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Effects of Sex and Prenatal Androgen Manipulations on Onuf's Nucleus of Rhesus Macaques

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Abstract

The role of gonadal steroids in sexual differentiation of the central nervous system (CNS) is well established in rodents, but no study to date has manipulated androgens prenatally and examined their effects on any CNS structure in a primate. Onuf's nucleus is a column of motoneurons in the sacral spinal cord that innervates the striated perineal muscles. This cell group is larger in males than in females of many species, due to androgens acting during a sensitive perinatal period. Here, we examined Onuf's nucleus in 21 adult rhesus monkeys, including control males and females, as well as males whose mothers had been treated with an anti-androgen or testosterone during gestation. We found a robust sex difference, with more motoneurons in control males than in females. The soma size of Onuf's nucleus motoneurons was also marginally larger in males. Treatment with the anti-androgen flutamide for 35–40 days during early gestation partially blocked masculinization of Onuf's nucleus: motoneuron number in flutamide-treated males was decreased relative to control and testosterone-treated males, but remained greater than in females, with no effect on cell size. A control motor nucleus that innervates foot muscles (Pes9) showed no difference in motoneuron number or size between control males and females. Prenatal testosterone treatment of males did not alter Onuf's nucleus motoneuron number, but did increase the size of both Onuf's and Pes9 motoneurons. Thus, prenatal androgen manipulations cause cellular-level changes in the primate CNS, which may underlie previously observed effects of these manipulations on behavior.

Keywords

spinal cord; brain; sex difference; testosterone; motoneuron; primate; Onuf's nucleus

Sexual differentiation in mammals depends on differential exposure of males and females to gonadal steroid hormones. Androgens produced by the fetal testes promote masculinization of the external genitalia (Wilson et al., 1993). Testosterone and its androgenic and estrogenic

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metabolites also underlie sex differences in the central nervous system (CNS) of rodents, often by acting during a critical developmental window (Forger et al., 2016). Little direct evidence is available regarding mechanisms of sexual differentiation of the CNS in primates, although studies of humans with disorders of sex development support a role for androgens in sexual differentiation of the human brain and behavior.

For example, genetically male individuals with complete androgen insensitivity syndrome (CAIS), have a normal female appearance and psychosexual development, as well as female-like white matter microstructure and neural activation when viewing sexual images or performing a mental rotation task (Hamann et al., 2014; Hines et al., 2003; van Hemmen et al., 2016, 2017; Wisniewski et al., 2000). Since CAIS individuals lack functional androgen receptors throughout life and are raised as females, however, it is not clear whether effects are due to developmental hormone exposure, adult hormone exposure, or to rearing. Conversely, human females exposed to excess androgens *in utero* as a result of congenital adrenal hyperplasia (CAH) show masculinization of toy preferences, play behavior, and spatial abilities (Berenbaum et al., 2012; Mueller et al., 2008; Hines, 2010; Nordenström et al., 2002; Pasterski et al., 2011; Puts et al., 2008). Neuroanatomical changes have also been reported in CAH patients, but appear to be related to the accompanying abnormality in glucocorticoids, rather than to developmental androgen exposure, per se (Merke et al., 2003; Mnif et al., 2013).

More controlled studies, in which androgen exposure has been experimentally manipulated prenatally have been conducted in rhesus monkeys. Manipulations of androgens beginning around gestational day (GD) 40 of a 164-day gestation altered the development of the external genitalia, whereas treatments beginning after GD100 altered physiology and play behavior (Goy et al., 1988). To date, however, no study has examined structural sex differences in the CNS after manipulating gonadal steroids prenatally in a primate.

One of the simplest and best-studied sexually dimorphic neural systems of mammals concerns the motoneurons that innervate the striated perineal muscles (for review see Sengelaub and Forger, 2008). In rats, two clusters of motoneurons in the lower lumbar spinal cord, the spinal nucleus of the bulbocavernosus (SNB) and the dorsolateral nucleus (DLN), send their axons via the pudendal nerve to innervate the bulbocavernosus and ischiocavernosus muscles, respectively (McKenna and Nadelhaft, 1986). These muscles attach to the base of the penis and play important roles in erection and ejaculation (Hart, 1972; Hart and Melese-d'Hospital, 1983; Karacan et al., 1983; Sachs, 1982). The bulbocavernosus and ischiocavernosus muscles are absent or greatly reduced in females, which also have fewer SNB or DLN motoneurons than do males (Breedlove and Arnold, 1980; Jordan et al., 1982).

The development of sex differences in the perineal muscles and their innervating motoneurons depends on perinatal exposure to androgens. SNB motoneurons and their target muscles initially form in rats of both sexes, but the neuromuscular system degenerates in females around the time of birth (Cihák et al., 1970; Nordeen et al., 1985; Sengelaub and Arnold, 1986). Motoneuron number in the SNB and DLN can be completely masculinized in females treated with androgens perinatally (Breedlove et al., 1983b; Jordan et al., 1982) and

is female-like in males with a genetic inactivation of androgen receptors (Breedlove and Arnold, 1982). The SNB is also demasculinized in males prenatally exposed to the anti-androgen flutamide (Breedlove and Arnold, 1983a), although copulatory behavior is normal in these males, presumably because sexual differentiation of the brain in rodents depends largely on estrogenic metabolites of testosterone (Schwarz and McCarthy, 2008).

In carnivorans and primates, motoneurons innervating the bulbocavernosus and ischiocavernosus muscles are found in Onuf's nucleus, a single motor pool located in the lower lumbar to upper sacral spinal cord (Onuf, 1900). Adult male dogs and hyenas have more Onuf's nucleus motoneurons than do females (Forger and Breedlove, 1986; Forger et al., 1996) and, in both cases, the sex difference is due to prenatal androgen exposure. Onuf's nucleus motoneuron number is male-like in female dogs exposed prenatally to testosterone propionate (Forger and Breedlove, 1986), and is completely female-like in male spotted hyenas exposed to an anti-androgen *in utero* (Forger et al., 1996).

Among primates, Roppolo and colleagues (1985) found no sex difference in the number of motoneurons in Onuf's nucleus of rhesus monkeys, based on retrograde labeling of the pudendal nerve, but only two males and two females were examined. A sex difference (male > female) in Onuf's nucleus cell number has been reported in Japanese monkeys (Ueyama et al., 1985) and humans (Forger and Breedlove, 1986), but neither study manipulated androgens. Remarkably, over 30 years have elapsed without a neuroanatomical demonstration that prenatal androgens alter Onuf's nucleus, or any other neuroanatomical structure in the spinal cord or brain of a primate, no doubt because the obstacles inherent in a study that requires prenatal treatments and post-mortem analyses in a primate are daunting. This study is a first step in addressing that gap, by examining Onuf's nucleus motoneurons in control female and male rhesus monkeys, as well as males exposed to supplemental androgens or anti-androgens prenatally.

Methods

Animals and treatments

The spinal cords of 21 rhesus macaques (*Macaca mulatta*) were used in this study. Control males and females were untreated, whereas treated males had been subjects in a previous longitudinal study of the effects of prenatal hormone manipulations on anatomy, physiology and behavior (Herman & Wallen, 2007; Herman et al., 2000, 2003, 2006; McFadden et al., 2006; Tomaszewski et al., 2001, 2005; Zehr et al., 2005). Details of housing and rearing conditions have been described previously (Herman et al., 2000, 2003). The animals were sacrificed prior to the start of this study. All were adult (4.8 – 20.6 years of age) and there was no significant difference between the groups in age at sacrifice (Table 1). Animal use adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of the Yerkes National Primate Research Center.

Details of the prenatal hormone treatments are published elsewhere (Herman et al., 2000). Briefly, timed-pregnant females received intramuscular injections of an androgen receptor blocker (30 mg/kg flutamide in DMSO, twice daily) or testosterone (20 mg testosterone

enanthate in sesame oil, weekly; 0.25 cc DMSO twice daily on all other days of treatment and in the afternoon of days when they received morning injections of testosterone). Treatment was started between GD35-40 ("early") for most of the subjects in this study and continued for 35–40 days. For two testosterone-treated subjects, treatment started between GD110-115 ("late") and also continued for 35–40 days. Differentiation of the external genitalia occurs from about GD40-GD80 in rhesus monkeys (Prahalada et al., 1997). The early flutamide treatments prevented full masculinization of the genitalia, with effects ranging from mild hypospadias to a markedly reduced penis length with a urethral opening separate from the glans (Herman et al., 2000). Supplemental testosterone treatment (early or late) increased penis length, but otherwise did not alter male genital development (Herman et al., 2000).

Final groups consisted of eight control females, six control males, three testosterone-treated males (N=1 early and 2 late), and four early flutamide-treated males.

Spinal cord processing

Vertebral columns were collected at sacrifice and immersion fixed in 10% buffered formalin. After at least several weeks of fixation, spinal cords were removed from the columns and fixed in formalin for an additional week or more. A 10mm length of the spinal cord including the lower lumbar and upper sacral segments was then dissected out and placed into 20% sucrose for 5–7 days. A freezing microtome was used to cut four series of 50 μ m cross sections. Two of the four series were mounted on gelatin-coated slides, stained with Klüver-Barrera (a combination Nissl/myelin stain), and coverslipped with Permount. The remaining sections were stored at -20°C in cryoprotectant.

Analysis of Motoneuron Number and Size

All analyses were performed by investigators blind to treatment group. Onuf's nucleus could readily be identified by its position and distinctive morphology. It is located at the base of the ventral horn in spinal segments lumbar 7 to sacral 2, medial to the lateral motor nucleus of those segments (Roppolo et al., 1985), and is further characterized by relatively small, closely packed motoneurons. Onuf's nucleus motoneurons possess a prominent network of longitudinal dendrites, which create a faint halo around the nucleus in Klüver-Barrera stained cross sections.

Counts of motoneurons in Onuf's nucleus were made unilaterally in two of the four series of sections of each animal, and were then doubled to estimate total cell number. In cases where the histology of one of the series was compromised, or if counts of the first two series differed from each other by $> 10\%$, a third series of sections was stained and counted. Counts were made at 400X magnification, and performed in accord with stereological principles and methods previously validated for the counting of motoneurons (Clarke and Oppenheim, 1995). Because the number of motoneurons is relatively small, all cells could be counted and random sampling was not required. To avoid double counting of split motoneurons, cells were counted only if they fit the morphological criteria of motoneurons (i.e., large, multipolar and darkly stained), and had a visible nucleus that came into focus within the thickness of the section.

We also counted motoneurons in Pes9, a cell group that innervates intrinsic muscles of the foot (Vanderhorst and Holstege, 1997). Pes9 is the primate homolog of the retrodorsolateral nucleus (RDLN) of rodents, which has often been used as a control nucleus in studies of the SNB (Jordan et al., 1982; Monks et al., 2003; Zup et al., 2003), and can be unambiguously identified as the only motoneuron cell group located in the lateral horn in spinal segments overlapping Onuf's nucleus (Sengul et al., 2013). Pes9 motoneurons were counted in 20 sections of every animal, starting from the caudal-most section in which Onuf's nucleus motoneurons were present, and continuing rostrally through the next 19 sections. Because Onuf's nucleus was present in an average of 21.3 sections in each of the four series of sections, our Pes9 counts approximated the rostro-caudal extent of Onuf's nucleus.

For analyses of motoneuron size, we used Stereo Investigator software (MBF Bioscience, Williston, VT) to trace outlines of the soma and nucleus of the motoneurons in every third section of Onuf's nucleus and Pes9. To avoid selection bias, every motoneuron in which boundaries could be distinguished was traced. On average, 47 somas (38 nuclei) were traced from each animal for Onuf's nucleus, and 81 somas (54 nuclei) were traced for Pes9; the number of somas is higher than the number of nuclei because soma boundaries were more often clearly discernable.

Statistics

One-way analysis of variance (ANOVA) was used to evaluate differences in age, motoneuron number and cell size between four groups: control females, control males, testosterone-treated males and flutamide-treated males. The "early" and "late" testosterone-treated males clustered tightly together for all measures and were therefore combined in the analyses below; early and late subjects are identified by different symbols in the figures. Planned comparisons using Fisher's Least Significant Difference were performed only following a significant overall ANOVA. Effects sizes were calculated using partial eta squared (η^2) for the overall ANOVAs and Cohen's d for pairwise comparisons.

Results

Onuf's nucleus motoneuron number and cell size

Onuf's nucleus was visible in Klüver-Barrera stained sections as a group of medium-sized motoneurons surrounded by a faint "halo" of myelinated fibers (Figure 1). In humans, Onuf's nucleus is split into dorsomedial and ventrolateral cell groups (Forger and Breedlove, 1986). A split was not consistently seen in Onuf's nucleus of rhesus monkeys and, when present, it was dorsolateral / ventromedial, as previously described (Roppolo et al., 1985). Pes9 motoneurons were identified as large motoneurons in the lateral horn of the sections containing Onuf's nucleus (Figure 1).

The number of Onuf's nucleus motoneurons differed by group ($F_{3,17} = 18.8$, $P < 0.0001$; $\eta^2 = 0.77$; Figure 2). Control males had more Onuf's nucleus motoneurons than did control females ($P < 0.0001$), with a very large effect size ($d = 3.26$). Motoneuron number was nearly identical in control males and males treated prenatally with testosterone (Figure 2; $P > 0.80$). Early flutamide treatment partially blocked masculinization of motoneuron number

in Onuf's nucleus: cell number was higher than in control females ($P < 0.002$, $d = 2.39$), but appeared reduced compared to control males (a nonsignificant P -value, but relatively large effect size: $P = 0.08$, $d = 1.15$). When flutamide-treated males were compared to control plus testosterone-treated males, the reduction in motoneuron number in the flutamide group was significant ($P < 0.05$), and the effect size was again large ($d = 1.35$).

Onuf's nucleus soma size also differed by group ($F_{3,16} = 6.2$, $P = 0.005$; $\eta^2 = 0.54$; Figures 3 and Figure 4A). Soma size was about 30% larger in control males than in females ($P = 0.05$, $d_s = 1.16$). Flutamide-treated males did not differ from control males on this measure ($P = 0.98$, $d_s = 0.02$), and had nonsignificantly larger cell size than did females with a large effect size ($P = 0.07$, $d_s = 1.22$). Strikingly, males receiving prenatal testosterone had larger Onuf's nucleus somas than all other groups ($P < 0.005$, $d_s = 3.38$ versus females; $P < 0.05$, $d_s = 1.50$ versus males; $P < 0.05$, $d_s = 1.61$ versus flutamide-treated males; Figure 3C). Mean nucleus size of motoneurons in Onuf's nucleus did not differ significantly among groups ($F_{3,16} = 1.7$, $P = 0.20$; $\eta^2 = 0.24$; Figure 4B).

Pes9 motoneuron number and cell size

Pes9 motoneuron number differed between groups in the overall ANOVA ($F_{3,17} = 3.6$, $P < 0.05$; $\eta^2 = 0.39$; Figure 5). As expected, control males did not differ from females in cell number ($P = 0.53$, $d_s = 0.33$). Relative to control males, however, Pes9 motoneuron number was reduced in both flutamide- and testosterone-treated males ($P = 0.014$, $d_s = 1.29$, and $P = 0.039$, $d_s = 1.54$, respectively).

There also was a significant effect of group on both Pes9 soma ($F_{3,17} = 13.0$, $P < 0.0005$; $\eta^2 = 0.70$) and nucleus size ($F_{3,17} = 25.6$, $P < 0.0005$, $\eta^2 = 0.82$; Figure 6). Control males did not differ from females (somas: $P = 0.61$, $d_s = 0.28$; nuclei: $P = 0.81$, $d_s = 0.12$) or from flutamide-treated males (somas: $P = 0.24$, $d_s = 0.79$; nuclei: $P = 0.66$, $d_s = 0.35$) on either measure. However, testosterone-treated males had larger Pes9 somas ($P < 0.0005$, $d_s = 2.98$ for each comparison) and nuclei ($P < 0.0005$, $d_s = 5.20$ for each comparison) than all other groups, with very large effect sizes.

Discussion

Our findings confirm a sex difference in Onuf's nucleus of rhesus monkeys, and suggest that this difference depends on differential prenatal androgen exposure in males and females. We also find that supplemental prenatal testosterone increases motoneuron cell size in Onuf's nucleus and Pes9 of male monkeys.

One limitation of our study is that control males and females were untreated, whereas treated males were born to females given twice daily injections for 35–40 days of gestation. This could be a concern if the injections caused significant stress in the pregnant females, because maternal stress can attenuate masculinization, at least in rodents (Ward, 1984; Anderson et al., 1985). Mitigating this concern, however, is that fact that the mothers of flutamide- and testosterone-treated animals received injections on an identical schedule, yet only the flutamide-treated group exhibited a decrease in Onuf's nucleus cell number. The testosterone-treated males were, if anything, hyper-masculine with respect to Onuf's nucleus

size. In addition, the pregnant females had been habituated to the handling procedure, and were trained to voluntarily enter the location where blood samples were collected prior to their becoming pregnant, and injections were delivered during pregnancy (Herman et al., 2000). Thus, the stress of the procedure is likely to have been mild, in contrast to the more severe stressors used to affect sexual differentiation in rodent studies. Finally, although vehicle-treated controls were unavailable for the current study, they were included in the original study design (Herman et al., 2000) and have been compared to untreated controls on several measures. No differences were found between untreated monkeys and vehicle-treated controls in either otoacoustic emissions or the timing of pubertal development (both of which are sexually differentiated; McFadden et al., 2006; Herman et al., 2006). Thus, while we cannot completely rule out an effect of injection regime on spinal motoneurons, this factor seems unlikely to have accounted for the differences reported here. The most likely explanation is that, as in other mammals, prenatal androgens influence sexual differentiation of Onuf's nucleus in rhesus monkeys and also have lasting effects on cell size in at least two motoneuron pools.

Sex Differences

Our findings clearly establish a sex difference in the spinal cord of rhesus monkeys: control males had 47% more Onuf's nucleus motoneurons than did females, with no overlap between the sexes. The previous negative finding (Roppolo et al., 1985), which compared Onuf's nucleus in just two rhesus monkeys of each sex, was likely underpowered and/or due to technical issues associated with the retrograde labeling method used to identify motoneurons. The magnitude of the sex difference in motoneuron number found here is larger than that in humans (25% more motoneurons in ventrolateral Onuf's nucleus of men; Forger and Breedlove, 1986), and similar to that in Japanese monkeys (~50%; Ueyama et al., 1985). Thus, Onuf's nucleus motoneuron number is sexually differentiated in primates, as it is in several rodent species, at least two carnivores, and one insectivore (Breedlove and Arnold, 1980; Forger and Breedlove, 1986, 1987a; Forger et al., 1996; Holmes et al., 2009; Polak and Freeman, 2010; Ulibarri et al., 1995; Wee and Clemens, 1987). As expected, we found no difference between control males and females in the number of motoneurons in Pes9, a cell group that innervates foot muscles. Similarly, there is no sex difference or, if anything, a slight female advantage, in motoneuron number in the homologous nucleus of rodents (the RDLN; Jordan et al., 1982; Monks et al., 2003; Moore et al., 1996; Tobin & Payne, 1991; Zup et al., 2003).

The cellular mechanisms underlying sex differences in the number of perineal motoneurons have been well studied in rodents. SNB motoneurons and their target muscles form in rat and mouse embryos of both sexes, but degenerate around the time of birth in females, unless they are provided with exogenous androgens (Breedlove and Arnold, 1983b; Cihák et al., 1970; Jacob et al., 2008; Nordeen et al., 1985; Park et al., 1999; Xu et al., 2001). Both the SNB motoneurons and their target muscles are spared in female mice with a deletion of the pro-apoptotic gene, *Bax*, or overexpression of the pro-survival protein, *Bcl-2* (Jacob et al., 2005, 2008; Zup et al., 2003), indicating that death of this neuromuscular system is by apoptosis. SNB cells of perinatal females can be rescued by the local application of neurotrophic factors to the region of SNB axon terminals, whereas the localized blockade of

trophic factor action prevents masculinization of the SNB (Forger et al., 1993, 1997; Xu et al., 2001). Taken together, the most likely interpretation is that androgens prevent apoptosis of the perineal muscles, which then rescues the innervating motoneurons via the production of neurotrophic factors.

The mechanisms underlying sexual differentiation of Onuf's nucleus in primates may be similar. The overproduction of neurons, followed by a period of naturally occurring cell death, is a widespread characteristic of vertebrate neural development. In humans, motoneuron cell death overlaps with androgen production by the fetal testes (Forger and Breedlove, 1987b; Soler-Botija et al., 2002). The androgen manipulations in the current study likely coincided with the prenatal period of motoneuron cell death in rhesus monkeys, and could have altered motoneuron survival either directly, or via effects on the target muscles.

We also found marginally larger Onuf's nucleus soma sizes in control male rhesus monkeys than in females. Ueyama et al. (1985) did not find a sex difference in Onuf's nucleus soma size of Japanese monkeys, but this was based on measurements in a single male and single female. There is no sex difference in the soma size of Onuf's nucleus motoneurons in adult spotted hyenas or dogs (Forger and Breedlove, 1986; Forger et al., 1996), but the size of SNB motoneurons is much larger in male than in female rodents (Breedlove and Arnold, 1981; Forger and Breedlove, 1987; Park et al., 2002; Ulibarri et al., 1995; Wee and Clemens, 1987). The weak (or absent) sex differences in cell size in Onuf's nucleus versus the robust sex differences in cell size in the SNB may be related to the more heterogeneous nature of Onuf's nucleus: the large majority of SNB motoneurons innervate highly sexually dimorphic muscles (McKenna and Nadelhaft, 1986), whereas Onuf's nucleus motoneurons innervate two dimorphic muscles (bulbocavernosus and ischiocavernosus) as well as two monomorphic muscles (the external anal and external urethral sphincter muscles; Thüroff et al., 1982; Yamamoto et al., 1978).

Effects of Prenatal Androgen Manipulations on Motoneuron Number

Onuf's nucleus motoneuron number was almost identical in control males and males treated with prenatal testosterone, which is consistent with observations in rodents (Breedlove et al., 1982; Park et al., 1999). Apparently, endogenous androgen production is sufficient to maximally spare perineal motoneurons from cell death. In contrast, early prenatal flutamide treatment partially blocked masculinization of Onuf's nucleus in rhesus monkeys: mean cell counts were lower than in control males and males given supplemental testosterone, but remained significantly higher than in females. The effect of early flutamide may have been incomplete because the critical period for androgen action extended beyond our treatments. Plasma testosterone is elevated in fetal male rhesus monkeys from about GD50 until birth (Resko, 1985), and our treatments overlapped with only the first portion of that period. Both early and late prenatal flutamide treatment of male rhesus monkey resulted in otoacoustic emissions that were intermediate between those of control males and females (McFadden et al., 2006), which also suggests a prolonged sensitive period for androgen action. In spotted hyenas, Onuf's nucleus motoneuron number was completely female-like in males given prenatal anti-androgen treatments that began early in gestation and extended almost to birth

(Forger et al., 1996), and we predict that the same would be true in rhesus monkeys. An alternative explanation for the partial effect of flutamide treatment is that the dose used did not completely block testosterone action. Although this dose prevented full masculinization of the genitalia in males, effects varied between individuals (Herman et al., 2000), and no other published study has examined prenatal flutamide treatments in rhesus monkeys to which we can compare our dosing. In either case, exposing mothers to flutamide early in gestation alters the sexual differentiation of Onuf's nucleus in their male offspring.

Androgen effects on Onuf's nucleus motoneuron number could also be tested by examining androgenized female monkeys. Although females exposed to testosterone either early or late in gestation were included in the original cohort (Herman et al., 2000), tissues from these animals were not available for the current study. Masculinization of the genitalia was fairly subtle in the testosterone-treated females (Herman et al., 2000), suggesting a sub-optimal dose or a critical period extending beyond the chosen treatment periods.

We found lower Pes9 motoneuron numbers in males treated prenatally with testosterone or flutamide than in control males or females, which was unexpected. It is possible that prenatal treatments altered the survival of Pes9 cells. Alternatively, this finding may be an artifact of our sampling method. We counted all motoneurons throughout the rostral-caudal extent of Onuf's nucleus, but restricted Pes9 counts to 20 sections of each animal. In larger animals, with longer spinal cords, we may have sampled a smaller proportion of Pes9. The spinal cord stops elongating early in postnatal life (e.g., approximately age 4 in humans; Martini et al., 2015). Interestingly, late androgen- and early flutamide-treated male rhesus monkeys had longer crown-rump lengths than did vehicle-treated controls during the first 18 months of life (Herman et al., 2000), whereas control males and females did not differ in this measure. Similarly, Onuf's nucleus extended over more sections in flutamide-treated males than in all other groups in the current study (data not shown). Because we focused on Onuf's nucleus during histological processing of the spinal cords, we do not have the full extent of Pes9 in all animals in order to address the question directly. The data available, however, support the possibility that lower Pes9 motoneuron number in flutamide- and testosterone-treated males is an artifact of sampling a smaller proportion of Pes9 in those groups.

Effects of Prenatal Androgen Manipulations on Motoneuron Cell Size

Prenatal testosterone supplementation of males robustly increased soma size in adulthood in both Onuf's nucleus and Pes9. This was also unexpected, although the finding has some precedent in the rat SNB (Breedlove and Arnold, 1983a). Neither SNB nor RDLN motoneurons express the androgen receptor until after the first postnatal week in rats (Jordan et al., 1997). Thus, prenatal androgens may alter adult motoneuron size indirectly, via action on the target muscles, which express the receptor before birth (Jordan et al., 1997). Alternatively, prenatal testosterone treatment could increase motoneuron size if it leads to increased circulating levels of androgens or increased sensitivity to androgens in adulthood. Many motoneurons in adult rats and rhesus monkeys express androgen receptors (Bonsall et al., 1985; Freeman et al., 1995; Simerly et al., 1990), and may respond to circulating androgen levels with changes in cell size and/or gene expression (Breedlove and Arnold, 1982; Forger et al., 1998; Leslie et al., 1991; Zuloaga et al., 2007). Although we did not

obtain plasma from our animals at sacrifice, testis size and testosterone levels were unaffected by prenatal testosterone treatment at the first (pubertal) breeding season, or the following year (Herman et al., 2006). Because rhesus monkeys are seasonal breeders, males go through annual cycles of extremely low testosterone levels in the nonbreeding season to 20-fold higher levels during the breeding season (Plant et al., 1974). Thus, any variations in adult testosterone levels between groups would occur against a backdrop of repeated marked variation in testosterone naturally experienced by male rhesus monkeys.

Implications for Behavior

Estrogenic metabolites of testosterone play a crucial role in sexual differentiation of the brain in rodents (Schwartz and McCarthy, 2008), but differentiation of the brain in primates is thought to be primarily androgen-mediated (Wallen, 2005; Thornton et al., 2009). If so, then the masculinization of behavior and physiology might be blocked in male rhesus monkeys exposed prenatally to flutamide. In partial support of this prediction, males exposed to flutamide prenatally are more female-like than are control males in the number of separation-rejection vocalizations during infancy (Tomaszycki et al., 2001), the rate of click-evoked optoacoustic emissions (McFadden et al., 2006), and the use of local markers in a spatial navigation task (Herman and Wallen, 2007). On the other hand, prenatal flutamide treatment did not alter interest in infants or the production of agonistic screams during the juvenile period (Herman et al., 2003; Tomaszycski et al., 2005). Sexual behavior (masturbation and mounting) around the time of puberty also did not differ by prenatal treatment (Herman et al., 2006), although ejaculatory performance could not be tested because the anti-androgen treatment altered the orientation of the penis such that intromission was difficult or impossible. Thus, some sexually differentiated traits in rhesus monkeys may depend on androgens produced postnatally, or be androgen-independent (e.g., due to estrogens or sex chromosome complement). Alternatively, because animals received flutamide either “early” or “late” in gestation, but not both, effects may require longer treatment or different doses of flutamide, as argued for the masculinization of Onuf’s nucleus, above. It is remarkable that 58 years after the organizational-activational hypothesis of sexual differentiation was proposed (Phoenix et al., 1959), we still know so little about the extent to which the primate brain and behavior are programmed by prenatal testosterone.

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Highlights

- Cellular-level effects of prenatal androgens on the CNS of a primate are examined
- A sex difference in cell number in Onuf's Nucleus of rhesus monkeys is established
- Prenatal anti-androgen treatment partially blocks masculinization of Onuf's nucleus
- Prenatal testosterone supplementation of males increases motoneuron cell size

Figure 1.

Photomicrograph of a Klüver-Barrera stained section through the upper sacral spinal cord of a rhesus monkey. The location of Onuf's nucleus (ON) and Pes9 motoneurons is indicated. Abbreviations: cc, central canal. Scale bar = 1 mm.

Figure 2.

Effect of sex and prenatal androgen manipulations on motoneuron number in Onuf's nucleus. Control females had fewer Onuf's nucleus motoneurons than all male groups. Prenatal testosterone treatment of males did not alter motoneuron number, and motoneuron number was intermediate in males treated with flutamide. Subjects in the Male + Testosterone group received testosterone either during an "early" (circle) or "late" (triangles) prenatal period. Different letters above the bars indicate significant differences between group means.

Figure 3.

Cell size in Onuf's nucleus is increased by prenatal testosterone treatment of males.

Photomicrographs depict the left ventral quadrant of a cross section through the spinal cord in a (A) control female, (B) control male, (C) testosterone-treated male, and (D) flutamide-treated male. Scale bars = 50 μ m.

Figure 4.

Soma size of Onuf's nucleus motoneurons depends on sex and prenatal testosterone exposure. **(A)** Control males had larger soma sizes than females, and males treated with testosterone prenatally had larger soma sizes than all other groups. Flutamide-treated males did not differ significantly from control males or control females on this measure. **(B)** Mean size of motoneuronal nuclei in Onuf's nucleus did not differ significantly by group. Subjects in the Male + Testosterone group received testosterone either during an "early" (circle) or "late" (triangles) period *in utero*. Different letters above the bars indicate significant differences between group means.

Figure 5.

Pes9 motoneuron number does not differ between control males and females, but is reduced in males treated with testosterone or flutamide *in utero*. Subjects in the Male + Testosterone group received testosterone either during an “early” (circle) or “late” (triangles) period *in utero*. Different letters above the bars indicate significant differences between group means.

Figure 6.

The size of Pes9 motoneurons in adulthood is increased by prenatal testosterone treatment of males. Soma (**A**) and nucleus (**B**) size of Pes9 motoneurons did not differ between females, males and flutamide-treated males. However, both soma and nucleus size was larger in testosterone-treated males than all other groups. Subjects in the Male + Testosterone group received testosterone either during an “early” (circle) or “late” (triangles) period *in utero*. Different letters above the bars indicate significant differences between group means.

Table 1

Mean age and age range (in years) of the monkeys in each group

GROUP	N	MEAN (SEM)*	RANGE
Control Female	8	12.6 (2.04)	5.5 – 20.6
Control Male	6	9.0 (1.74)	4.8 – 15.2
Testosterone-treated Male	3	13.1 (2.36)	7.6 – 19.1
Flutamide-treated Male	4	12.4 (0.03)	4.8 – 20.6

* There was no significant difference between groups in mean age at sacrifice (ANOVA: $F_{3,17} = 0.862$, $P = 0.48$; $\eta^2 = 0.13$). All pairwise comparisons between groups were also non-significant.