Research report

Motoneurons innervating guinea pig perineal muscles are sexually dimorphic in size but not number

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Abstract

Sexual differentiation occurs prenatally in guinea pigs but extends into the postnatal period in rats. Steroids affect the development of two motoneuron nuclei of the rat lumbar spinal cord that innervate sexually dimorphic perineal muscles. The spinal nucleus of the bulbocavernosus (SNB) innervates the bulbocavernosus (BC) and levator ani (LA) muscles while the dorsolateral nucleus (DLN) innervates the ischiocavernosus (IC). In male rats, perinatal testosterone prevents degeneration of these muscles and results in a sex difference in both motoneuron size and number in adulthood. For comparative purposes, we examined the guinea pig motoneurons innervating these muscles, as well as those innervating the retractor penis (RP) and retractor clitoris (RC), muscles that have no counterpart in rats. Injections of horseradish peroxidase localized the BC/LA and IC motoneurons of guinea pigs to discrete columns in spinal levels L6 and S1, with the BC/LA motoneurons occupying a more medial position. The RP/RC motoneurons were found in L5. Motoneuronal soma area was larger in males in all examined motor pools, as was nuclear area of BC/LA and IC motoneurons. Although raw counts suggested a sex difference in cell number in the motor columns containing BC/LA and IC motoneurons, either of two different correction procedures for split nuclei error eliminated the sex difference in cell number, emphasizing the importance of such corrections when comparing neurons of different size.

Keywords: Levator ani; Bulbocavernosus; Ischiocavernosus; Steroid; Testosterone

1. Introduction

Examination of the perineal muscles of rats has provided insights into the mechanisms of sexual differentiation of the nervous system. Adult male rats have two striated penile muscles that have no counterpart in the adult female: the bulbocavernosus (BC) and the ischiocavernosus (IC). These muscles and the levator ani (LA) are all present and attach to the clitoris in female rats at birth. All three muscles have been reported to involute in females shortly after birth [9,17], but the LA has more recently been reported to persist in female rats with approximately one-tenth as many fibers as in the male LA [21]. In adult male rats, the BC acts during reproduction to form and remove copulatory plugs [31] and mediate cuplike reflexive erections [16]. The BC and LA are innervated by the spinal nucleus of the bulbocavernosus (SNB) [5], also called the dorsomedial nucleus [32]. The BC, present in both sexes at birth, degenerates in females within a few days unless the animal receives androgen [9]. Early androgen treatment yields more SNB cells in adult females [4], while early androgen deprivation in males leads to the loss of the BC and fewer SNB cells [3]. The sexual dimorphism in motoneuronal number arises through androgenic sparing of cells from ontogenetic death [2,27]. The site of androgen action is apparently the muscle, with the neurons being spared as the indirect beneficiaries of muscle survival [12–14].

The IC, which mediates penile flips in male rats [16], is innervated by the dorsolateral nucleus (DLN) of the lumbar spinal cord [5,32]. Males have more DLN neurons than do females, and treating neonatal females with testosterone increases motoneuron number in the rostral half of the nucleus [19,20].

SNB and DLN motoneurons are also larger in males than in females [6,20]. Sex differences in the size of SNB somata, while also influenced by androgen, apparently arise from a different and independent mechanism than...
that which produces differences in motoneuronal number. For instance, adult androgen treatment increases the size but not the number of SNB cells [6]. Late postnatal androgen treatment (day 7–11) of female rats does not affect adult SNB cell number but does increase cell size in adulthood [4]. The critical period for androgen’s masculinizing effect on cell size extends at least a week after the critical period for the effect on cell number [23].

Homologues to the SNB–BC system have also been examined in a few species that undergo sexual differentiation primarily during the prenatal period. In dogs, cats and primates (including humans), the BC and IC persist in adult females but are much smaller than in males. These muscles, along with other striated perineal muscles, are innervated by Onuf’s nucleus in the sacral spinal cord [28]. In both humans and dogs, Onuf’s nucleus contains significantly more neurons in males than in females; in dogs, this difference is eliminated by prenatal androgen treatment of females [15]. The total number of pudendal nerve motoneurons is reported to be greater in males than in females in macaque monkeys [34] but not in cats [33]. None of these studies found a sex difference in the size of Onuf’s nucleus neurons.

In short, sexual dimorphism in perineal motoneuron number, while most extensively studied in the rat, has been demonstrated in a variety of species, including humans. Sexual dimorphism in perineal motoneuron size, however, has so far been found only in rodents. Since it is primarily in the rat that the mechanisms underlying sexual differentiation are studied, it is reasonable to ask whether the same factors that produce dimorphism postnatally in the rat act prenatally to produce comparable sex differences in other species. Among rodents, the guinea pig differs from rats, and resembles humans, by its almost exclusively prenatal sexual differentiation; for this reason the guinea pig has been considered an important animal model for the study of hormonal effects of the developing human brain [7,18]. If guinea pigs display sexual dimorphism in motoneurons innervating the perineal striated muscles, they could be a suitable animal model for investigating the applicability of the SNB model to species that undergo prenatal sexual differentiation.

The goals of the studies presented here were (1) to localize the motoneurons innervating these various muscles in male and female guinea pigs, and (2) to examine the identified spinal motoneurons to determine whether they are sexually dimorphic in size or number.

2. Materials and methods

2.1. Muscle terminology

Post-mortem dissections showed that male guinea pigs have perineal muscles that closely resemble those of rats (Fig. 1a, c): the BC wraps around the base of the penis and

![Figure 1](image-url)
and extends laterally to the pelvic girdle (Fig. 1c). In this study, we refer to this latter muscle, which has no apparent homologue in adult female guinea pigs, as the IC. For convenience, we will refer to the midline-oriented muscle that Cooper and Schiller [11] call the IC as the retractor penis (RP) in males and the retractor clitoris (RC) in females (Fig. 1a, b), simply because the attachments suggest a retracting action. However, without embryological studies, we cannot be certain of the identity of these muscles, and we are aware of no muscles with such attachments in rats.

2.2. Retrograde labeling of motoneurons

Adult albino guinea pigs were obtained from Charles River or Simonsen laboratories and housed in same-sex pairs in polyurethane cages with wood shavings for bedding. Animals were maintained on a 14 L:10 D photoperiod and had continuous access to water and Purina guinea pig chow. Fresh vegetables (bell pepper or lettuce) were provided twice per week.

The retrograde tracer horseradish peroxidase (HRP) was used to localize the motoneurons innervating the muscles of interest. Animals were deeply anesthetized with ketamine cocktail (21 mg ketamine, 2.4 mg Rompun, 0.3 mg acepromazine/ml, 0.07 ml/100 g body weight, s.c.) and the muscles exposed by incisions and blunt dissection. Incisions were made in either the abdominal wall or the perineal sac (see Fig. 1). Twenty males received bilateral injections (2.0 to 5.0 μl per side) of 30% HRP (Sigma type VI in water) in either the BC/LA (9), the IC (6) or the RP (5); seventeen females were injected in the LA (7) or RC (10). Three males received asymmetrical injections in which the IC was injected on one side and the BC on the other. Since the IC is small and immediately adjacent to the BC, cotton was packed between the two muscles to minimize spread of HRP into the BC whenever the IC was injected. Care was taken during BC injections to choose a medial site not immediately adjacent to the IC.

After 2–3 days, the animals were killed by overdose of sodium pentobarbital (0.6 mg/100 g body weight), then perfused through the heart with physiological saline followed by a 1% paraformaldehyde–glutaraldehyde solution. Spinal cords were removed, postfixed 1–3 h in 1% paraformaldehyde–glutaraldehyde, stored overnight in buffered sucrose at 4°C, then frozen-sectioned in the frontal plane at 50 μm. Alternate sections were reacted with tetramethyl benzidine to visualize HRP [26] and counterstained with neutral red.

2.3. Measurements of size and number of motoneurons in Nissl stain

BC, IC and LA motoneurons

Animals, obtained and housed as before, were killed by overdose of sodium pentobarbital and perfused intracar-
columns. For counting, the caudal-most section in which the medial and lateral motoneuronal columns were readily distinguishable was selected as an anchor point. All medial and lateral column motoneurons with a visible cell nucleus were counted in the anchor section and in 19 subsequent alternate rostral sections. Any cells seen in the dorsomedial SNB-like position were counted as part of the medial column, since such cells were labeled only by BC or LA injections of HRP. Counts were also made of the single cluster of motoneurons for 10 sections caudal to the anchor point; these motoneurons were termed the “merged” neurons. Counting by these criteria included most HRP-filled motoneurons. Raw counts were corrected for split nuclei by the methods of Abercrombie and Konigsmark because spherical motoneuronal nuclei fit the assumptions of both methods [22]. Areas of somata and nuclei were measured from camera lucida drawings of twelve randomly selected motoneurons per cluster per animal with a digitizing pad and microcomputer. All counts, tracings and measurements were done without knowledge of the animal’s sex. Measures of soma area, nuclear area and motoneuron number were evaluated by 2-way ANOVA (sex by spinal nucleus), with \( n = 10 \) males and 10 females.

**RP / RC motoneurons**

Since these cells did not form a recognizable nucleus, it was not possible to count or measure them reliably in Nissl-stained tissue. Therefore, 6 males and 6 females were given a saturating bilateral injection (10–15 \( \mu l \)) of HRP in the RP or RC. Animals were killed and processed as before, but frozen-sectioned in the longitudinal plane. All sections were stained with neutral red and all labeled cells were counted. Somata and nuclei area were measured from 12 HRP filled cells per animal as before. Raw counts were corrected for split nuclei area by both the Abercrombie and Konigsmark methods.

RP and RC muscles were removed from animals in both experiments and stored in formalin. Muscles were trimmed, dried and weighed from 6 randomly selected animals of each sex. Muscle weights, soma area, nuclear area and RP/RC motoneuron number were compared by Student’s \( t \)-test.

3. Results

3.1. Location of motoneuron pools

In males injected in either the BC, IC or LA, HRP-labeled motoneurons were seen in the L6 and S1 regions, at approximately the caudal border of the lumbar enlargement. Most S1 cells formed a single cluster in the base of the ventral horn, though some cells occupied a more dorsomedial position reminiscent of the SNB location in rats and a few motoneurons were seen midway between these ventral and dorsomedial positions (Fig. 2a). More

![Fig. 3. Photomicrograph showing HRP-filled MNs (small arrowheads) in the L6 spinal segment of a male injected in the left IC and the right BC. Notch in dorsal horn indicates the side ipsilateral to the IC injection. Note that the MNs filled by BC injections occupy the more medial of two MN columns (hollow arrows), while IC MNs occupy the more lateral (filled arrows).](image-url)
Table 1
Comparison of mean soma and nuclear area (\(\mu m^2\)) ± SEM of motoneurons innervating perineal muscles

<table>
<thead>
<tr>
<th></th>
<th>merged (a)</th>
<th>medial MNs (b)</th>
<th>lateral MNs (a)</th>
<th>RP/RC MNs (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>male soma</td>
<td>942 ± 44</td>
<td>982 ± 47</td>
<td>969 ± 44</td>
<td>1292 ± 181</td>
</tr>
<tr>
<td>female soma</td>
<td>786 ± 44 (^*)</td>
<td>799 ± 24 (^*)</td>
<td>813 ± 35 (^*)</td>
<td>908 ± 127 (^*)</td>
</tr>
<tr>
<td>male nuclei</td>
<td>223 ± 18</td>
<td>254 ± 24</td>
<td>263 ± 32</td>
<td>163 ± 13</td>
</tr>
<tr>
<td>female nuclei</td>
<td>141 ± 6.1 (^*)</td>
<td>142 ± 6.6 (^*)</td>
<td>160 ± 7.6 (^*)</td>
<td>148 ± 14</td>
</tr>
</tbody>
</table>

\(^*\) Significantly different from males, \(P < 0.05\).

3.2 Motoneuron number and size

**BC / IC / LA**

Analysis by two-way ANOVA (sex by nucleus) showed a main effect of sex on soma and nuclear area (\(P < 0.05\)). Males had larger soma and nuclei in all three clusters (the lateral and medial rostral divisions, and the caudal "merged", Table 1). While the raw motoneuronal counts showed a significant main effect of sex (\(P < 0.05\)), both the Abercrombie and Konigsmark correction procedures eliminated the difference (\(P > 0.05\), Table 2).

**RP / RC**

The number of HRP-labeled RP/RC motoneurons did not differ between the sexes either before or after correction for split nuclei error (Student’s \(t\)-test, \(P > 0.05\), Table 2). As with the BC, IC and LA motoneurons, there was a significant sex difference in soma size favoring males (\(P < 0.05\), Table 1); however, nuclear size did not differ significantly between the sexes (\(P > 0.05\)). There was also a prominent dimorphism in the mass of these muscles; RP’s had a mean paired weight of 151 ± 17 mg compared to 4.9 ± 0.9 mg for the RC.

4. Discussion

Sex differences in raw counts of these guinea pig perineal motoneurons are apparently artifacts of the larger cell size in the male. Since the nuclei of motoneurons are larger in males, they are more likely to be split by sectioning and therefore counted twice. Abercrombie’s correction technique adjusts the estimate of total cell number by assuming spherical nuclei and gauging the probability of bisecting profiles from large versus small spheres in sections of a given thickness; Konigsmark uses the same...
we counted only cells labeled by HRP and found no sex obvious sex difference in motoneuron number in the area comparisons. The only motor group that had no sex difference in nucleus size also had no sex difference in raw counts of motoneuron number. This finding suggests that the differences in nucleus size gave rise to the differences in raw number, an idea that has been supported by the Abercrombie and Konigsmark corrections. Although alternate methods of neural counting have been proposed [10], the Abercrombie and Konigsmark methods are appropriate for this study since the spinal motoneuron nuclei are very nearly spherical and section thickness was the same for all comparisons.

Guinea pigs clearly differ from rats in their lack of an obvious sex difference in motoneuron number in the area of the spinal cord innervating the sexually dimorphic striated perineal muscles. To our knowledge, the guinea pig is the only rodent so far examined in which such a difference in cell number has not been found. It is entirely possible that the nuclei investigated also innervate other, sexually monomorphic muscles, just as the SNB in rats innervates the non-sexually dimorphic external anal sphincter [25]. Indeed, this is almost certainly the case in the lateral motoneuron nucleus of guinea pigs, where the female has as many motoneurons as the male despite the lack of an IC. Some of those neurons presumably innervate other muscles in the female, and probably in the male as well. If the number of motoneurons innervating monomorphic muscles does not differ between the sexes, but are included in the analysis, as was the case in the thionin-stained material in this study, differences in the number of motoneurons innervating sexually dimorphic muscles could be masked. However, this possibility fails to explain the lack of sex difference in the RP/RC motoneurons, where we counted only cells labeled by HRP and found no sex difference in number despite a female mean muscle mass that was only 3% of its male counterpart. We felt that such saturating doses of HRP could only be reliably delivered in relatively small muscles of simple trajectory (such as the RP/RC) and not in large muscle of complicated trajectory such as the BC, LA or IC. Thus, sex differences in the number of perineal motoneurons in guinea pigs appear subtle or nonexistent. Since guinea pig prenatal sexual differentiation does not lead to the same type of perineal motoneuron sexual dimorphism seen in other prenatally differentiating species, a guinea pig model is unlikely to shed much light on questions concerning the mechanisms underlying such dimorphisms in Onuf’s nucleus.

However, guinea pigs do show a marked sex difference in perineal motoneuron size, an effect that has not been reported in dogs, humans [15], cats [33], or monkeys [34]. Conclusions in the last two species may be considered tentative, since Ueyama et al. [33,34] examined only one animal of each sex. In guinea pigs, measuring soma size in a sample of twelve randomly selected motoneurons per animal revealed a sex difference favoring males in all four motoneuron pools. This indicates a robust sex difference, which could be a result of hormonal action, the males’ larger muscle size, the males’ larger overall body size or some combination of the three.

However, the soma size difference in guinea pig perineal motoneurons raises interesting questions regarding the relationship between neuron size, target size and overall body size. In a number of sexually dimorphic regions of the central nervous system, sex differences in soma size have been shown to depend on different mechanisms than do differences in cell number. In the rat, different critical periods exist for the masculinization of the SNB cell number and size, and cell size, but not number, can be influenced by testosterone levels throughout life [4,6,23]. Soma size in another rat motoneuron group, the retrodorsolateral nucleus (RDLN), increases slightly with adult androgen treatment, although the size of one of its target muscles, the flexor digitorum brevis (FDB), is not altered by adult androgen [24]. While we have not investigated the cause of the sex difference in motoneuron size in the guinea pig, the rat models suggest that androgen or one of its metabolites acts both during development and in adulthood to increase motoneuron size in males. At present it is not clear whether androgenic size increases in SNB motoneurons are caused by direct action on the neurons, indirect action on their target muscles, or elsewhere [1,30].

There is an additional possibility: larger motoneurons in males may not be the result of innervating specific sexually dimorphic muscles but part of an overall trend that makes the entire male guinea pig nervous system larger than its female counterpart. The medial preoptic nucleus (MPN) of guinea pigs contains an anteriorly placed compact subnucleus (MPNa) that has a greater number of neurons and twice the volume in females as in males [7]. However, individual neurons in that region are larger in males, just as they are in the rest of the MPN [8]. The difference in neuron number is thought to be the result of a hormonally controlled sex difference in neurogenesis and neural migration, while the size difference is speculated to be related to the larger size of the male brain [8]. Such a difference, while clearly a type of sexual dimorphism, would be widespread rather than restricted to areas of the nervous system directly involved in sexual behavior.

If such a trend really exists in the guinea pig, it is possible that the size difference in motoneurons has nothing to do with the fact that they innervate the sexually dimorphic pelvic muscles, but is the inevitable result of residing in a male guinea pig’s larger body and central
nervous system. In such a case, some factor(s) in the male guinea pig, either systemically available or derived from the generally larger neuronal targets, would act in the central nervous system to increase soma size [29].

Whether the size difference in perinatal motoneurons is caused by the innervation of dimorphic muscle or the generally larger size of male guinea pigs remains to be seen. Comparisons of male and female motoneuronal size in general neuromuscular systems could be useful tests of the “overall size difference” hypothesis of Byne et al. [8]. Examining the effects of androgen manipulations in both development and adulthood on the size of motoneurons could provide clues as to how such dimorphisms arise.

References


