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Prenatal flutamide alters sexually dimorphic nuclei in the spinal cord of male rats

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The effects of prenatal exposure to the antiandrogen flutamide on two sexually dimorphic nuclei of the lumbar spinal cord, the dorsolateral nucleus (DLN) and the spinal nucleus of the bulbocavernosus (SNB), were investigated. Rat dams were given daily injections of 5 mg flutamide or vehicle alone from day 11 through 21 of pregnancy. The spinal cords and perineal morphology of their male and female offspring were examined in adulthood. Flutamide reduced the number of SNB and DLN neurons, reduced the somal and nuclear area of SNB neurons, and reduced the weight of the perineal muscles in males. Flutamide produced no effect in females. No sexual dimorphism was found in the mean somal area of DLN neurons, but a sexual dimorphism was found in the distribution of somal areas in our samples; females had proportionately more large neurons than males. Flutamide-treated males also had proportionately more large neurons than control males but fewer than females. A sexual dimorphism was found in the nuclear areas of DLN neurons but flutamide did not influence this trait.

INTRODUCTION

The dorsolateral nucleus (DLN) and the spinal nucleus of the bulbocavernosus (SNB) of the spinal cord are sexually dimorphic. Males have more neurons than females in both of these structures, and males have larger somata in the SNB[2,3,11].

Several pieces of evidence suggest that the sexually dimorphic characteristics of the SNB are the result of perinatal androgen levels. Exposure of female rats to testosterone propionate (TP) during prenatal development masculinizes the SNB by increasing the number and size of its neurons[4,19,20]. Conversely, depriving males of androgenic stimulation by prenatal administration of the antiandrogen flutamide decreases the number and size of SNB neurons[5]. Similarly, prenatally stressed males, whose plasma testosterone levels are altered during fetal life[26,27], have an abnormally low number of neurons in the SNB in adulthood[10].

The hormonal mechanism leading to the masculinization of the DLN has not been completely described but is also believed to depend on exposure to androgen during perinatal ontogeny. Prenatal administration of TP to female rats has been shown to masculinize (increase) the number of neurons in the DLN[11,20,23]. Males who were exposed to prenatal stress had fewer DLN neurons than controls, presumably because prenatal stress reduces plasma testosterone during an ontogenetic period important for the differentiation of the DLN[10]. A direct assessment of DLN morphology in males deprived of normal androgen exposure by chemical agents during prenatal ontogeny has not been reported.

In the present study, the DLN and SNB of adult male and female rats whose mothers were injected with flutamide during pregnancy were compared to control males and females. Neurons were counted, and the somal and nuclear areas were measured in a sample of neurons in both the DLN and SNB.

MATERIALS AND METHODS

Subjects

The spinal cords of 32 male and 25 female offspring of Sprague–Dawley rats (Harlan S-D, Madison, WI) bred in the Villanova laboratory were studied. Mothers were allowed to adapt for two weeks after arrival from the supplier before being bred. All animals were given ad libitum access to water and food (Purina rat chow) in a vivarium maintained at 21–22°C with a reverse 12/12 h

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dark/light cycle (lights on at 23.00 h).

Apparatus

The data were collected using a Newvicon video camera (Model 65; Dage-MTI Inc., Michigan City, IN) attached to a Nikon Optiphot microscope. Images of individual histological sections were displayed on a 19 inch Ikegami monitor. The areas of the neurons and their respective nuclei were measured by using a Bausch and Lomb hipad digitizer connected to an Apple IIe computer which used a Unicomp software package (Southern Micro Instruments, Atlanta, GA).

Procedure

Twenty-two nulliparous Sprague–Dawley females in behavioral estrus were placed with males until an observer noted two ejaculations. Day 0 of gestation was the dark period in which the ejaculations occurred and the following light period. The dams were assigned randomly either to the flutamide group (n = 10), which received injections of 5 mg of flutamide (Schering Corporation, Bloomington, NJ) suspended in 0.15 cc propylene glycol vehicle, or to the control group (n = 12), which received injections of the same vol. of vehicle alone. Daily injections were given subcutaneously from day 11 through 21 of gestation.

All animals were weaned into mixed sex groups of six animals on day 21 or 22 of age and were observed for sexually dimorphic play behavior as juveniles. As adults, the males were tested for saccharin preference and were given tests for male copulatory behavior. The details and results of these behavioral tests are provided elsewhere[8]. All animals remained gonadally intact throughout the experiment.

At 120 days of age, 16 control males, 16 flutamide-treated males, 16 control females, and 9 flutamide-treated females were given an overdose of chloral hydrate with pentobarbital and were perfused with saline followed by a 10% formalin solution. Between one and four individuals were selected from each litter. The spinal cord was removed and fixed in 10% formalin-saline. The cord was frozen and sectioned at 50 μm. All serial sections were mounted and stained with thionin.

Counts of neurons were made on both sides of the cord using 200× magnification. Only densely stained neurons in which the nucleus of the cell could be clearly seen were counted. All counts were corrected according to Konigsmark's method[3,14]. The number of cells in the DLN was obtained by counting the neurons in the 26 caudal-most sections containing the DLN. The number of cells in the SNB was obtained by counting the number of neurons within 200 μm of the central canal of the spinal cord. The count proceeded from the caudal-most appearance of the SNB until the rostral point at which no additional neurons were found for two consecutive sections on either side of the spinal cord.

Twenty neurons in the DLN and twenty neurons in the SNB of each animal (10 from each side of the spinal cord in both cases) were selected randomly from those that had a clearly visible cell boundary and a clearly visible nucleus. The areas of these neurons and their nuclei were measured. A mean was calculated for the ten neurons measured on each side of the spinal cord. The means from the right and the left side were then averaged for each animal. The nuclear areas for each animal were calculated in the same fashion as the somal areas. The investigator who counted the neurons and made the area measurements was blind to the animals' gender and prenatal treatment.

At sacrifice, the presence or absence of a vaginal orifice was noted in all males. The ischiocavernosus (IC)/bulbocavernosus (BC)/levator ani (LA) muscle complex of the perineum were removed in the males by teasing the LA away from the colon and cutting the IC muscle. The muscles were fixed for approximately 18 months in a 10% formalin-saline solution. Although it would be interesting to know if the hormonal effects on the IC muscle and DLN were related, the IC muscles were invariably damaged in the course of dissection, thus precluding any such analysis. The IC muscles were separated from the rest of the complex, and the remaining BC/LA muscles were cleaned, blotted dry, and weighed.

RESULTS

DLN neurons

The mean number of DLN neurons in control and flutamide-treated males and control and flutamide-treated females is displayed in Fig. 1. The number of DLN neurons was analyzed in a 2 × 2 (sex × prenatal treatment) analysis of variance. This analysis revealed that males had significantly more neurons than females ($F_{1,53}$ = 85.7, $P < 0.001$), that flutamide-treated animals had fewer neurons than vehicle-injected controls ($F_{1,53}$ = 58.7, $P < 0.001$), and that there was a significant sex × treatment interaction ($F_{1,53}$ = 37.0, $P < 0.001$). A post-hoc Newman–Keuls test revealed that flutamide-treated males had significantly fewer neurons than control males ($P < 0.01$) but were not significantly different from females. There was no significant differences between the flutamide-treated females and control females.

The mean DLN somal areas in control and flutamide-treated males and females are shown in Table I. Somal areas of DLN neurons were analyzed in a 2 × 2 (sex × treatment) analysis of variance. This analysis revealed no significant differences due to sex, treatment, or their interaction (all Fs < 1.00).

Although there were no significant differences in mean somal areas, we observed that some animals had a number of extraordinarily large DLN neurons (larger than 1650 μm^2). To determine if these unique neurons were differentially distributed as a function of sex or treatment, we examined the frequency distribution of somal areas in the samples measured in each group. The DLN somal areas sampled from all animals in each group were pooled and originally sorted into 150 μm^2 bins. In order to make the data easier to analyze, the first three bins of neurons were combined and categorized as being small neurons (150–899 μm^2), the next three bins were

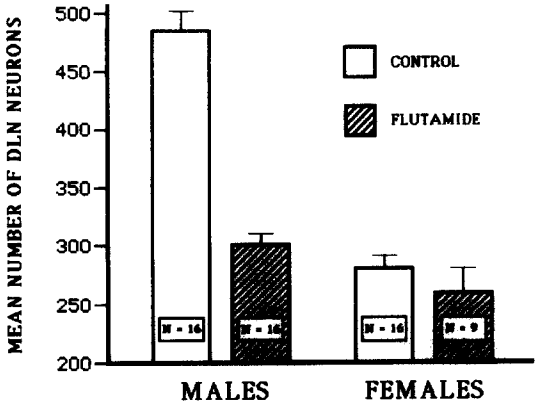

Fig. 1. Mean number of dorsolateral nucleus (DLN) neurons in control and flutamide-treated male and female rats. Bars represent S.E.M.

TABLE I

The mean (± S.E.M.) areas (μm^2) of SNB and DLN somata and their respective nuclei in control and flutamide-treated male and female rats

Twenty neurons were sampled in each animals. Also shown are the mean (± S.E.M.) weights (g) of the BC/LA muscle complex in the males.

	Males		*Females*	
	Control $n = 16$	*Flutamide* $n = 16$	*Control* $n = 16$	*Flutamide* $n = 9$
DLN somal area	850.35 (± 28.31)	861.62 (± 31.54)	841.16 (± 51.83)	796.06 (± 46.36)
DLN nuclear area	215.28 (± 4.55)	210.13 (± 4.05)	189.23 (± 5.72)[a,b]	186.11 (± 8.93)[a,b]
SNB somal area	1456.92 (± 24.93)	1122.03 (± 35.60)[a]	759.18 (± 18.10)[a,b]	701.60 (± 23.52)[a,b]
SNB nuclear area	252.25 (± 3.30)	214.00 (± 4.00)[a]	179.76 (± 4.19)[a,b]	167.71 (± 7.34)[a,b]
BC/LA weight	1.4956 (± 0.0285)	0.6292 (± 0.0524)[a]	–	–

[a] Significantly different from control males; [b] significantly different from flutamide males.

combined and categorized as medium neurons (900–1649 μm^2), and the remaining bins were combined and categorized as large neurons (1650 μm^2 and larger). The average frequency of occurrence of each type of neuron in each group is displayed in Table II.

A separate 2 × 2 ANOVA (sex × treatment) was performed on the frequency data for each size class of neurons. Females had a greater frequency of large neurons than males in our samples ($F_{1,53} = 25.3$, $P < 0.001$), treatment produced no significant main effect ($F < 1.0$), but the interaction between sex and treatment was significant ($F_{1,53} = 6.9$, $P < 0.01$). Flutamide-treated males had proportionately more large neurons than did control males ($t_{30} = 2.37$, $P < 0.05$), but had fewer large neurons than did control females ($t_{30} = 3.48$, $P < 0.001$). Flutamide and control females did not differ from one another in the frequency of large neurons ($t_{23} = 1.50$, $P > 0.10$). Males had a greater frequency of medium sized neurons in our samples than did females ($F_{1,53} = 11.9$, $P < 0.001$), treatment produced no significant main effect ($F < 1.0$), but the interaction between sex and treatment was significant ($F_{1,53} = 9.0$, $P < 0.005$). Flutamide-treated males had fewer medium neurons than control males ($t_{30} = 3.16$, $P < 0.003$) but more medium neurons than control females ($t_{30} = 2.40$, $P < 0.05$). Flutamide and control females did not differ from one another in the frequency of medium neurons ($t_{23} = 1.31$, $P > 0.20$). The frequencies with which small neurons occurred in our samples were not significantly different among any of the groups (all Ps > 0.25).

TABLE II

The mean frequency (± S.E.M.) of occurrence of DLN neurons with small (150–899 μm^2), medium (900–1649 μm^2), or large (1650 μm^2 or larger) somal areas in a sample of twenty neurons measured in each control male and female and each flutamide treated male and female

	Size of Neurons		
	Small	*Medium*	*Large*
Control males ($n = 16$)	12.75 (± 0.66)	6.63 (± 0.62)	0.63 (± 0.20)
Flutamide males ($n = 16$)	14.06 (± 0.43)	4.38 (± 0.35)	1.56 (± 0.34)
Control females ($n = 16$)	13.69 (± 0.79)	2.88 (± 0.52)	3.44 (± 0.42)
Flutamide females ($n = 9$)	13.44 (± 0.75)	4.11 (± 0.87)	2.44 (± 0.47)

The mean nuclear areas of DLN neurons are shown in Table I. A 2 × 2 (sex × treatment) analysis of variance revealed a significant sex difference ($F_{1,53} = 19.8$, $P < 0.001$); males had larger nuclear areas than did females. The main effect for treatment and the sex × treatment interaction were not significant (both Fs < 1.00).

SNB neurons

The mean numbers of SNB neurons in each of the four groups are displayed in Fig. 2. A 2 × 2 (sex × treatment) analysis of variance revealed that males had significantly more neurons than females ($F_{1,53} = 768.5$, $P < 0.001$), that flutamide-treated animals had significantly fewer neurons than controls ($F_{1,53} = 321.4$, $P < 0.001$), and that there was a significant sex × treatment interaction ($F_{1,53} = 372.9$, $P < 0.001$). A Newman–Keuls test revealed that flutamide-treated males had significantly fewer SNB neurons than control males ($P < 0.01$) but had more than control females ($P < 0.01$). Flutamide-treated females did not differ significantly from control females.

The mean somal areas of SNB neurons in all groups are displayed in Table I. A 2 × 2 (sex × treatment) analysis of variance revealed that males had significantly larger somal areas than females ($F_{1,53} = 388.1$, $P < 0.001$), that flutamide-treated animals had significantly

smaller somal areas than controls ($F_{1,53}$ = 47.8, P < 0.001), and that the sex × treatment interaction was significant ($F_{1,53}$ = 23.9, P < 0.001). The Newman–Keuls test showed that the mean somal area of the flutamide-treated males was significantly smaller than that of control males (P < 0.01) but was significantly larger than that of control females (P < 0.01). Flutamide-treated and control females were not significantly different from one another.

The mean areas of the nuclei of SNB neurons in flutamide-treated and control males and in flutamide-treated and control females are also displayed in Table I. A 2 × 2 (sex × treatment) analysis of variance revealed that males have significantly larger nuclear areas than females ($F_{1,53}$ = 172.3, P < 0.001), that flutamide-treated animals had significantly smaller nuclear areas than did controls ($F_{1,53}$ = 30.9, P < 0.001), and that the sex × treatment interaction ($F_{1,53}$ = 8.4, P < 0.005) was significant. The Newman–Keuls test showed that the nuclear areas of flutamide-treated males were significantly smaller than in control males (P < 0.01) but were larger than in control females (P < 0.01). The female groups did not differ significantly from each other.

Perineal morphology

The mean weights of the BC/LA muscle complex in flutamide-treated and control males are presented in Table I. The BC/LA muscle complex was considerably lighter in flutamide-treated males than in control males (t_{30} = 14.52, P < 0.001). An orifice leading into a blind vagina was noted in 14 of the 16 flutamide-treated males. This structure was not present in any of the control males.

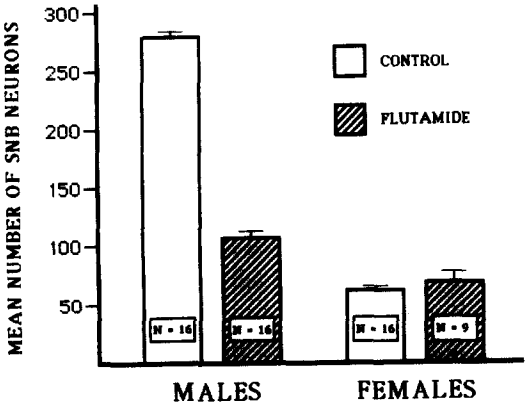

Fig. 2. Mean number of spinal nucleus of the bulbocavernosus (SNB) neurons in control and flutamide-treated male and female rats. Bars represent S.E.M.

DISCUSSION

The results clearly show that in male rats, normal masculinization of the DLN and SNB is dependent upon exposure to prenatal androgens. Flutamide, which blocks androgen receptors, reduced the number of neurons in the DLN to the levels characteristic of females. The present findings complement and extend Breedlove and Arnold's[5] observations that flutamide reduces the number, somal area, and nuclear area of SNB neurons, and also reduces the weight of perineal muscles. In their study prenatal flutamide exposure was combined with neonatal castration and a direct comparison with females was not provided.

The precise mechanism through which the anti-androgenic action of flutamide reduces the number of neurons in the DLN and SNB of males is unclear. It is unlikely that flutamide interferes with the birth of DLN and SNB neurons in males. Ventral spinal cord motoneurons are postmitotic by day 14 of gestation[7], and sex differences in plasma testosterone levels are not detectable in fetal rats until day 18 of gestation[28].

Since neurons seem to migrate from the DLN to the region of the SNB during prenatal development[22], flutamide could have reduced the number of DLN neurons by facilitating their migration to the SNB. Specifically, flutamide could have blocked the prenatal action of dihydrotestosterone (DHT)[18], an androgen that appears to inhibit the migration of DLN neurons to the SNB[1,24]. However, since flutamide males had fewer SNB neurons than control males, this scenario seems unlikely.

The most likely mechanism through which flutamide could have diminished the number of DLN and SNB neurons is by preventing androgen from rescuing the motoneurons from cell death. This explanation is particularly compelling in the light of existing studies of the ontogeny of these two nuclei. The sexually dimorphic number of DLN and SNB neurons is primarily due to differential patterns of neuronal death. Both males and females lose DLN and SNB neurons, but females lose more than males[9,19,23]. Perinatal administration of TP to females reduces the loss of both SNB and DLN neurons so that the final numbers approximate those of males[19,23]. Conversely, Breedlove and Arnold[5] have shown that exposure of fetal males to flutamide blocks endogenous androgens from maintaining SNB neurons. The present study replicates that finding and further demonstrates that the same is true for DLN neurons.

We found no sex difference in the mean cross sectional area of DLN neurons. The lack of a sex difference in the mean somal area of DLN neurons is consistent with a previous finding in our laboratory[12] but is at variance with a report by McKenna and Nadelhaft[16] in

which DLN neurons were found to be larger in males than females. McKenna and Nadelhaft[16] used retrograde tracers to identify the DLN neurons, whereas we used a thionin stain. Critical differences in methodology may explain the discrepant results.

Although there were no differences in the mean somal size of DLN neurons, we discovered a sex difference in the distribution of somal areas in our samples. Notably, control females had over five times as many large neurons than did control males, and flutamide-treated males had a number between control males and females. The presence of proportionately more large neurons in the DLN of females and flutamide-treated males may suggest that these neurons innervate a structure shared by these two groups which is not found in control males. It is tempting to postulate that one function of the large DLN neurons is to innervate muscle fibers located in the wall of the lower vagina. Unfortunately, there is no definitive description of the presumptive homologues of the ischiocavernosus and bulbocavernosus muscles in the female rat. McKenna and Nadelhaft[16] describe these muscles as being too fine in the female to distinguish clearly from connective tissue in gross dissection. By means of electrical stimulation, these investigators have localized bulbocavernosus muscle fibers originating from the lateral and ventral surface of the vagina and ischiocavernosus fibers originating on the ischial ramus. The spinal cord nuclei innervating these fibers in the female have not been identified. Most of the flutamide-treated males in the present study had developed the caudal portion of the vagina, but it was abnormal compared to the vagina of a control female (i.e. shorter and smaller). The presence of this abnormal feminine structure could explain why the flutamide males had proportionately fewer of the large neurons than did females.

Since a few large neurons were found in control males, we must conclude that some of these neurons serve a function unrelated to the innervation of a feminine structure. For example, neurons innervating the urethral sphincter originate in the DLN[16,21]. Since both females and flutamide-treated males have fewer motoneurons that innervate the IC muscles, proportionately more of the twenty DLN neurons sampled in each of these animals would include neurons innervating the urethral sphincter. If urethral sphincter neurons are larger than other neurons in the DLN, this could account for the greater proportion of large neurons in females and flutamide-treated males. To assess this possibility, we measured in eight control males 20 lateral and 20 medial DLN neurons, which are presumed to innervate the urethral sphincter and IC muscles, respectively[16,17,21]. The lateral DLN neurons were significantly smaller than the medial neurons ($t_7 = 3.61$, $P < 0.01$). These data are at odds with McKenna and Nadelhaft's[16], who found that neurons innervating the urethral sphincter are slightly larger than neurons innervating the IC muscles. The conflicting results of our laboratory and McKenna and Nadelhaft[16] may be due to the use of very different methodological approaches. McKenna and Nadelhaft identified neurons by applying two different retrograde tracers, horseradish peroxidase for the urethral sphincter neurons and Fast blue for the IC neurons. We identified neurons on the basis of their anatomical location. McKenna and Nadelhaft's approach has the advantage of identifying the source of innervation with certainty. Our method has the advantage of using the same stain on both medial and lateral neurons before making measurements and making statistical comparisons. Pending an unequivocal characterization of the relative size of different types of DLN motoneurons, it is not possible to conclude whether the greater proportion of large neurons found in our females is a sampling artifact or a previously undescribed subpopulation of sexually dimorphic neurons.

If there truly is a greater number of large DLN neurons in females and flutamide males relative to control males, this result suggests several possible conclusions, First, the genotype does not directly determine a greater number of large DLN neurons in females than in males; flutamide males do not have a female genotype, yet they have more large DLN neurons than control males. Secondly, these large neurons are associated with an absence of androgenic stimulation during early development. Androgenic stimulation during fetal development may promote cell death in the subpopulation of large DLN neurons in normal males. Although such an effect of steroids has not yet been described in mammals, Truman[25] has provided evidence that steroids can trigger the self destruction of cells in the course of ontogeny in insects. Alternatively, males may have proportionately fewer of these large DLN neurons than females because androgens cause these large neurons to migrate out of the DLN during early development. It is further possible that testosterone may cause large DLN neurons to differentiate into a smaller neuron type in males, or testosterone could prevent growth of these particular DLN neurons in males. These latter explanations not only account for the presence of more large DLN neurons in females but also for the presence of more medium sized neurons in control males than in flutamide-treated males and females.

Our finding that the nuclear areas of DLN neurons are larger in males than in females is consistent with Jordan, Breedlove, and Arnold[11] and with previous results in our laboratory[12]. However, we found no evidence for an effect of prenatal flutamide exposure on the

nuclear area of DLN neurons in males. The lack of a flutamide effect suggests that prenatal androgens are not involved in the sexually dimorphic appearance of the nuclei in adult animals. Since Jordan, Breedlove, and Arnold[11] also found that neonatal TP had no effect on the nuclei of DLN neurons, androgens probably do not exert an organizing effect on this characteristic. Since no aromatase activity can be detected in the spinal cord[15], and very few DLN cells show any estrogen binding[2,6,13], this sexually dimorphic trait probably is not caused by estrogenic action. Thus, the sexually dimorphic size of the nuclei of DLN neurons may arise purely through the activational influences that androgens exert in adulthood.

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