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Perineal Musculature and Its Innervation by Spinal Motoneurons in the Male Rabbit

Effects of Testosterone

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Abstract

Striated muscles in the perineum, which include the ischiocavernosus (IC), bulbocavernosus (BC), and levator ani (LA), were identified in an attempt to understand motor regulation of penile erection in the male rabbit. The IC surrounded the corpus spongiosum of the penis whereas the BC attached to the dorsum of the penis at the midline. The LA encircled the rectum and attached to the base of the penis. This anatomy suggested that the IC plays the primary role in the erection in the rabbit, whereas the BC may cause flips of the erected penis. Spinal motoneurons that innervate the IC were identified by retrograde labeling by horseradish peroxidase (HRP). As in other mammals, spinal labels from the IC appeared ipsilaterally in the ventral horn that encompassed the sixth lumbar (L 6) and the first sacral segments. HRP injections into the BC labeled a small number of cells bilaterally at the same spinal levels. Rabbits are peculiar in having the IC motoneurons scattered among other motoneurons, unlike the rat and other rodents that have their IC motoneurons aggregated to form a spinal nucleus. Castration caused significant decreases in both the wet weight of IC muscles and the size of IC motoneurons within 2 weeks. Testosterone supplement following castration maintained the IC muscle weight and the neuronal size. Neither castration nor testosterone supplement induced changes in the number of IC motoneurons. (J Nippon Med Sch 2000; 67: 164—171)

Key words: penile erection, spinal cord, testosterone, retrograde labels, rabbit

Introduction

The rabbit perineal musculature and vasculization of the corpus cavernosum in the penis have been suggested to bear certain structural resemblances to those in man, rather than those in rodents[1]. Although sexual behavior is less understood in the rabbit than in the rat, at least the rabbit is distinctively different from the rat and more resembles man: e.g. the rabbit ejaculates at every intromission whereas the rat requires several intromissions to achieve an ejaculation[2]. Thus, the rabbit would be suited for the study of hormonal and pharmacological regulation of human penile erection.

Studies in the rat have shown that both vascular and striated muscle effector systems manifest for the initiation and maintenance of penile tumescence. The integrity of the striated muscle effector system is required to accomplish a rigid erection, presumably by bulbospongiosus muscle exerting a greater force on the bulbus penis[3]. Although autonomic control of vas-

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cular smooth muscle in the corpus cavernosum of the penis has been studied in the rabbit[4-6], the striated muscle effector system in this species has escaped scrutiny.

In humans, dogs, and rats, the bulbocavernosus (BC) and ischiocavernosus (IC) muscles attach to the crus of the penis and are involved in erection and ejaculation[7-9]. Relative sizes of the BC and IC muscles differ between species, and the anatomy and nomenclature of these muscles in the rabbit have not attracted much attention.

Spinal distribution of perineal motoneurons also differs strikingly between species. In the rat, the BC and IC muscles are innervated by two distinctively separate groups of motoneurons, the spinal nucleus of bulbocavernosus and the dorsal linear nucleus, respectively. In the cat, dog and human, neurons innervating the BC and IC muscles are found in a single cell group in the ventrolateral quadrant of the ventral horn of the sacral spinal cord. The nucleus, often referred to as the Onuf's nucleus[10], also includes motoneurons for anal and urethral sphincters[11-13]. In these species, the Onuf's nucleus is a well-defined, compact column of relatively small motoneurons[14-16]. Although HRP injection into the external anal sphincter in the rabbit labeled a cluster of motoneurons in spinal segments S_2 and S_3 [17], not much is known about spinal motoneurons for striated muscles responsible for penile erection in this species.

Castration of the rabbit is followed by a pronounced decline in penile erection and ejaculation, which is reversed by treatment with testosterone[18,19]. Both vascular and striated muscle effector systems are targets of the testosterone action in the rat; the striated muscle effector system must be intact to obtain an earlier restoration of penile erection by testosterone treatment[3]. Thus, the striated muscle effector system is prominently involved in testosterone-dependent erection, although disputes about the myogenic[20] or neuronal[21] route of testosterone action persist.

In the present study, we identified striated muscles that attach to the rabbit penis and accomplished retrograde identifications of spinal motoneurons that innervate these muscles. Changes induced by castration and testosterone provided clues to distinguish particular perineal muscles and spinal motoneurons. Because the perineal muscles in male rabbits differed considerably from those in the rat and certain other species, we focused our discussion on comparative aspects.

Materials and methods

(1) *Animals*

The male white house rabbits (2.5～3.5 kg), *Oryctolagus cuniculus sp.*, used in this study were purchased from Saitama Experimental Animal Supply Inc. (Saitama). They were maintained in a controlled environment at 23℃ with 12-hr light-dark cycle (lights off at 11 a. m.) and were allowed free access to laboratory chow and water at all times. Animal housing and all procedures conformed to "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council, 1996.

The rabbits were sacrificed by an overdose of pentobarbital sodium (Abbott, 60 mg/kg body weight, in a 50 mg/m*l* solution). Three of them were used for anatomical identification of perineal musculature. Another cohort of intact (n=3) and castrated (n=8) animals were used for neural tracing studies. Castrations were carried out under anesthesia with an *i.v.* injection of pentobarbital sodium (30 mg/kg body weight), which was supplemented by an *i.m.* dose of ketamine hydrochloride (Sankyo, 25 mg/kg body weight, in a 50 mg/m*l* solution). They were allowed 2 weeks to recuperate. Five of the 8 castrated rabbits received *s.c.* injections of testosterone propionate (Wako, 5 or 10 mg /day, in a 50 mg/m*l* solution in oil) every day during this period. The remaining 3 were injected with oil vehicles.

(2) *Perineal anatomy*

The location, size and configuration of attachment to the base of the penis were determined for the BC, IC and levator ani (LA) muscles in the carcass (**Fig. 1**). A pair of perineal muscles, each originating from the ischium and attached to the base of the penis, was identified as the IC. Another midline-oriented muscle attached to the dorsum of the glans penis was identified as the BC, but as described in detail in the results, its mass appeared too diminutive to play any major role in the erection in the rabbit.

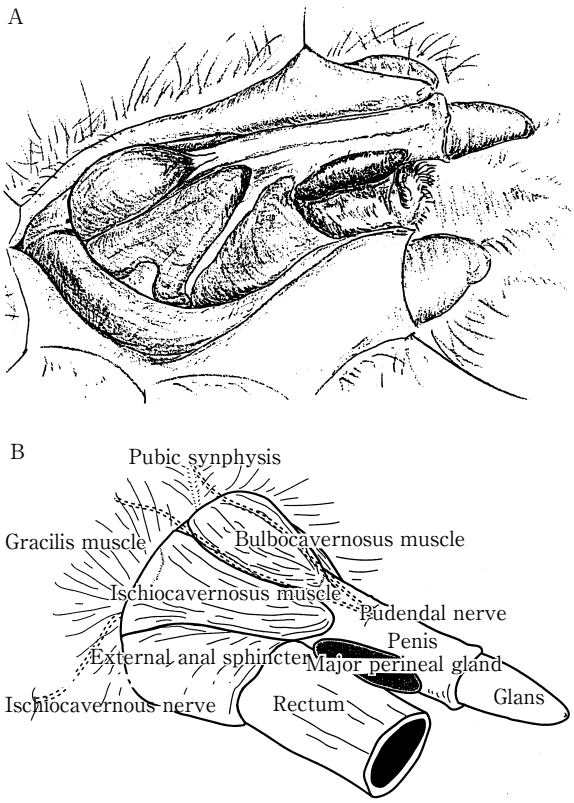

Fig. 1 Perineal striated musculature of the male rabbit. Dissected view from upper right (A) and its scheme (B).

(3) *Retrograde neuronal tracing*

Horseradish peroxidase grade 1 C (HRP, Toyobo, Tokyo) was dissolved at a concentration of 50% (w/v) in 0.9% saline, which contained 2% (w/v) dimethyl sulfoxide. The rabbits were given a similar formula of anesthetics to that used for the castration. The right IC muscle was exposed by an inguinal incision in the anesthetized animal and 10 μl each of HRP solution was injected at 5 points in the muscle along its longitudinal axis at regular intervals of 5 mm. The wound was sutured and covered. For the identification of any topographic difference in the spinal distribution of IC motoneurons that innervate subdivisions of the IC, a single injection of 10 μl of HRP was made in either the proximal or distal portion of the IC in 6 rabbits. BC motoneurons were labeled in 3 rabbits by a single injection of 10 μl of HRP in the midline.

The animals survived for 3 days and were then given an overdose of pentobarbital sodium (60 mg/kg body weight). Transcardial perfusion with 0.9% saline (1,000 ml) was followed by a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH=7.4, 1,500 ml). The lower lumbar and upper sacral portion of the spinal cord was removed and postfixed in the same fresh fixative for an additional 4 hours and then soaked in a progressive series of 10 and 30% sucrose in the phosphate buffer for 3 days. Frozen sections (50 μm) were cut in either the transverse or parasagittal plane and stored in the phosphate buffer at 4℃ for no more than 48 hours. Visualization of HRP labels was made using 3,3', 5,5'-tetramethylbenzidine (TMB) as chromogen, following the protocol described by Mesulam[22] that attempted to minimize the tendency for excessive crystallization of the TMB product and the instability of the reaction over time. Briefly, the sections were reacted with TMB for 20 minutes at 4℃, then rinsed in 0.01 M acetate buffer, mounted on gelatinized slides, dried overnight and counterstained with 0.5% Neutral Red.

(4) *Histological evaluation*

In slices of the spinal cord, cross sections of the cell bodies with HRP-labels were identified in which their nuclei showed up in their largest sizes. Locations of cell bodies were plotted in transverse or parasagittal sections of the spinal cord. Morphometric analyses of individual neurons were accomplished by using camera lucida. The contour of the cell body and major dendrites were microscopically traced by altering the focus in each section. The camera-lucida drawings were analyzed by NIH image 1.60 to determine cellular dimensions.

(5) *Perineal musculature*

The Ischiocavernosus and right gastrocnemius muscles (as a control) in all animals were removed before perfusion under anesthesia with an overdose of pentobarbital. The wet weights of these muscles were determined. In different experimental groups, the mean wet weight of the IC muscle and the left gastrocnemius muscle was calculated in each animal, and the group means (the average of the wet weight of the IC muscles and the left gastrocnemius muscle in individual animals for each group) were compared, using the number of animals per group as n. Prior to comparison, mean muscle weights were adjusted to the body weight in each group.

(6) *Androgen extraction and assay*

Prior to perfusion by fixatives, left ventricular blood was collected, heparinized and centrifuged. The plasma was stored at −20℃ until androgen extraction. For the extraction of androgen, the plasma was diluted, added to with diethyl ether, centrifuged, and chilled in a mixture of dry ice and methanol. The supernatant was warmed and shaken to dispel diethyl ether, added to with 1 ml distilled water, and kept at −80℃. The concentration of testosterone in the solution was determined by solid phase ^{125}I radioimmunoassay, using a Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA).

(7) *Statistics*

The statistical analysis was made by two-tailed *t* test with a significance limit of $p<0.05$. When recalculated, the mean wet weights of the IC muscle and the left gastrocnemius muscle were compared among the intact group, the castrated group and the castrated, androgen treated group. A two-way analysis of variance (ANOVA) was employed. Similarly, the mean areas of the somata and dendrites of the IC motoneurons in each group were compared. All values are presented in the text as means and their standard deviations.

Results

1. Perineal musculature in the rabbit

Three muscles were identified to attach to the penis in the rabbit, in a close resemblance to the human perineal anatomy (**Fig. 1**). A pair of muscles spanned symmetrically from the ischium to the base of the penis in each side; the second, a vestigial muscle, attached to the midline of the dorsum of the penis; the third originated from the base of the penis and encircled the rectum. Following the nomenclature in human anatomy, we identified the first muscle as the ischiocavernosus' the second as the bulbocavernosus, and the third as the levator ani.

2. Distribution of IC motoneurons

HRP injection to the ischiocavernosus labeled motoneurons in the L_6, L_7 and S_1 segments, which corresponded to the caudal part of the lumbar enlargement. The labels were ipsilateral to the injected muscle. In the transverse section of the spinal cord counterstained by Neutral Red, labeled neurons were scattered in the ventral horn among other large, presumed motoneurons (**Fig. 2**). Rostrally in the L_6 segment, the labeled neurons were in the dorsomedial quadrant of the ventral horn. They passed gradually into the ventrolateral quadrant in the more caudal sections. It was noted, however, that at any rostrocaudal level, the labeled neurons did not aggregate to form a discrete nucleus in parasagittal (**Fig. 2**) or transverse (**Fig. 3**) sections.

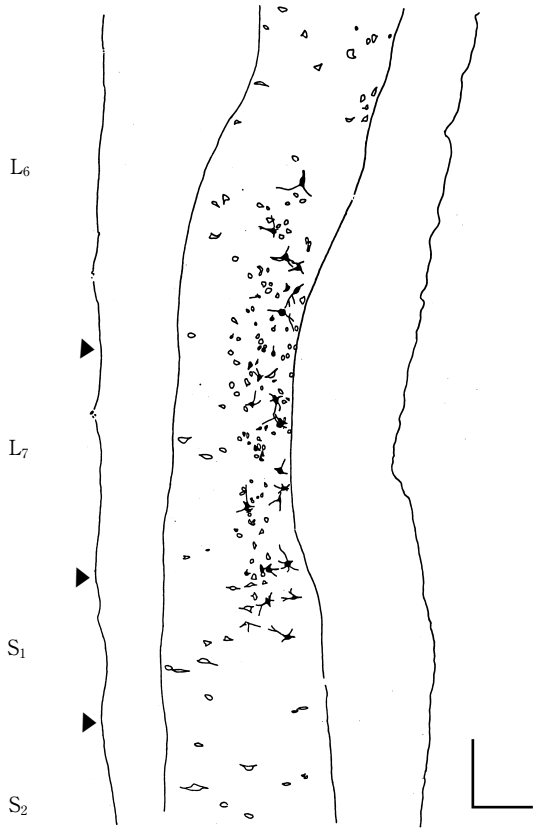

Fig. 2 Rostro-caudal distribution of labeled neurons on a parasagittal section (dorsal is left) following HRP injections to ischiocavernosus muscle. Initial parts of dendrites of some neurons, which exhibit Golgi-like labeling, are shown with the contour of other labeled cell bodies in a parasagittal section of the spinal code of a castrated and testosterone-treated rabbit. Unfilled cell bodies are other motoneurons of the same level of the spinal cord. Note that labels aggregate at caudal L_6 to mid S_1.Scale bars: 400 μm.

Injection of HRP to the bulbocavernosus also resulted in labels intermingled with other neurons in

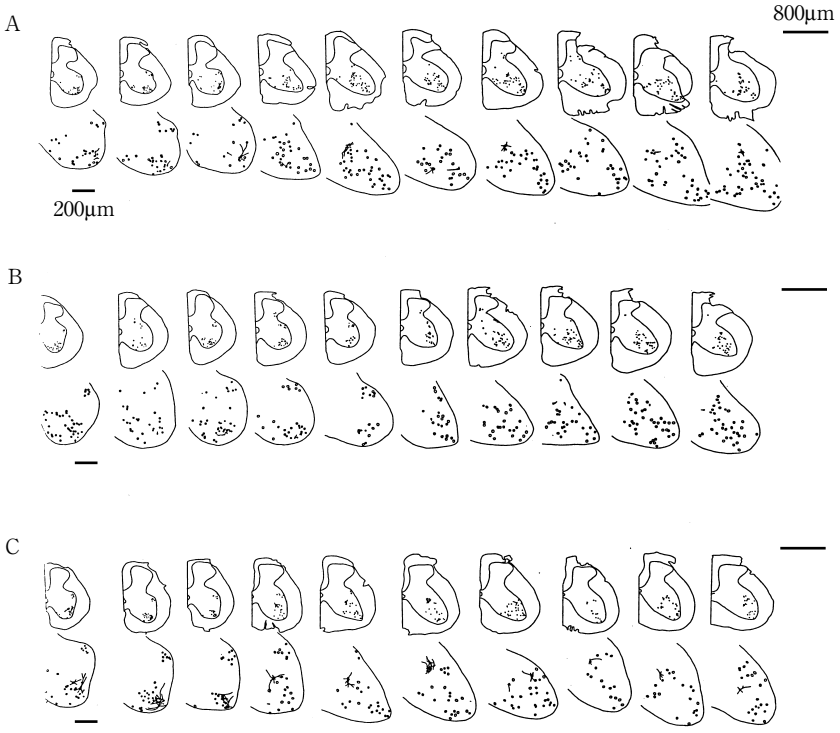

Fig. 3 Localization of labeled spinal motoneurons following HRP injections to the ischiocavernosus muscle is shown in serial transverse sections of the spinal cord (dots with thorns in the upper panel of each pair). The ventral horn is enlarged in the lower panel. Other dots in each panel are other motoneurons at the same level of the spinal cord. Representative serial sections are shown for (A) intact, (B) castrated, and (C) castrated and testosterone-treated rabbits. Scale bars: 200 μm.

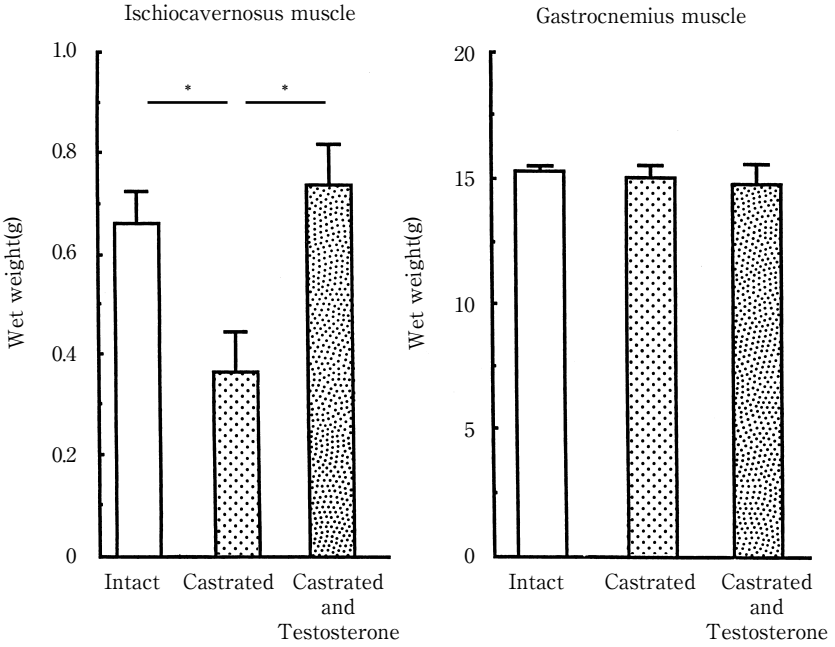

Fig. 4 Wet weight of ischiocavernosus and left gastrocnemius muscles in the male rabbit. Effects of castration and testosterone treatment are shown (*P<0.05).

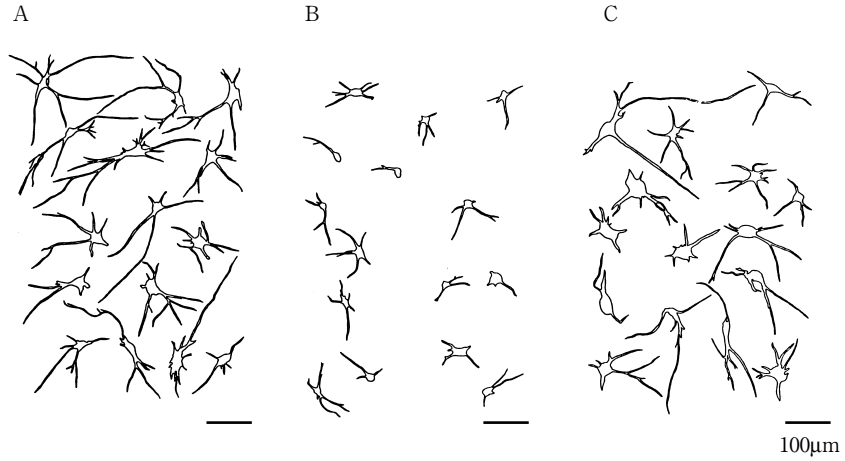

Fig. 5 Camera-lucida drawings of HRP-labeled somata and initial parts of dendrites of representative ischiocavernosus motoneurons in intact (A), castrated (B) and castrated and testosterone-treated (C) male rabbits.

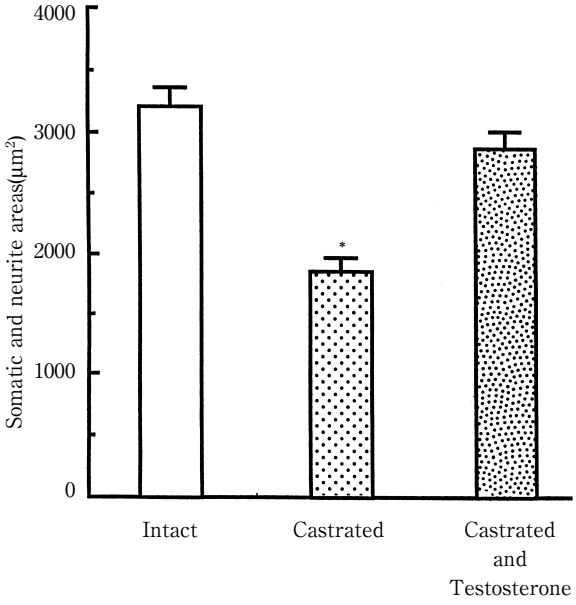

Fig. 6 Effects of castration and testosterone-treatment on somatic and dendritic area of HRP-labeled ischiocavernosus motoneurons (*P<0.05).

the ventral horn at the L_6–S_1, similar to the labels by ischiocavernosus injections, although bulbocavernosus labels were bilateral (data not shown).

3. Effect of castration and androgen treatment on IC muscles

Within 2 weeks following castration, the wet weight of the freshly dissected ischiocavernosus muscle decreased from 0.66 ± 0.06 g (mean ± SD) (n=22) to 0.35 ± 0.12 g (n=6) (P<0.01). Daily injections of testosterone during the 2-week period following castration maintained the ischiocavernosus at 0.74 ± 0.08 g (n=6) (P<0.01 *vs* castrated animals) (**Fig. 4**). No difference was detected in the weight of the left gastrocnemius muscle in the intact, castrated, and castrated and testosterone-treated animals.

The serum testosterone was 15.00 ± 1.41 ng/m*l* in intact animals (n=5). It was undetectable at 2 weeks following castration (n=5). Daily injection of testosterone increased the titer to 46.20 ± 21.50 ng/m*l* (n=5) at the time of dissection.

4. Effect of castration and androgen treatment on IC motoneurons

During the same period following castration, the area of neuronal somata and principal dendrites of the ischiocavernosus motoneurons decreased to 1867.06 ± 102.91 μm² (n=115) from 3207.33 ± 159.85 μm² (n=1631) in intact controls. Daily testosterone injection increased the area to 2873.26 ± 142.79 μm² (n=142). No significant difference was detected between the groups in the number of the ischiocavernosus motoneurons (**Fig. 5 & 6**).

Discussion

Confusion persists in the nomenclature of rabbit perineal musculature. The ischiocavernosus in the present study spanned between the ischium and the penile base in symmetry. The bulbocavernosus is a

vestigial muscle that attached to the midline of the dorsum of the penis. Our ischiocavernosus is the sub-ischiocavernosus in Barone[23]. The bulbocavernosus was often amassed together with the urethralis and ischiocavernosus to form the bulboglanduralis. Our nomenclature followed that in the human anatomy, because the rabbit perineum bore an obvious resemblance to that in the human[9].

The present study provided morphological evidence that the ischiocavernosus is the major striated muscle responsible for penile erection in the rabbit. The perineal musculature in the male differed distinctively between the rabbit and the rat. Unlike the rat, the rabbit ischiocavernosus is larger than the bulbocavernosus as in the human[9]. Whereas the regressed rabbit bulbocavernosus attaches to the dorsum of the penis in the midline, the rat bulbocavernosus is larger than the ischiocavernosus and makes a pair on both sides of the base of the penis. A muscle that attaches to the dorsum of the penis and extends back to the pubis as the rabbit bulbocavernosus has been identified in the guinea pig[24] and hyena[21]. To our knowledge, no muscles with such attachments exist in the rat. The guinea pig muscle is termed the ischiocavernosus[24] or the retractor penis[21], but its attachments to the penis and the pelvic girdle are similar to the rabbit ischiocavernosus.

The mass of both bulbocavernosus and ischiocavernosus, but not that of the gastrocnemius, depended on testosterone in the rabbit as in the rat[3, 25] or mouse[26]. Androgen receptors in striated muscles are particularly high in the sexual muscles.

Following injections of HRP into the ischiocavernosus, retrogradely labeled neurons were found in the spinal segments L_6–S_1. Unlike in other mammals, ischiocavernosus motoneurons were scattered within the ventral horn in the rabbit, without constituting any distinct nucleus. HRP injections to the bulbocavernosus resulted in similarly dispersed labels in the same region. In the rat, the ischiocavernosus and bulbocavernosus motoneurons form distinct and separate nuclei in the L_5 and L_6 segments, which are termed the spinal nucleus of the bulbocavernosus and dorsal linear nucleus, respectively. In the cat, dog and human, the Onuf's nucleus contains motoneurons of the bulbocavernosus and ischiocavernosus as well as those of anal and urethral sphincters[11–13]. In the rabbit, motoneurons of the external anal and urethral sphincters make up a separate nucleus in the S 2 and S 3 segments, which has been accounted to be homologous to the Onuf's nucleus in other species[17]. Thus the architecture of perineal innervation in the rabbit bears certain peculiarity among species.

The scattered distribution of ischiocavernosus motoneurons in the present study is not due to artifacts, because multiple injections to different regions of the muscle consistently produced a similar pattern in different animals. The compact architecture of the rat spinal nucleus of the bulbocavernosus has been associated with neuronal interconnections through chemical synapses and gap junctions to achieve erection[27]. Granted that such a neural link operates for penile erection, it is not known how such functional linkage is achieved between dispersed motoneurons in the rabbit.

Crystallization of TMB product did not hamper morphometric analysis in the present study. Castration of the rabbit was followed by a pronounced reduction in the HRP-labeled neuronal soma size, which is reversed by testosterone treatment. Neuronal response to testosterone indicates that the rabbit ischiocavernosus motoneurons share a similar characteristic with those in the rat[12, 28–30]. Testosterone-induced changes in the neuronal soma size could be either direct or secondary myotrophic action of the steroid[29].

In conclusion, the rabbit provides a good model for the study of motor control of penile erection insofar as it is borne in mind that the distribution of the spinal motoneurons for the erection in this species follows a peculiar pattern.

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(Received, December 13, 1999)
(Accepted, December 28, 1999)