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Immunoreactivity of Androgen Receptor Protein in Sexually Dimorphic Spinal Motonucleus in Neonatal Male Rats

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The spinal motonucleus of the genitofemoral nerve regulating scrotal temperature can also be related to prenatal and neonatal testicular descent by gubernacular change in rats, and a sexually dimorphic-like bulbocavernosus/dorsolateral motonucleus. There is a hypothesis that neonatal androgen affects these motonuclei, and induces development of sexual organs through neural stimulation. Until now, the accumulation of isotope-labelled androgen and the immunoreactivity of androgen receptor protein in each sexually-dimorphic spinal motonucleus have been revealed in adult rats but they have not been established in rats during neonatal periods. To investigate the presence of the androgen receptor in spinal sexually-dimorphic motonuclei in the neonatal period, we evaluated the androgen receptor immunoreactivity of these motonuclei.

In Sprague-Dawley male rats, the lumbar spinal cords were resected at postnatal days 3, 10 and 30, and stained immunohistochemically using polyclonal antibody of androgen receptor protein. The immunoreactivity of androgen receptor protein was observed in the cells of the genitofemoral motonucleus from the 13th thoracic to the 2nd lumbar spinal cord and the bulbocavernosus/dorsolateral motonucleus was observed from the 4th to 5th lumbar spinal cord in all age groups. The proportional areas of both motonuclei at days 3 and 10 on cross-section were larger than at day 30. The motonuclei at days 3 and 10 were similar in all age groups. With the above results, the presence of androgen receptor protein was confirmed in the genitofemoral and bulbocavernosus/dorsolateral motonucleus from neonate to day 30. The larger proportional area of these motonuclei in neonates may indicate an active role for these motonuclei during the neonatal period. Although the immunoreactivity does not directly imply the presence of a functional receptor, neonatal androgen could be responsible for the development of sexual organs through the spinal motonucleus.

Key Words: Genitofemoral nerve, spinal motonucleus, neonate, androgen receptor

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Cremasteric muscles, an important muscle responsible for testicular temperature control, are innervated by the genitofemoral nerve (GFN). Bulbocavernosus muscle, an essential structure in maintaining erection and urethral sphincteric action, is innervated by the motonucleus of GFN. The spinal nucleus of bulbocavernosus/dorsolateral nucleus (SNB/DLN) is another important spinal motonucleus innervating perineal muscles. Both GFN and SNB/DLN show sexual dimorphism (Gorski, 1966;

Gorski *et al.* 1978; Breedlove and Arnold, 1980; Jacobson *et al.* 1981; Breedlove *et al.* 1982; Breedlove and Arnold 1983a,b; Fishman and Breedlove, 1988; Sengellaub *et al.* 1989) These nuclei can be altered morphologically by changing the androgen milieu during the neonatal period, and such changes continue to exist until adulthood (Breedlove and Arnold, 1983a, 1983b; Han and Choi, 1995). Therefore any abnormality associated with these nuclei is presumed to be closely related to androgen.

The most common congenital abnormality presumed to be affected by the motonuclei is cryptorchidism. Cryptorchidism is a testicular failure to fully descend to the scrotum during the late fetal and infantile periods. Many theories describe the pathophysiology of cryptorchidism, and the most prominent theories are abnormality of the gubernaculum testis and androgen deficiency. However, Beasley and Hutson proposed that GFN, which innervates gubernaculum during the fetal and infantile period, may be responsible for the testicular descent and that the motonucleus of GFN itself is controlled by an unknown factor (Beasley and Hutson, 1988). Previously, we have observed that if GFN in rats is isolated and severed, the actual length of the gubernaculum decreases (Han and Choi, 1991), which seems to be in strong support of the theory of Beasley and Hutson (1988). We have also observed that during the neonatal period androgen changes the morphology of GFN (Han and Choi, 1995), and proposed that androgen may play a key role in testicular descent by developing the GFN motonucleus and the subsequent differentiation of gubernaculum. Androgen receptors were found on the gubernaculum itself (George and Peterson, 1988; Heyns and Pape, 1991; Husmann and Macphaul *et al.* 1991), thus it is not clear whether androgen affects GFN or gubernaculum, or both.

Another nucleus that shows sexual dimorphism is SNB/DLN. It also exhibits morphological changes after the administration of androgen during the neonatal period (Breedlove and Arnold, 1983b), and since it is easier to observe than GFN, such effect that androgen regulate the size and the cell number of the nucleus in rats have been examined more extensively. Also in SNB/DLN, it is not clear whether the exact site of action is the spinal motonucleus or the muscles it innervates (Breedlove and Arnold, 1983c; Breedlove, 1986; Fishman and Breedlove, 1988).

For the motonuclei present in the central nervous system, the time of androgen response is varied (Gorski *et al.* 1978; Breedlove and Arnold, 1980). Therefore, to evaluate the effect of androgen on the spinal motonucleus, various intervals in the fetal and neonatal periods should be included in the search for the androgen receptor. But the spinal cord is a complex bundle of nerves and isolating certain groups of nerves or motonuclei and measuring the density of androgen receptors is almost impossible. Thus, staining androgen receptors on intact spinal cord is the only plausible course. We have attempted to immunohistochemically stain androgen receptors on the motonuclei of GFN and SNB/DLN during different periods of development.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were used. Day 0 was designated as the day of birth. Immunohistochemical staining of androgen receptors were performed on days 3, 10, and 30.

Confirmation of androgen receptors

Under ether anesthesia, the right auricle was opened and 4°C and 0.1 M phosphate buffer saline (PBS) was irrigated through the left ventricle. The fixative, PBS solution containing 4% paraformaldehyde and 0.5% glutaraldehyde, was again irrigated at 4°C. Then, under the operative microscope, laminectomy was performed and the spinal cord from T13 to L5 was removed and put in the same fixating solution overnight. On the next day, sections of 50 μm in thickness were made using a vibratome. Sections from the day 3 and day 10 groups were fixed on the glass slides, and sections from the day 30 group were immersed in PBS for another 2 days.

Slides were washed twice for 10 minutes each before undergoing immunohistochemical staining. To minimize the nonspecific binding of biotinylated goat anti-rabbit antibody, slides were treated in 5% normal goat serum (NGS) for 30 minutes at room

temperature. Each slide was then washed for 5 minutes with PBS. To block the activity of endogenous biotin, slide was washed for 30 minutes and incubated for 5 minutes with avidine containing solution and biotin washing solution. The polyclonal antibody containing 1% NGS was diluted to 1:50 concentration and stored for one day. The biotinylated goat anti-rabbit immunoglobulin G was diluted to 1:500 with PBS and exposed to the slide for 30 minutes at room temperature. Then avidin-biotin-horseradish complex was administered for 30 minutes and washed off with PBS for 30 minutes. For chromogen, 0.02% diaminobenzidine and 10 mM imidazole were used. 0.5 M Tris buffer solution and 0.01% H_2O_2 solution was used as the last step and dehydration was completed.

RESULTS

Genitofemoral motonucleus (Fig. 1)

In all three groups (days 3, 10, 30), the immunoreactivity for androgen receptors was found at the lamina VIII (Molander *et al.* 1984) located on the anterior horn of T13 through the L2 level. From the cross-section, the immunoreactive cells were found in the column of Clarke (Molander *et al.* 1984). The area of androgen receptor activity showed a tendency to decrease as a rat aged. However, the intensity of staining was increased as a rat aged. At day 3 and 10, the immunoreactivity was found only at the cytoplasm, but the activity was observed both at the nucleus and cytoplasm at day 30.

SNB/DLN (Fig. 2)

In all three groups (days 3, 10, 30), the immunoreactivity for the androgen receptors was found at the lamina IX (Molander *et al.* 1984) located on the anterior horn and medial aspect of L2 through the L5 level. From the cross-section, the immunopositive cells were found at the column of Clarke (Molander *et al.* 1984). Cells that stained positively were found at the lamina IX of the anterior horn

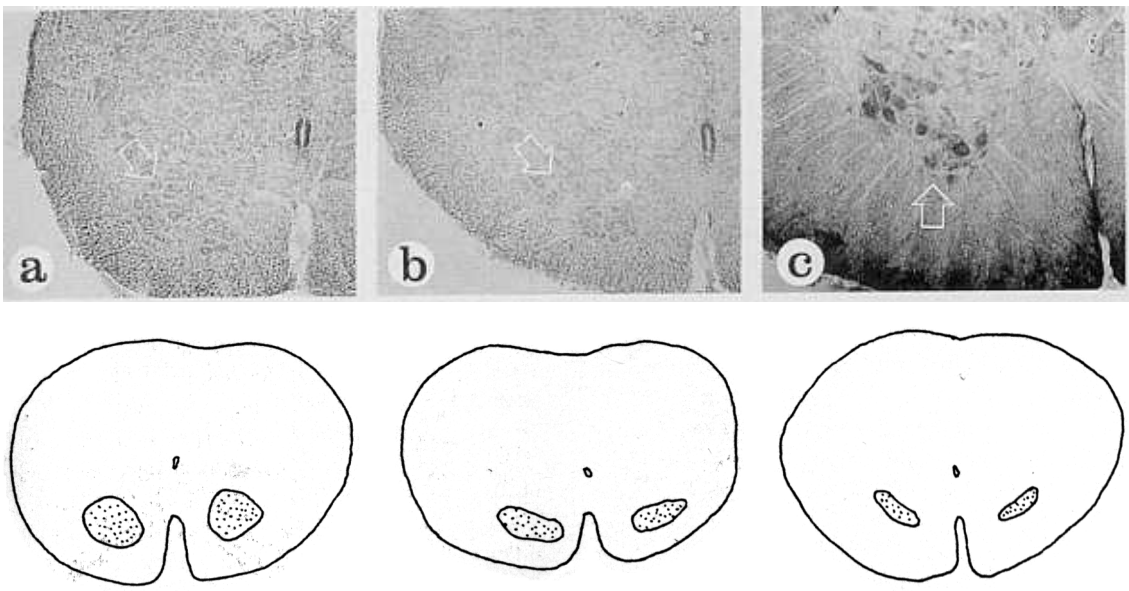

Fig. 1. Androgen receptor protein immunoreactivity of the L1 rat spinal cord. A, postnatal day 3: B, postnatal day 10: C, postnatal day 30: Black arrow indicates the spinal motonucleus of genitofemoral nerve. The bottom of each figure shows the area of androgen receptor protein immunoreactive cells on the anterior half of the spinal cord for each observation time. Immunoreactive cells of postnatal days 3 and 10 mainly contain dark-colored dots in cytoplasm and those of postnatal day 30 contain dark-colored dots in both nucleus and cytoplasm. 1:50 dilution of androgen receptor protein antiserum was used in these experiments. All photographs are enlarged at the same magnification (×40).

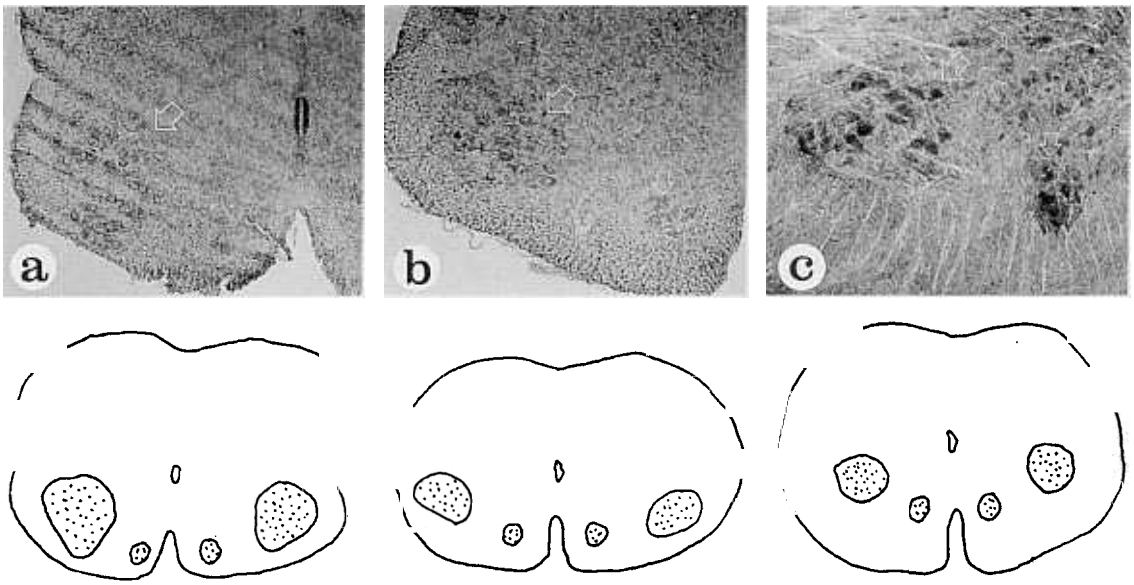

Fig. 2. Androgen receptor protein immunoreactivity of L1 rat spinal cord. A, postnatal day 3: B, postnatal day 10: C, postnatal day 30: Long black arrow indicates the spinal motonucleus of SNB. Short black arrow indicates the spinal motonucleus of DLN: The bottom of each figure shows the area of androgen receptor protein immunoreactive cells on the anterior half of the spinal cord for each observed time: Immunoreactive cells of postnatal days 3 and 10 mainly contain dark-colored dots in cytoplasm and those of postnatal day 30 contain dark-colored dots in both nucleus and cytoplasm: 1:50 dilution of androgen receptor protein antiserum was used in these experiments. All photographs are enlarged at the same magnification (×40).

which was presumed to be the area of SNB. Similar cells were found at the lamina IX of the ventrolateral aspect. However no immunoreactive cells were found at the retrodorsolateral nucleus, which is another nucleus responsible for perineal muscles. For SNB androgen receptors, the area of receptor activity did not show any change according to age. For the positive cells identified as DNL, the size and number of cells decreased as a rat aged. However the intensity of staining increased as a rat aged. At days 3 and 10, the immunoreactivity was found only at the cytoplasm, but the activity was observed both at the nucleus and cytoplasm at day 30.

DISCUSSION

The morphological change of the gubernaculum testis plays a major role in testicular descent which aids the passage through the inguinal canal (Wensing, 1987). Androgen (Elder *et al.* 1982; Han *et al.* 1992) and GFN (Beasley and Hutson, 1988; Han *et al.* 1992) are the suspected culprits that brings such change of gubernaculum. Cryptorchidism can be corrected experimentally by administering androgen in rats (Rajfer and Walsh, 1977; Habenicht and Neumann, 1983), and when antiandrogen such as cyproterone acetate and flutamide was administered during the fetal period, cryptorchidism could be induced. The receptors for androgen can be found at the gubernaculum (George and Peterson, 1988; Heyns and Pape, 1991; Husmann and Macphaul, 1991), and antiandrogen can change the morphology and histologic composition of gubernaculum (Han *et al.* 1992). Accordingly, it can be hypothesized that the testicular descent is mediated by the interaction of androgen and gubernaculum. Lewis briefly described the non-palpable testis in rats when GFN was harmed before postnatal day 10 (Lewis, 1948). Beasley and Hutson microscopically severed the rat psoas muscle which is believed to

harbor GFN, and observed the testicular atrophy and failure of testicular descent (Beasley and Hutson, 1988). However, their experiment also injured the testicular vessels and sciatic nerves (Hadziselimovic *et al.* 1988). We specifically severed the GFN under an operative microscope and observed the atrophy of vas deference and the length of testicular vessels, thereby confirming the observation made by Beasley and Hutson (1988).

GFN innervates crematerie muscles, aids in controlling testicular temperatures during adulthood and also innervates gubernaculum in the late fetal and neonatal periods. The spinal motonucleus of GFN is located at the anterior horns of L1 and L2. The number and size of cells constituting the mononucleus exhibit sexual differences (Kojima and Sano, 1984; Nagy and Senba, 1985; Han and Choi, 1995), and can be altered by the administration of androgen (Han and Choi, 1995).

Androgen, gubernaculum and GFN interact in the testicular descent. First, androgen produced from the fetal and neonatal testis affects gubernaculum and thereby the descent of the testis. In this situation it is not certain whether androgen exerts directly on the gubernaculum or indirectly through GFN. However it is known that androgen receptors are found on gubernaculum, thus androgen may act directly on gubernaculum. Second, testicular androgen during fetal and neonatal periods acts on the mononucleus of GFN, and therefore gubernaculum- mediated descent occurs. In this case it is also not certain whether androgen acts initially on the mononucleus or gubernaculum.

Observing the SNB/DLN, which shows a similar sexual dimorphic as the GFN motonucleus, can aid in elucidating the actual mechanism of GFN. When androgen is removed during fetal and neonatal periods, the number and size of the SNB/DLN mononucleus decreases and the result is feminization. If exogenous androgen is administered, the opposite phenomenon occurs (Breedlove and Arnold, 1983a). However, this phenomenon does not occur in mature rats (Breedlove and Arnold, 1983b). It means that only the developing SNB/DLN motonucleus responds to androgen manipulation. Whether the effect of androgen is direct or indirect via the action to the muscles is not certain. There is evidence that when androgen is tagged with radioactive isotopes and administered, the radioactivity can be found at the SNB/DLN. Conflicting reports claim that when SNB/DLN was destroyed and androgen was administered in neonatal female rats, the muscles innervated by SNB/DLN showed full male-level growth (Fishman and Breedlove, 1988). In addition, SNB/DLN does not show androgen concentrations in the neonatal period as in the adult period.

We have seen immunoreactivity of androgen receptors both in neonatal and adult rates at GFN and SNB/DLN motonuclei. This is evidence that androgen is a primary substance affecting these motonuclei. Also in the neonatal period when the testis descends under the influence of GFN (Beasley and Hutson, 1988; Hadziselimovic *et al.* 1988; Han and Choi, 1991) and gubernaculum (Han *et al.* 1992), the presence of androgen immunoreactivity proves that androgen acts on the motonuclei, and by the changes on the motonuclei, the target tissue undergoes subsequent morphologic changes. Although it is not clear that the androgen receptor is fully functional, a recent paper confirmed the close relationship between androgen receptor immunoreactivity and the function of spinal motonucleus (al-Shamma and Arnold, 1995). However, the exact relationship of androgen receptors, both in the motonuclei and gubernaculum, should be elucidated in the future.

Organs under the influence of androgen show the characteristic change of androgen receptors during growth (Takake *et al.* 1990). For example, androgen receptors in the prostate increase in benign prostatic hyperplasia, and the number of penile androgen receptors decreases after puberty. Gubernaculum shows a significant decrease in the number of androgen receptors immediately after birth (George and Peterson, 1988; Heyns and Pape, 1991; Husmann and Macphaul, 1991). We have seen an increase in the immunoactivity of androgen receptors at postnatal day 30 compared to birth. It is known that androgen receptor activity is usually found in the cytoplasm in the central nervous system (Wagari *et al.* 1993). The interesting point is that the immunoreactivity of androgen receptors in the motonucleus of neonatal rats is also found at the cytoplasm. Another interesting observation is that the number and size of cells in the motonuclei of GFN and DLN is greater during the neonatal period than at postnatal day 30.

The difference in the size of the motonucleus of GFN may be responsible for the neonatal descent of testis, and the difference in DLN is important in the development of the urethral sphincter, which shows a definite sexual difference (McKenna and Nadelhaft, 1986). The significance of androgen receptors at the column of Clarke should be elucidated in the future.

In summary, cells stained positively for the androgen receptor were found both at the motonucleus of GFN and SNB/DLN in postnatal days 3, 10, and 30. The number of cells stained positive decreased as time elapsed at the motonucleus of GFN and DLN, but no change in number was observed with SNB. Conclusively, the existence of androgen receptors were confirmed in the neonatal motonuclei of GFN and SNB/DLN. The motonucleus of GFN was controlled by androgen, and thereby controlled the testicular descent. The neonatal DLN affects the urethral sphincter muscles, which exhibits a definite sexual difference.

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