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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.

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 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

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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 and Arnold, 1983b; Jordan et al., 1982) and is female-like in males with a genetic inactivation of androgen receptors (Breedlove and Arnold, 1981). 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 et al. (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.

1. Methods

1.1. 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 and Wallen, 2007; Herman et al., 2000, 2003, 2006; McFadden et al., 2006; Tomaszycki 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.

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.

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 ml 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.

1.2. 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 10 mm 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 μm 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.

1.3. 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 lumber 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