

# Breeding Status Affects Motoneuron Number and Muscle Size in Naked Mole-Rats: Recruitment of Perineal Motoneurons?

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**ABSTRACT:** Naked mole-rats live in large colonies and exhibit a strict reproductive hierarchy. Each colony has one breeding female and one to three breeding males; all other individuals are nonreproductive subordinates. Subordinates show a remarkable lack of sex differences in behavior and anatomy, but can become reproductive if removed from the colony. We recently reported that the striated perineal muscles and their innervating motoneurons, which are sexually dimorphic in all other mammals examined to date, are not dimorphic in subordinate naked mole-rats. Here we asked whether sexual differentiation of this neuromuscular system occurs when a subordinate becomes a breeder. The size and number of cells within Onuf's nucleus (homologue of the rat spinal nucleus of the bulbocavernosus) as well as perineal muscle volume were examined in subordinate and breeding naked mole-rats of both

sexes. Sex differences in perineal motoneurons were not observed, regardless of social status. To our surprise, however, counts of motoneurons in Onuf's nucleus were increased ~30% in breeders of both sexes. This was accompanied by a reciprocal decrease in cells in Onuf's nucleus that were characterized by small soma size, and lacked a clear nucleus or nucleolus. Although not exhibiting typical motoneuron morphology, some of these small cells were positive for the motoneuron marker, SMI-32. The neuronal changes correlate with increased perineal muscle volumes in breeders. We propose that small, relatively undifferentiated cells are recruited to the pool of large Onuf's nucleus motoneurons when subordinate naked mole-rats become breeders. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 66: 1354–1364, 2006

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## INTRODUCTION

Virtually all previous work on sexual differentiation of the nervous system has focused on species in

which direct reproduction is the only means of passing on one's genes. In cooperatively breeding species, some individuals forgo or delay reproduction to assist in the rearing of genetically-related offspring (Solomon and French, 1997). Consequently, one might expect differences in the cellular mechanisms, pattern, or timing of sexual development among species with these different reproductive strategies.

Naked mole-rats (*Heterocephalus glaber*) exhibit the most extreme form of cooperative breeding in a mammal. Each colony is comprised of a single breed-

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ing female (the queen), one to three breeding males, and a large number of nonreproductive “subordinates,” who assist in foraging, colony defense, maintenance of the tunnel system, and caring for the young (Jarvis, 1981; Brett, 1991; Lacey et al., 1991; Lacey and Sherman, 1991). Subordinates can become reproductive if a breeder dies, or if they are removed from the colony and paired with an opposite-sex mate (Faulkes et al., 1990; Margulis et al., 1995). Once a breeding animal is established, however, it is rarely overthrown and, as a result, the large majority of individual naked mole-rats never achieve reproductive status (Brett, 1991).

Subordinate naked mole-rats exhibit no sex differences in behavior or body size, and the external genitalia are remarkably monomorphic (Jarvis, 1981; Lacey et al., 1991; Peroulakis et al., 2002). Sexual differentiation of the nervous system may also be suppressed. The perineal motoneurons and their target muscles are sexually dimorphic in every other mammal examined to date. In mice and rats, motoneurons in the spinal nucleus of the bulbocavernosus (SNB) and the dorsolateral nucleus of the lumbar spinal cord innervate striated perineal muscles that attach to the penis (Sachs, 1982; Hart and Melese-D’Hospital, 1983). These muscles are innervated by a single cell group known as Onuf’s nucleus in other species, including humans (Onuf, 1899; Sato et al., 1978; Thüroff et al., 1982; Forger et al., 1996). Males have more perineal motoneurons than do females, and the target muscles are absent or reduced in size in females of many species (Breedlove and Arnold, 1980; Forger and Breedlove, 1986; Wagner and Clemens, 1989; Ulibarri et al., 1995; Forger et al., 1996). These sex differences in mice and rats are due to death of the muscles and motoneurons in females during perinatal life (Cihak et al., 1970; Nordeen et al., 1985; Jacob et al., 2005).

We previously determined that the perineal muscles are innervated by a single motor pool (Onuf’s nucleus) in naked mole-rats, and found no sexual dimorphism in any feature of this neuromuscular system (Peroulakis et al., 2002). However, only subordinates were available for the previous study. This prompted the speculation that some features of sexual differentiation in naked mole-rats might occur only if an individual becomes a breeder. To test this prediction, we examined perineal muscles and motoneurons in animals assigned to breeding-pair or subordinate conditions. A novel and unpredicted result of this analysis was the finding of an increased number of large Onuf’s nucleus motoneurons in breeders of both sexes.

## MATERIALS AND METHODS

### Animals

Naked mole-rat colonies were maintained at the University of Connecticut, Storrs. All animals were descended from individuals captured in Kenya, as described previously (Peroulakis et al., 2002). Colonies were maintained in polypropylene tubs (with Plexiglas™ lids) containing corncob bedding and connected by lengths of acrylic tubing. Animals were fed ad libitum on a diet consisting of sweet potato supplemented with apples, carrots, squash, and oatmeal. Animal rooms were maintained on a 12:12 light/dark photoperiod, and room temperature maintained at 28–30°C. Naked mole-rats are very long-lived and can survive for over 20 years in captivity (Jarvis and Bennett, 1991). Subjects in this study were between 3 and 17 years of age and weighed between 31.7 and 66.2 g. Breeders were created by removing subordinates from their colonies and pairing them with opposite-sex mates; all breeders had been paired 4 to 8 years prior to sacrifice and had produced at least one litter. There were no significant differences in age or body weight between breeders and subordinates.

### Motoneuron Number and Size

For analysis of muscle and motoneuron morphology, groups consisted of six subordinate males, four subordinate females, four breeding males, and three breeding females. Animals were deeply anesthetized and decapitated; the vertebral columns and perineums were dissected out and immersion-fixed in formalin. Tissues were then transferred to Bouin’s solution and embedded in paraffin. Lumbosacral spinal cords were coronally sectioned at 10  $\mu$ m, mounted on gelatin subbed slides, and stained with Klüver-Barrera.

Darkly stained cells that were large, multipolar, with an unstained nucleus and clearly visible nucleolus were considered motoneurons and were counted throughout the rostral-caudal extent of Onuf’s nucleus, as previously (Peroulakis et al., 2002). For comparison, we also examined motoneurons in the retrodorsolateral position at the same spinal level. Although the target muscles of these cells have not been determined in naked mole-rats, we refer to this cell group as the retrodorsolateral nucleus (RDLN). In mice and rats, motoneurons in this region innervate muscles of the hindfoot (Nicolopoulos-Stourmaras and Iles, 1983; Forger et al., 1997). RDLN motoneurons are not sexually dimorphic in size or number, and are not affected by perinatal hormone treatments in rats (Jordan et al., 1982; Tobin and Payne, 1991). Motoneuronal nucleoli in both cell groups were counted unilaterally in alternate sections.

Cell size was determined from camera lucida tracings of at least 25 motoneurons in Onuf’s nucleus and the RDLN of each animal. Cells were traced in sections that were evenly spaced along the rostral-caudal extent of each nucleus and all cells within a chosen section were traced to avoid experimenter bias. On average, cells were traced from seven sections (range: 5–9 sections) in each animal. Tracings were made on a Cross Pad and imported into SigmaScan for analysis of mean cross-sectional areas of the

somas, nuclei, and nucleoli. Subsequently, we also assessed the number of “small cells” in Onuf’s nucleus (see later). These cells are much smaller than the motoneurons included in the counts described earlier, and do not have a visible nucleolus (Fig. 2). Somas were traced for all small cells; nuclei were traced for these cells only when the nuclear membrane could be clearly discerned by focusing through the section. Raw cell counts were corrected for sampling ratio, and for split nucleoli (motoneurons) or somas (small cells) according to the method of Königsmark (1970).

Motoneuron counts have been validated when performed as described above (Clarke and Oppenheim, 1995). Given the finding of a novel cell type (small cells) within Onuf’s nucleus, however, we sought to verify our findings by performing stereological cell counts. Motoneurons and small cells in Onuf’s nucleus were therefore counted in a subset of the animals (four subordinates and four breeders) using an adaptation of the optical disector method and StereoInvestigator software (MicroBrightfield, Williston VT). All cells within Onuf’s nucleus were counted unilaterally in alternate sections (sampling is not necessary because of the small number of cells involved). A guard zone of 1  $\mu\text{m}$  was used at the top and bottom of each section, and the number of cells of each type that came into focus within the section was recorded.

## Morphology of the Perineal Muscles

Perineums were sectioned at 10  $\mu\text{m}$  and mounted on gelatin-subbed slides. Sections were stained with Gomori’s trichrome to enhance the detection of muscle striations. We previously identified the levator ani (LA), ischiocavernosus (IC), and urethral muscle (UM) as targets of Onuf’s nucleus motoneurons in naked mole-rats (Peroulakis et al., 2002). The UM may be homologous to the bulbocavernosus muscle of other mammals, which wraps around the bulb of the penis. The term bulbocavernosus is avoided, however, because naked mole-rats do not have a penile bulb (Peroulakis et al., 2002). The LA, IC, and UM muscles were traced in sections spaced 400  $\mu\text{m}$  apart through the perineum. Volume was determined by summing cross-sectional areas and multiplying by the distance between traced sections.

## Immunocytochemistry for the Motoneuron Marker SMI-32

SMI-32 is a mouse monoclonal antibody (Sternberger Monoclonals Incorporated, Lutherville, MD) that binds to nonphosphorylated epitopes in neurofilament H and specifically labels motoneurons within the spinal cord of mice (Bar-Peled et al., 1999). To determine the percentage of large motoneurons and small cells in Onuf’s nucleus that are positive for SMI-32, spinal cord sections from three male and three female subordinates were stained with Kliver-Barrera and all cells within Onuf’s nucleus were traced by camera lucida. On the basis of the tracings, cells were designated as large motoneurons or small cells. Sections were then destained and immunocytochemistry for SMI-32 was performed as described previously (Jacob et al., 2005). Briefly, sections were incubated over-

night in SMI-32 (1:100), followed by goat anti-mouse secondary antibody (1:150) and ClonoPAP (1:200; a peroxidase-mouse antiperoxidase complex, Sternberger Monoclonals Incorporated). Cells were evaluated “by eye” and considered positive if they were moderately or darkly stained. Sections stained for SMI-32 were then compared to tracings to determine the identity of SMI-32 labeled cells (i.e., large motoneurons or small cells). SMI-32 staining in the spinal cord of naked mole-rats is confined to the location of motor pools and the distribution of label appears very similar to that previously described in mice (Fig. 3). All staining was abolished when the primary antibody was omitted.

## Data Analysis

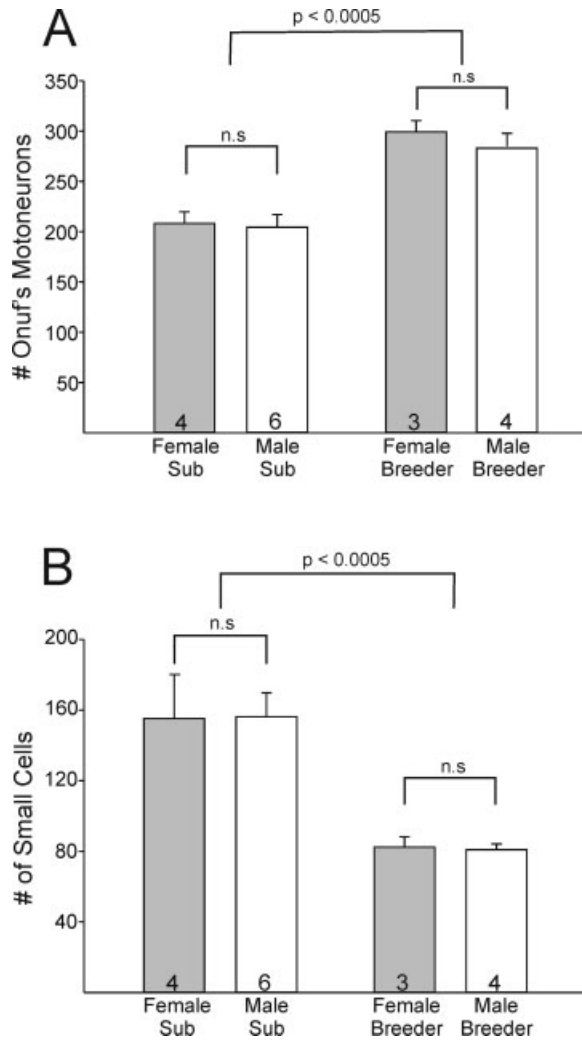
Cell numbers, cell sizes, and perineal muscle volumes were analyzed by two-way ANOVAs (sex-by-status), followed by planned comparisons using Fisher’s Least Significant Difference. Cell numbers were assessed using both raw cell counts and Königsmark corrected counts. The pattern of main effects, interactions, and all planned comparisons were identical, and only the corrected counts are reported below. Means were reported  $\pm$  SEMs and  $p < 0.05$  was considered statistically significant.

## RESULTS

### Motoneuron Number in Onuf’s Nucleus Is Increased in Breeders

Counts of large, multipolar cells confirmed the absence of a sex difference in the number of Onuf’s nucleus motoneurons in subordinate naked mole-rats [Fig. 1(A)]. We found, however, a marked increase in motoneuron number in breeders, which was significant for both males ( $p = 0.001$ ) and females [ $p < 0.0005$ ; Fig. 1(A)]. By ANOVA, these results were reflected in a significant main effect of breeding status ( $p < 0.0005$ ), no main effect of sex ( $p > 0.4$ ), and no sex-by-status interaction ( $p > 0.6$ ). We also observed a marginally significant main effect of status on Onuf’s nucleus motoneuron soma size ( $p = 0.050$ ), with larger somas in breeders (Table 1). There were no effects of sex or status on the sizes of motoneuron nuclei (Table 1) or nucleoli (not shown).

To determine whether the effect of breeding status on motoneuron number was specific to Onuf’s nucleus, we examined the RDLN, a motor pool just dorsal and lateral to Onuf’s nucleus in the lumbosacral spinal cord. There was a tendency for more RDLN motoneurons in females, but this was not significant ( $p < 0.06$ ). Importantly, however, there was no effect of status ( $p > 0.3$ ), and no sex-by-status interaction ( $p > 0.4$ ) on motoneuron number in the RDLN. There also was no effect of sex or status, and no sex-by-status interaction on RDLN cell size (all  $p$ ’s  $> 0.4$ ).



**Figure 1** (A) Mean ( $\pm$  SEM) number of motoneurons in Onuf's nucleus of subordinates and breeders. There was a marked increase in motoneuron number in breeders, which was significant for both males ( $p = 0.001$ ) and females ( $p < 0.0005$ ). (B) Mean number of small cells within Onuf's nucleus. There was a significant main effect of breeding status ( $p < 0.0005$ ), with fewer small cells in breeders, no main effect of sex, and no sex-by-breeding status interaction. n.s., not significant. The number of animals in each group is indicated at the base of each bar.

Thus, reproductive status does not have a generalized effect on motor pools in the lumbosacral cord.

### Number of Small Cells Within Onuf's Nucleus Is Decreased in Breeders

The increase in motoneuron number in Onuf's nucleus of breeders was surprising. Motoneuron genesis is normally complete during embryonic development (Altman and Bayer, 1984) and, as far as we are aware,

the birth of new motoneurons has not previously been established for any adult mammal. We therefore turned our attention to a population of cells within Onuf's nucleus previously assumed to be interneurons. These cells do not fulfill the normal criteria for inclusion in motoneuron counts as they are oval or spindle-shaped, one-third the size of motoneurons, and generally have no clear nucleus or nucleolus (Fig. 2). However, a nuclear membrane could be discerned in many cases by focusing up and down at high power, suggesting that these are whole cells, and not fragments of motoneurons from adjacent sections.

Counts of "small cells" in Onuf's nucleus revealed a highly significant main effect of breeding status ( $p < 0.0005$ ) with fewer of these cells in breeders [Fig. 1(B)]. This decrease was significant for both males and females ( $p < 0.012$  in each case). Moreover, the decrease in small cell number could numerically account for the observed increase in the number of large Onuf's nucleus motoneurons in breeders [compare Fig. 1(A,B)]: when both populations of cells are combined, there are no significant effects of sex ( $p > 0.8$ ), breeding status ( $p > 0.3$ ), or sex-by-status interaction ( $p > 0.9$ ) on total cell number in Onuf's nucleus. As was the case for large motoneurons, mean soma size of small cells was marginally larger in breeders than in subordinates ( $p = 0.053$ ), whereas nucleus size did not vary by sex or breeding status (Table 1).

The changes in cell number with breeding status were confirmed using the optical disector method. In counts of all cells within Onuf's nucleus of four subordinates and four breeders (all males), breeders had significantly more large motoneurons ( $p < 0.005$ ) and significantly fewer small cells ( $p < 0.01$ ) in Onuf's nucleus than did subordinates (data not shown). We also asked whether there was a similar population of small cells in the RDLN. We did find some thionin-stained profiles within the RDLN of the approximate size of the small cells in Onuf's nucleus. Some of these appeared to be soma fragments of large motoneurons from adjacent sections, but in other cases a nucleus could be discerned. However, there was no effect of sex or status ( $p$ 's  $> 0.8$ ), and no sex-by-status interaction ( $p > 0.5$ ) on the number of small cells in the RDLN (data not shown). There also was no effect of sex ( $p > 0.3$ ) or status ( $p > 0.5$ ), and no sex-by-status interaction ( $p > 0.8$ ) on the size of small cells in the RDLN.

### SMI-32 Labels Some Small Cells in Onuf's Nucleus

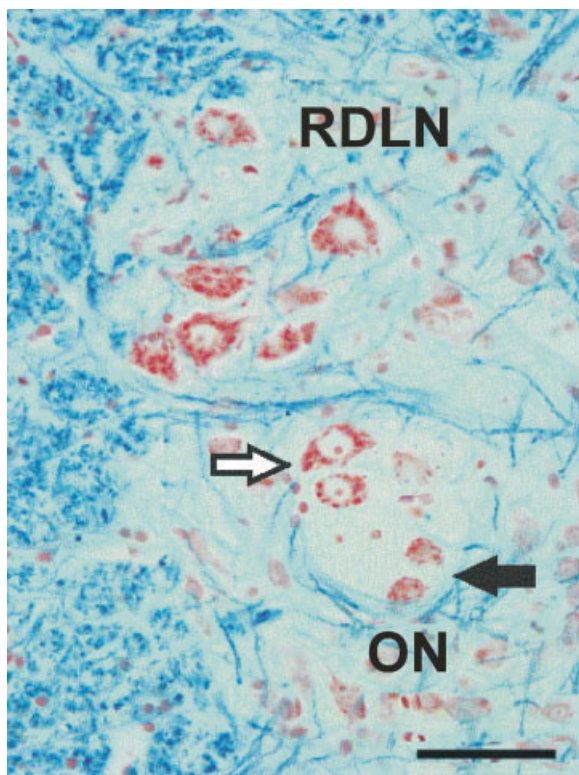
We next considered two possibilities: (1) the small cells in Onuf's nucleus of subordinate naked mole-

**Table 1** Mean ( $\pm$ SEM) Cross-Sectional Areas of Somas and Nuclei of Large Motoneurons and Small Cells in Onuf's Nucleus of Subordinate (Sub) and Breeding (Breed) Naked Mole-Rats

	Motoneurons		Small Cells	
	Soma ( $\mu\text{m}^2$ )	Nucleus ( $\mu\text{m}^2$ )	Soma ( $\mu\text{m}^2$ )	Nucleus ( $\mu\text{m}^2$ )
Sub females ( $n = 4$ )	388 $\pm$ 39	117 $\pm$ 5	124 $\pm$ 8	50 $\pm$ 4
Sub males ( $n = 6$ )	342 $\pm$ 19	98 $\pm$ 10	126 $\pm$ 8	61 $\pm$ 9
Breed females ( $n = 3$ )	411 $\pm$ 38	109 $\pm$ 15	138 $\pm$ 6	48 $\pm$ 7
Breed males ( $n = 4$ )	437 $\pm$ 3	127 $\pm$ 2	148 $\pm$ 9	54 $\pm$ 3
ANOVA				
Sex	n.s.	n.s.	n.s.	n.s.
Status	$p = 0.050$	n.s.	$p = 0.053$	n.s.
Interaction	n.s.	n.s.	n.s.	n.s.

n.s., not significant.

rats are atrophic motoneurons, some of which are triggered to grow in breeders, or (2) the small cells are not motoneurons, but may be induced to differentiate to a motoneuronal phenotype by the switch in



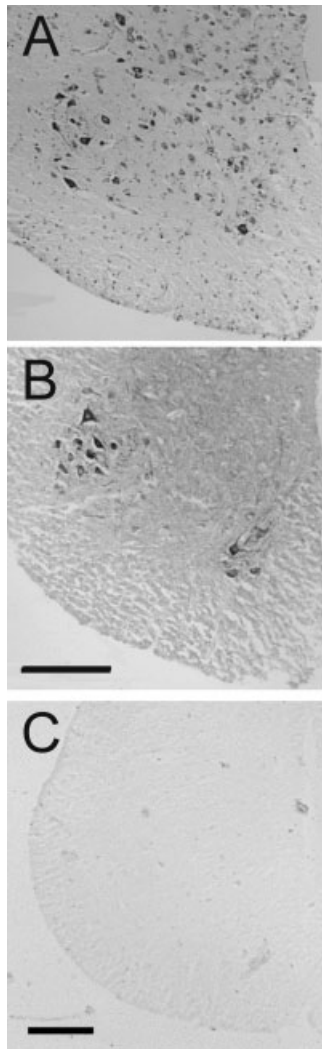
**Figure 2** Photomicrograph of Onuf's nucleus (ON) in a subordinate naked mole-rat. Both typical motoneurons (open arrow) and small cells (closed arrow) can be seen. The small cells are on average one-third the size of motoneurons and in most cases do not have a clear nucleus or nucleolus, although a nucleus can often be discerned by focusing through the cell. The retrodorsolateral nucleus (RDLN) can be seen just dorsolateral to Onuf's nucleus. Scale bar = 50  $\mu\text{m}$ .

breeding status. As a first test of these possibilities, we examined spinal cord sections of subordinate naked mole-rats that were sequentially Nissl stained, destained, and immunolabeled using the monoclonal antibody, SMI-32. As in other rodents, we find that SMI-32 selectively labels motoneurons in the spinal cord naked mole-rats (Fig. 3). Approximately 65% of the cells in Onuf's nucleus classified as small cells based on Nissl staining were moderately or darkly labeled by SMI-32 (Fig. 4). Although this is lower than the proportion of large motoneurons that were rated as positive under the conditions of our assay (82%;  $\chi^2 = 16.89$ ,  $df = 1$ ;  $p < 0.005$ ), a substantial fraction of small cells may be considered motoneurons by this criterion.

### Perineal Muscle Size Is Increased in Breeders

We also examined the volumes of Onuf's nucleus target muscles, the LA, UM, and IC, of breeder and subordinate naked mole-rats. As reported previously (Peroulakis et al., 2002), there was no effect of sex on LA muscle volume in subordinates. However, we find a main effect of breeding status ( $p < 0.05$ ), with larger LA muscles in breeders (Figs. 5 and 6). We also find a sex-by-status interaction ( $p = 0.01$ ), due to the fact that LA volume increased more than two-fold in breeding females ( $p < 0.005$  compared to female subordinates), but did not change in males [Fig. 5(A)]. The UM also was increased in volume in breeding animals [main effect of status,  $p < 0.005$ ; Figs. 5(B) and 6], with no main effect of sex and no sex-by-status interaction. IC volumes did not vary significantly by sex or breeding status, although there was a trend for smaller IC muscles in females ( $p < 0.06$ ; not shown).

Breeding females are often among the largest animals in the colony (Jarvis et al., 1991). Although



**Figure 3** SMI-32 selectively labels motoneurons in the lumbo-sacral spinal cord of the naked mole-rat. (A) A thionin stain reveals all Nissl-positive cells in a transverse section of the lower lumbar spinal cord in a subordinate naked mole-rat. The left ventral quadrant is shown. (B) A neighboring section immunostained with SMI-32. (C) Control section in which the primary antibody was omitted during immunocytochemistry. Scale bars: (a) and (b) = 100  $\mu\text{m}$ , (c) = 200  $\mu\text{m}$ .

there were no significant effects of sex or breeding status on body mass, breeding females did have the greatest mean body mass of the groups in this study. To determine whether increases in the perineal muscles of breeders could be accounted for solely on the basis of body size, we analyzed LA and UM volume data after correcting for body weight at sacrifice ( $\text{mm}^3/\text{g}$ ). The volume of the LA muscle remained significantly larger in breeders after correcting for body mass ( $p < 0.04$ ) and, again, post hoc tests revealed a significant increase for breeding females ( $p < 0.02$ ), but not males

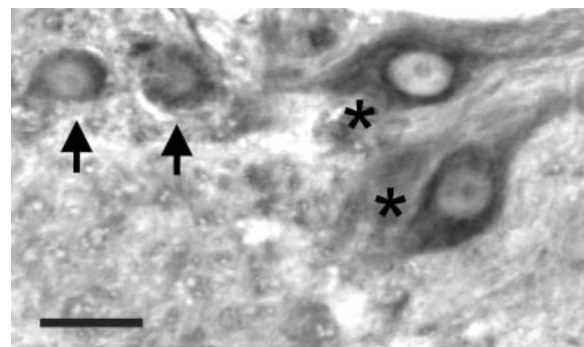
( $p > 0.10$ ; not shown). However, the increase in UM muscle size in breeders was no longer significant after correcting for body mass ( $p = 0.09$ ; not shown).

## DISCUSSION

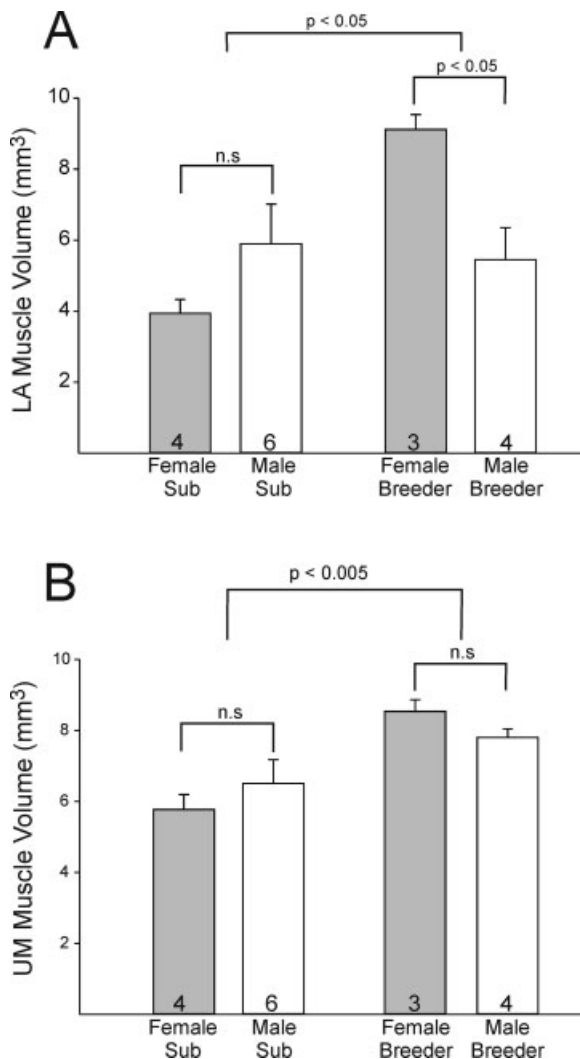
Social signals profoundly influence sexual development in naked mole-rats, with the queen and her consorts suppressing reproduction in all subordinates (Faulkes et al., 1990; Faulkes and Abbott, 1991). Although the mechanism of suppression is not known, increases in gonadal hormone levels and the onset of reproductive behaviors are observed when a subordinate is removed from the colony and housed with an opposite sex mate (Faulkes et al., 1990; Faulkes et al., 1991; Faulkes et al., 1994). Here we demonstrate that the change in social role from subordinate to breeder also affects motoneurons and their target muscles: breeders exhibited an increase in the number of large Onuf's nucleus motoneurons, a decrease in the number of small cells within the nucleus, and increases in the size of the UM and LA muscles.

### Relative Absence of Neural Sex Differences

Although we found considerable plasticity in perineal muscles and motoneurons, there were no sex differences in motoneuron number or motoneuron cell size (soma, nucleus, or nucleolus) in breeding or subordinate naked mole-rats. This confirms our previous observations in subordinates (Peroulakis et al., 2002) and stands in contrast to other mammals examined to date, where the size and/or number of motoneurons innervating perineal muscles are greater in males (Breedlove and Arnold, 1980; Ueyama et al., 1985;



**Figure 4** High magnification view of SMI-32 immunolabeled cells in Onuf's nucleus of a subordinate naked mole-rat. Large motoneurons (asterisks) and small cells (arrows) are seen. Scale bar = 25  $\mu\text{m}$ .



**Figure 5** Perineal muscle volumes in subordinates and breeders. (A) There was a main effect of breeding status on LA volume, with larger muscles in breeders ( $p < 0.05$ ). There was also a sex-by-breeding status interaction ( $p = 0.01$ ), due to the fact that LA size increased in breeding females but not in males. (B) The UM also was increased in volume in breeding animals (main effect of status,  $p < 0.005$ ). There was no effect of sex and no sex-by-status interaction on UM muscle size. n.s., not significant.

Forger and Breedlove, 1986; Wagner and Clemens, 1989; Freeman and Breedlove, 1995; Ulibarri et al., 1995; Forger et al., 1996).

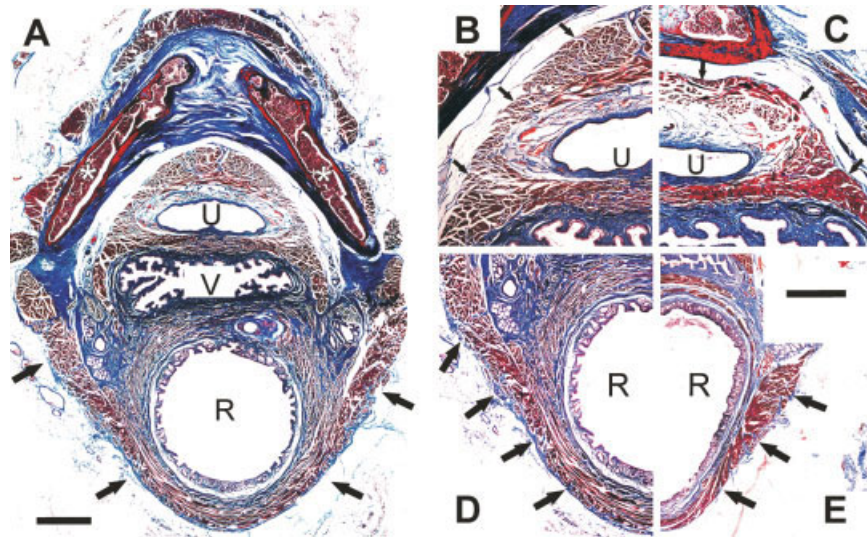
From an adaptive standpoint, a lack of sex differences among subordinate naked mole-rats makes some sense. The large majority of naked mole-rats are expected to remain subordinate throughout their lifetime, and male and female subordinates exhibit identical behaviors within the colony (Lacey and Sherman, 1991). Thus, for most members of the species, sex differences may not be adaptive, and might

even interfere with colonial duties. All breeders start out as subordinates. An absence of sex differences in perineal motoneurons of breeding naked mole-rats may result from an early developmental decision, since sexual differentiation of perineal motoneurons in other species depends primarily on differential exposure to gonadal androgens during early development (Breedlove and Arnold, 1983a; Breedlove and Arnold, 1983b; Forger and Breedlove, 1986; Forger et al., 1996). Although no information is available on the fetal or neonatal endocrinology of naked mole-rats, the results presented here suggest a testable hypothesis: given the paucity of morphological and behavioral differences between the sexes, one might predict either similar exposure to androgens in males and females during a critical period in development, or a relative insensitivity of developing tissues to differences in the hormonal milieu.

### Increased Size of Muscles and Motoneurons

Hormones circulating in adulthood generally do not alter fundamental sex differences in cell number, but androgens do increase the size of perineal motoneurons and muscle fibers in adult mice, rats, gerbils, and hamsters (Wainman and Shipounoff, 1941; Venable, 1966; Breedlove and Arnold, 1981; Forger and Breedlove, 1987; Fraley and Ulibarri, 2002; Hegstrom et al., 2002). Thus, differences in androgen levels between subordinate and breeding naked mole-rats could contribute to our findings. Breeding male naked mole-rats have approximately two-fold higher urinary testosterone levels than do subordinates (Clarke and Faulkes, 1998), and testosterone may also be elevated in breeding females (Faulkes et al., 1990; Clarke and Faulkes, 1997). However, in other rodents, and in contrast to the present findings, perineal motoneurons remain identifiable in low androgen conditions and motoneuron counts based on traditional criteria do not change (Breedlove and Arnold, 1981; Forger et al., 1992; Watson et al., 2001). Additionally, androgen levels in breeding female naked mole-rats are only about one-tenth those reported for breeder males (Clarke and Faulkes, 1997; Clarke and Faulkes, 1998), yet motoneuron and muscle sizes were at least as large in female as in male breeders in the current study. In fact, breeding females had the largest perineal muscles of any group, and the LA of breeder females was actually larger than that of breeder males.

This suggests that the changes we observed may not be solely hormone dependent, but due to breeding experience or social status, per se. Social signals may



**Figure 6** (A) Low power view of a cross section through the perineum of a breeding female. Muscles and bone are red, while connective tissue is blue. Arrows indicate the LA, which loops around the rectum. The UM can be seen surrounding the urethra on all sides. Asterisks indicate public bone. (B, D) Higher power views of the UM and LA (arrows) of the breeding female shown in (A). (C, E) UM and LA muscles of subordinate females (arrows). Scale bar in (A) = 1 mm; scale bar in (E) = 500  $\mu\text{m}$  for (B, C, D, E). Abbreviations: U, urethra; V, vagina; R, rectum.

be as important as gonadal hormones in determining physiological and behavioral changes in species with a strict reproductive hierarchy. For example, gonad-independent changes in neuron size and/or neuronal gene expression have been reported in teleost fishes undergoing sex-role changes in response to social cues (Semsar and Godwin, 2003). In a naked mole-rat colony, mutual genital nuzzling is a behavior displayed almost exclusively by breeders. This behavior occurs at all times of the female's ovulatory cycle and persists during pregnancy, or even after gonadectomy of both members of the breeding pair (Lacey et al., 1991; Goldman et al., 2006). Thus, genital nuzzling reflects social status, rather than circulating hormone levels or current reproductive condition. Interestingly, stimulation of the anogenital region affects development of the SNB neuromuscular system in neonatal male rats (Moore et al., 1992), suggesting the possibility that the genital nuzzling characteristic of naked mole-rat breeders could contribute to the changes in perineal muscles and motoneurons seen here.

The increased size of the perineal muscles in female breeders was unexpected and raises the question of function. The BC and LA control penile reflexes in other species (Hart, 1972; Sachs, 1982; Hart and Melese-D'Hospital, 1983; Karacan et al., 1983), but the role of these muscles in females is not clear. The BC is absent and the LA is vestigial in female rats and mice (Wainman and Shipounoff, 1941; Venable, 1966; Tobin and Joubert, 1991). A

small BC that serves to constrict the vagina is found in female dogs and humans (Miller et al., 1964; Shafik et al., 2002). Naked mole-rats do not have a striated muscle that specifically encircles the vagina. However, the LA loops around the rectum and vagina, attaching at the corpora cavernosa clitoris (Peroulakis et al., 2002), and contraction of this muscle likely constricts the vagina. The LA may therefore assist in extruding pups during delivery, or in maintaining the integrity of the perineal floor. Enlarged perineal muscles may therefore support the enormous reproductive load of naked mole-rat queens, which can give birth to hundreds of pups during their long reign (Jarvis, 1991). Similarly, lengthening of the lumbar vertebrae is a morphological specialization observed in queens that is thought to be related to increased pup-carrying capacity (O'Riain et al., 2000).

### Increased Motoneuron Cell Counts: Late Differentiation of Motoneurons?

The marked increase in the number of large Onuf's nucleus motoneurons in breeders of both sexes was the most surprising finding of this study. Position in a dominance hierarchy influences the survival of newly generated neurons in the dentate gyrus of rats (Kozorovitskiy and Gould, 2004), but a mechanism involving neurogenesis seems unlikely here. The generation of spinal motoneurons, including perineal motoneur-

ons, is normally complete during embryonic development (Breedlove et al., 1983; Altman and Bayer, 1984), and we are not aware of any demonstration of adult motoneurogenesis in a mammal. Neural progenitor cells reside in the gray matter of the spinal cord of adult mice and rats (Namiki and Tator, 1999; Horner et al., 2000; Yamamoto et al., 2001; Azari et al., 2005; Chi et al., 2006). These cells may proliferate and migrate in response to neural injury or disease, but, to date, there is no evidence that newly generated cells differentiate into motoneurons.

We find that the increase in large motoneuron number in Onuf's nucleus of breeding naked mole-rats is associated with a reciprocal, 50% decrease in the number of small cells. At least some of the small cells in subordinate naked mole-rats express the motoneuron marker, SMI-32. Taken together, we favor the explanation that small cells in Onuf's nucleus are relatively undifferentiated motoneurons that are recruited to the pool of large, multipolar motoneurons when an animal changes social status. To our knowledge, no such phenomenon has previously been described for a mammal. Our findings are reminiscent of reports in bullfrogs, where the late differentiation of so-called 'Type-L' cells is thought to augment motoneuron number in the lumbar spinal cord as the animal grows (Farel, 1987; Farel et al., 1993). Type L cells do not meet the normal criteria for motoneurons, but express motoneuron-specific markers (Farel et al., 1993), and are similar in appearance to the small cells in Onuf's nucleus [Fig. 2, present report and Fig. 12 in (Farel, 1987)].

Alternative explanations cannot be ruled out, however, and several questions remain. For example, some small cells remain in breeders, and the identity of these cells is not known. It will be important to probe the cells of Onuf's nucleus in subordinates and breeders with multiple motoneuron markers, as well as markers for other cell types in order to confirm the phenotype of these cells. It will also be important to determine whether small cells project their axons to the perineum and synapse with specific perineal muscles. If so, this would suggest one possible mechanism for the observed changes: growth of the UM and LA muscles, as seen in breeders, may trigger the differentiation of small cells by providing additional synaptic sites and/or increased availability of neurotrophic factors. A similar phenomenon could explain observations in male gerbils, where an increase in SNB motoneuron number is reported through puberty, concomitant with increases in target muscle size (Fraley and Ulibarri, 2001; Siegford and Ulibarri, 2004).

All breeders in the current study had been paired long term. Thus, the time course of the changes in

muscles and motoneurons associated with breeding status is not known, nor do we know whether the changes are reversible. These questions could be addressed by examining perineal muscles and motoneurons shortly after pairing, when the breeding pair reliably exhibits genital nuzzling, but has not yet produced a litter. Moreover, because breeders retain their status for several months after castration (Goldman et al., 2006), it may also be possible to tease apart effects of gonadal hormones from effects due to social status, *per se*.

Although remarkable progress has been made in identifying the molecular mechanisms of motoneuron differentiation during embryonic development (Jacob et al., 2001; Price and Briscoe, 2004), knowledge of later stages in motoneuron development is limited, and based on a small number of model systems. The present findings suggest delayed differentiation of motoneurons innervating the perineal muscles, and raise the question of whether environmental or experiential factors may trigger the late differentiation of motoneurons in other mammalian neuromuscular systems.

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