucleus in one study (Heritage et al., 1980), but was subsequently not observed in the same area in another study which used the same formaldehyde induced fluorescence technique combined with autoradiography (Heritage et al., 1980), highlighting the difficulties associated with the detection of steroid concentrating cells and those that may express the cognate receptors, as studies do not always concur.

The discrepancies between data in the present study and those previously reporting AR mRNA and AR immunoreactivity in cranial nerve nuclei (Yu and McGinnis, 2001; Simerly et al., 1990) can not be easily explained. One suggestion may be that while AR mRNA is expressed in these nuclei, mRNA editing and subsequent translation of the modified transcript produced a protein that had the PG-21 antibody recognition sequence deleted (i.e., the first 21 amino acids, or even a portion of it), and thus we were never able to detect it in the above stated regions. It may also be likely that AR mRNA is inherently unstable, expressed in low levels, or is translated only during certain physiological processes associated with cellular injury such as that observed after nerve crush (for example, see Jones, Brown, Damaser, 2001). Under the microscope it is evident that the RDLN in the lumbar spinal cord does not contain a substantial amount of androgen receptor protein as revealed by ICC using the PG21 antibody (see figure 1). In addition, the localization of AR-IR appears to by cytoplasmic in nature. However, in cranial nerve nuclei, nuclear and cytoplasmic AR were not observed, supporting the notion that these areas do not contain detectable amounts of AR, at least when compared to the RDLN.

Summary

In summary, Fos immunoreactivity was associated with male consumatory sexual behavior in the dorsal cochlear nucleus, nucleus of the trapezoid body, the lateral paragigantocellular reticular nucleus, nucleus gigantocellularis, linear nucleus, and raphe

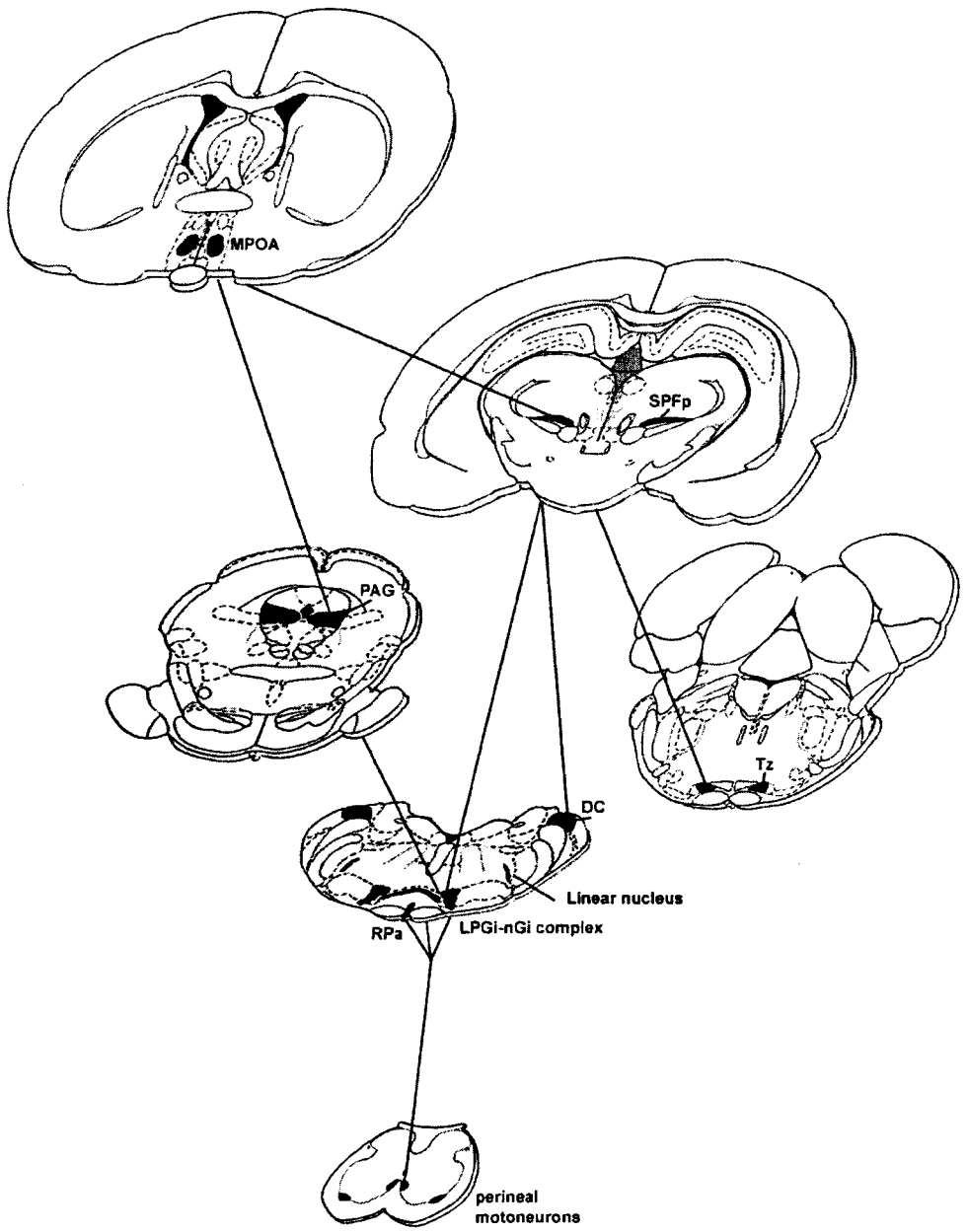
pallidus; ambiguous results were obtained in the posterodorsal and reticulo-tegmental regions, as well as in the intermediate reticular nucleus. Areas that contained androgen receptor immunoreactivity included the raphe pallidus, lateral paragigantocellular nucleus, and nucleus gigantocellularis implicating cell groups in the ventral medulla as being involved in the regulation of copulation via androgen receptor dependant mechanisms. It is suggested that the augmentation of Fos in these regions is potentially due to the active inhibition of penile reflexes observed in the post ejaculatory refractory period, however, the design of the study does not permit the temporal dissociation of Fos stimulation, and thus staining may also be due to other appetitive and consumatory activities. Finally, Fos is suggested to be associated with auditory information arising primarily from the male rat prior to, or just after ejaculation in the dorsal cochlear nucleus and nucleus of the trapezoid body; Fos may also have been stimulated in the linear nucleus via precopulatory behaviors.

Figure 12 is a pictorial representation of the Fos results in the caudal ventral medulla (i.e., the LPGi-nGi and RPa), the DC, and the Tz, as listed above. In addition, this figure depicts the connectivity of areas in the present study, in which copulation induced Fos immunoreactivity was observed, with regions of the PAG, SPFp, and MPOA based on previous anatomical studies (see Murphy and Hoffman, 2001; Coolen, Veening, Wells, Shipley, 2003; Greco et al., 1998). Information from the LPGi-nGi complex is sent to regions within the lateral PAG, and then to the MPOA. This circuit is thought to regulate the display of penile erections (Murphy and Hoffman, 2001). Moreover, the MPOA and PAG are known to also contain androgen receptors, as has been reported previously, and thus the descending circuit regulating penile reflexes appears to responsive to gonadal steroids such as testosterone as the LPGi-nGi complex was also found to contain androgen receptors in the present study. However, information from the LPGi-nGi complex, arising mainly from lumbar spionothalamic cells (LSt; Truitt and Coolen, 2002) may also travel to the medial SPFp. It is thought that the information sent to the medial SPFp is important for the display of ejaculation- lesions of this region, the spinothalamic tract, or the LSt cells results in a lack of ejaculation following mating.

Previous studies have shown that auditory information, from both the DC and Tz, is sent to the lateral SPFp, and then to such areas as the MPOA. Thus, the SPFp is important for not only processing and transmitting ejaculation related information, but may also integrate this information with auditory stimuli resulting from mating that arises in the DC and Tz.

Figure 12:

Summary Figure



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