Evidence That Androgen Acts Through NMDA Receptors to Affect Motoneurons in the Rat Spinal Nucleus of the Bulbocavernosus

Cynthia L. Jordan,1 Scott E. Christensen,2 Robert J. Handa,3 Jennifer L. Anderson,3 Wendy A. Pouliot,3 and S. Marc Breedlove1

1Neuroscience Program and Psychology Department, Michigan State University, East Lansing, Michigan 48824, 2Department of Psychology, University of California, Berkeley, Berkeley, California 94720-1650, and 3Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado 80523

In adult male rats, spinal nucleus of the bulbocavernosus (SNB) motoneurons shrink after castration and are restored in size after androgen treatment. Sixty-day-old Sprague Dawley males were castrated and implanted with SILASTIC capsules containing testosterone (T) or nothing, and osmotic minipumps continuously infusing MK-801, a noncompetitive NMDA receptor antagonist, or saline. Twenty-five days later, bulbocavernosus muscles were injected with the retrograde tracer cholera toxin-horseradish peroxidase conjugate (CT-HRP) to label SNB cells. As seen previously, among saline-treated rats, SNB somata of T-treated castrates were significantly larger than those of castrates receiving blank capsules (p < 0.0001). MK-801 treatment blocked this effect of T on the SNB. MK-801 had no effect on non-androgen-responsive spinal motoneurons in the neighboring retrodorsolateral nucleus (RDLN), nor did the drug affect SNB soma size in the absence of androgen treatment. Motoneuronal soma size in Nissl stain revealed the same pattern of results seen with CT-HRP fills. In situ hybridization indicated that SNB motoneurons express mRNA for the NMDA receptor subunits R1, R2a, and R2b. Castration reduced the expression of R1 mRNA in SNB motoneurons, an effect that was blocked by androgen replacement in castrates. R2A and R2B mRNA expression in SNB cells was not affected by androgen manipulations. Likewise, androgen manipulations had no effect on the expression of any NMDA receptor subtypes in RDLN motoneurons. These results suggest that androgen affects the size of SNB motoneurons by influencing their expression of the NMDA receptor, and therefore the response of the motoneurons to endogenous glutamate.

Key words: spinal nucleus of the bulbocavernosus; neural plasticity; NMDA receptor; androgen; motoneurons; MK-801

The NMDA receptor has been implicated in many instances of neural plasticity, including estrogen-induced morphological changes of hippocampal pyramidal cells. Specifically, estradiol administration causes a rapid increase in the spine density of CA1 pyramidal cells; selective antagonism of the NMDA receptor attenuates these effects. Likewise, the estrogen-dependent morphological changes of CA1 pyramidal cells have been directly correlated with an enhanced sensitivity to NMDA receptor-mediated synaptic input (Woolley et al., 1997). Androgenic modulation of the NMDA receptor has also been reported for the CA1 region of the hippocampus, albeit to a lesser extent. Kus et al. (1995a) reported a decrease in MK-801 receptor binding within CA1 for androgen-treated castrates versus untreated castrates. Androgens also protect CA1 cells from the excitotoxic effects of NMDA (Pouliot et al., 1996). Together these findings suggest that sex steroids and NMDA receptors can interact to bring about changes in neuronal morphology and physiology.

Genes encoding for the NMDA receptor are expressed throughout motoneurons of the rat lumbar spinal cord (Toelle et al., 1995). The presence of NMDA receptors in this region suggests, but by no means proves, that this receptor may participate in the androgen-induced neural response of SNB motoneurons. To assess a possible role of the NMDA receptor in the effects of androgen on SNB soma size, MK-801, a selective NMDA receptor antagonist, was concurrently administered with chronic androgen treatment to castrated adult male rats. We found evidence that such treatment does indeed interfere with the influence of androgen on SNB motoneuronal somata size, but...
does not affect soma size of neighboring, non-androgen-responsive motoneurons. We also found that androgen treatment caused SNB motoneurons to increase expression of the gene for one NMDA receptor subtype. Thus androgen may affect SNB morphology by affecting the sensitivity of the motoneurons to endogenous glutamate.

MATERIALS AND METHODS

Twenty-eight male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in our laboratory 2 weeks before treatment. At 55–60 d of age, rats were anesthetized with ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, 10 mg/ml acepromazine mg/ml; 0.09 ml/100 gm body weight) and castrated through scrotal incisions. Animals were also given subcutaneous implants of 2 cm-long (effective release length) SILASTIC capsules (1.6 mm inner diameter; 3.2 mm outer diameter; constructed as in Smith et al., 1977) containing either crystalline testosterone (T) or nothing (blank) and an osmotic mini-pump containing either MK-801 or saline vehicle alone. For implantation, an incision ~5 cm long was made in a shaved, cleaned area of skin between the scapulas, and a subcutaneous pocket was formed via blunt dissection. The infusion end of the minipump was placed away from the site of incision, and the incision was closed with four or five wound clips. The reservoir capacity of the osmotic minipumps used (Alzet model 2ML; Alza, Palo Alto, CA) is ~2 ml and provides a 2.5 μl/hr constant infusion rate for 28 d. MK-801 hydrochloride (Research Biochemicals, Natick, MA) was dissolved in sterile saline to 0.95–1.02 M in a cryostat. The sections were thaw mounted on to Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) and stored at ~80°C in slides boxes with dessicant until in situ hybridization was performed.

Spinal cord sections were examined for NMDA R1, R2a, R2b, R2c and R2d mRNA levels using in situ hybridization as previously described (Kus et al., 1995b). Synthetic oligonucleotide probes were used for detecting NMDA receptor subunit mRNAs. Their sequences were as follows: NMDA R1: 5' CTT CCG AGC AGT CGG AAC CTC CAC CTC TAC CTT GCT CGT GCT GAG CAG GAG CAG GAG CAG GAA CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT CAG CAG GGT CAG CAG CAA GGT CAG CAG GGT
RESULTS

As expected, there were no significant differences in body weights of groups on the day of surgery. Separate two-way ANOVAs conducted on body weights measured on days 14 and 28 of treatment confirmed that the MK-801-treated animals gained weight more slowly than saline-treated animals. However, there was a significant interaction between androgen and drug for both days (day 14: $F_{(1,24)} = 7.40, p < 0.05$; day 28: $F_{(1,24)} = 4.28, p < 0.05$). The blank/MK-801-treated group gained significantly less weight than the saline groups on both treatment days (blank/MK-801 vs T/saline, and blank/MK-801 vs blank/saline, two-tailed $t$ test, $p$ values < 0.05); however, MK-801-treated castrates receiving T did not differ significantly from the saline groups. Thus, MK-801 treatment reduced weight gain, but only in castrates not receiving androgen replacement.

Also as predicted, the seminal vesicle weights of blank-treated castrates were significantly smaller than those of T-treated castrate males ($p < 0.0001$; main effect of androgen) (Table 1). There were no main effects of MK-801 treatment, nor any significant interaction of drug and hormone treatment on seminal vesicle weight. BC/LA weights showed a very similar response (Table 1) with the same pattern of statistical significance. Thus, T exerted a strong androgenic effect on seminal vesicle and anabolic effect on BC/LA muscle weights, neither of which was significantly affected by MK-801 treatment.

The soma area analysis of CT-HRP-labeled SNB motoneurons (Fig. 1) revealed a main effect of androgen ($F_{(1,20)} = 35.39; p < 0.0001$) and an interaction between androgen and drug treatment ($F_{(1,20)} = 9.05; p < 0.007$). Post hoc comparisons revealed that T/saline-treated castrates had significantly larger somata than T/MK-801-treated castrates ($p < 0.05$). However, T/MK-801-treated castrates were not significantly different from blank/MK-801 castrates ($p > 0.05$). Finally, blank/MK-801 and blank/saline-treated castrates did not significantly differ, indicating that MK-801 did not reduce SNB soma size in the absence of T treatment. Thus, the statistically significant interaction of MK-801 and androgen treatment seems to be caused by MK-801 treatment blocking the effect of androgen on SNB soma size.

This interaction between androgen and drug treatment was also apparent in the size of Nissl-stained SNB soma ($F_{(1,21)} = 4.93; p < 0.05$) from the same animals. SNB soma from T/saline castrates were significantly larger than those from T/MK-801 and blank/saline castrates ($p < 0.05$ for both); no other group differences were found (Table 2). So again MK-801 treatment masked the effect of T replacement on SNB soma size in castrates. As we have seen previously (Rand and Breedlove, 1995), the apparent size of SNB somata is smaller in Nissl-stained material than in HRP-filled material, presumably because the HRP fill offers a more complete view of the soma and primary dendrites.

As predicted, the androgen treatment had no significant effect on the size of RDLN soma ($F_{(1,21)} = 0.28; p > 0.50$), and the size of Nissl-stained somata was approximately the same in the SNB and RDLN. However, there was no significant main effect of MK-801 treatment ($F_{(1,21)} = 0.21; p > 0.50$) nor an interaction of MK-801 and androgen ($F_{(1,21)} = 2.95; p > 0.10$) for RDLN measures (Fig. 2). Thus, MK-801 had no effect on the size of non-androgen-responsive RDLN motoneurons from these animals.

In situ hybridization revealed that spinal motoneurons expressed NMDA receptor 1a, 2a, and 2b, as reported by others. However, R1 expression was greater in SNB motoneurons than in RDLN motoneurons. Furthermore, androgen manipulations affected R1 expression levels in SNB cells (Fig. 3). SNB motoneurons from control-treated castrates displayed less labeling than did SNB cells from either sham-operated males or castrates treated with T ($p$ values < 0.05). Androgen had no effect on NMDA R1 expression in RDLN motoneurons. The other two

---

Table 1. Effect of T and MK-801 on mean ± SEM seminal vesicle weight (left) and mean weight of BC/LA target muscles of the SNB (right)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline (gm)</th>
<th>MK-801 (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.20 ± .02</td>
<td>0.18 ± .01</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.85 ± .15</td>
<td>1.97 ± .09</td>
</tr>
</tbody>
</table>

Mean areas ± SEM of Nissl-stained SNB motoneuron somata

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline (µm²)</th>
<th>MK-801 (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>472.26 ± 19.96*</td>
<td>489.34 ± 23.8 (5)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>561.79 ± 24.23 (7)</td>
<td>485.96 ± 11.06 (6)</td>
</tr>
</tbody>
</table>

$N$ is the number of animals indicated in parentheses. An ANOVA revealed the same interaction between androgen and drug treatment that was detected in the analysis of CT-HRP-labeled somata depicted in Figure 1. Androgen increased SNB soma size only in the absence of MK-801 treatment.

*Significantly different from T/Saline castrates ($p < 0.05$).
NMDA receptor subunits were not affected by androgen manipulations in either the SNB (Fig. 4) or the RDLN (data not shown). There was no specific hybridization for the NMDA R2c or R2d message in either motoneuronal pool in any group (data not shown).

DISCUSSION
MK-801 blocked the effects of testosterone on SNB soma size. The MK-801 treatment only affected cell size in animals treated with T, indicating that its effect is dependent on the presence of androgen. MK-801 had no effect on the androgen-unresponsive RDLN motoneurons from the same spinal sections, indicating that the drug does not have a nonspecific effect of shrinking motoneurons. Indeed, among castrates not receiving T, the mean size of motoneurons in each nucleus was slightly, but not significantly, larger in MK-801 animals than in those receiving saline. These findings suggest that the NMDA receptor may play a role in the cellular mechanism by which androgen exerts morphological changes in adult motoneurons of the SNB.

A number of alternative explanations can be considered. First, the MK-801 treatment may have disrupted normal binding of testosterone to the androgen receptor, which led to a subsequent reduction in the androgen-mediated change in soma size. However, the seminal vesicles and the BC/LA muscles of the T-treated animals were identical among the drug and vehicle treatments, indicating that MK-801 does not interfere with androgen receptor binding, at least in peripheral structures. This does not rule out the possibility that in SNB motoneurons, MK-801 somehow acts on NMDA receptors to alter androgens binding to their receptor in those motoneurons. The current results could also be explained if the MK-801 treatment suppressed normal androgen receptor expression. Again the peripheral structure weights do not support this explanation: global reduction of androgen receptor expression by MK-801 would be expected to reduce peripheral structure weights for MK-801 animals receiving testosterone. Last, it is theoretically possible that the changes in SNB soma size seen after androgen treatment are attributable to the binding of T to NMDA receptors and not to androgen receptors. However, Watson et al. (2001) examined rats mosaic for wild-type and defective androgen receptors and found that functional androgen receptors were necessary for SNB motoneurons to respond to androgens with an increase in size. The same study found that bound androgen receptors reside within the nucleus, indicating that the androgen receptors induce somatic changes in the SNB through a genomic action.

So it appears likely that NMDA receptors and androgens normally interact to influence SNB morphology. There are several possible means by which this interaction could occur. First, although we know that androgens act directly on SNB motoneurons to increase their soma size, we do not know the locus at which MK-801 acts to interfere with this effect. One possibility is...
that androgens directly affect NMDA receptor expression or activity within the SNB.

There is precedent for this idea. In the rat hippocampus, estradiol treatment in ovariectomized rats increases the number of NMDA receptor binding sites in the hippocampus (Weiland, 1992). Gazzaley et al. (1996) showed that the mechanism for this estradiol-dependent change is likely attributable to an increased production of the NMDA R1 subunit protein. Morphologically, estradiol increases spine density of CA1 pyramidal cells, an effect that is dependent on NMDA receptor activation (Woolley and McEwen, 1993). Subcutaneous injections of MK-801 blocked the increase in spine density of these cells in the presence of estradiol (Woolley and McEwen, 1994). Likewise, long-term potentiation is increased as estrogen levels increase naturally within the rat estrous cycle (Warren et al., 1995). Last, Woolley and et al. (1997) showed that estradiol treatment of CA1 pyramidal cells increased their sensitivity to synaptic input mediated by the NMDA receptor. This increase in sensitivity was significantly correlated with the estradiol-induced increase in spine density, suggesting that both the steroid and drug were acting directly on these neurons.

The role of androgens in NMDA receptor expression has also been examined in the CA1 pyramidal layer of rats, but only at the level of ligand binding. Interestingly, Kus et al. (1995a) found that NMDA receptor binding was reduced in this region for male castrates receiving androgen compared with nontreated castrates. However, within the ventral and medial regions of the lateral septum, NMDA receptor binding was increased by androgen treatment of castrates (Kus et al., 1995b). An interaction of androgens and NMDA receptors in the neurophysiology of hippocampal CA1 cells was suggested when chronic dihydrotestosterone propionate administration was found to protect such cells from an NMDA-induced irreversible depolarization (Pouriot et al., 1996). Both androgens and estrogens, then, cause neurophysiological changes in hippocampal pyramidal cells via an NMDA receptor mechanism. These findings strengthen the possibility that androgens and the NMDA receptor may interact in the rat SNB system. Our data directly confirm this possibility.

There have been several reports that spinal motoneurons possess NMDA receptors (Kalb et al., 1992; Toelle et al., 1995; Croul et al., 1998), including a recent report of NMDA R1 immunoreactivity in SNB motoneurons (Gougis et al., 2002). The current findings indicate that NMDA receptor genes are indeed expressed by SNB motoneurons and that androgen manipulations alter the level of NMDA receptor expression by these cells. Taken together, these findings suggest a specific mechanism of action. When androgen induces SNB motoneurons to increase the expression of NMDA receptor genes, this may increase the sensitivity of the cells to glutamate stimulation of the resultant receptors. It may be this activation of NMDA receptors that then causes SNB motoneurons to enlarge (Breedlove, 1997). Because the same androgen treatments that increase SNB somata size also increase SNB dendritic extent (Kurz et al., 1986) and the penile reflexes (Hart 1973) mediated by SNB target muscles, NMDA receptors may also mediate these effects of androgen (Leedy et al., 1987; Matsumoto et al., 1988). There are no studies of the physiological effects of NMDA receptor activation specifically in SNB motoneurons. However, studies of other spinal motoneurons indicate that activation of NMDA receptors increases spontaneous activity (McCrimmon et al., 1989; Abdrachmanova et al., 2002).

It is interesting that neighboring RDLN motoneurons also express NMDA receptor genes, but do not modulate the expression of this gene when androgens are manipulated. Likewise, RDLN motoneurons do not respond to androgens by increasing their somata size nor do they respond to the NMDA receptor antagonist MK-801, either in the presence or absence of androgens. These results suggest that not all NMDA-receptive neurons modulate receptor expression in the face of changes in androgen levels. Perhaps the ability of androgen to modulate NMDA receptor expression is a requirement for neurons to respond morphologically to the hormone. Of course, this begs the question of why some neurons change NMDA expression after androgen manipulations, whereas other neurons do not. It cannot be a simple matter of whether the neurons possess androgen receptors, because RDLN motoneurons possess androgen receptors, as shown by autoradiography (Breedlove and Arnold, 1983) and immunocytochemistry (Freeman et al., 1995). Both of these reports suggest that RDLN motoneurons have fewer androgen receptors than do SNB motoneurons, so there may be some threshold effect, in which a particular concentration of androgen receptors is required for androgens to modulate NMDA expression. On the other hand, there are some cases when rat motoneurons differ markedly in their morphological response to androgen, but show no apparent difference in androgen receptor complement (Jordan, 1997). So androgen receptors may be necessary, but are not sufficient, to confer androgen-induced plasticity to motoneurons.

The study of sex steroid-NMDA receptor interactions has been primarily restricted to the rat hippocampus; we report here that a similar interaction may occur in the spinal cord. These findings suggest that steroids may commonly act through NMDA receptors to modulate neuronal plasticity throughout the neuraxis, a hypothesis that might be usefully tested in other neural systems affected by steroid hormones.

REFERENCES

Breedlove SM (1997) Adult sexual experience alters neuronal morphol-

Breedlove SM, Arnold AP (1986) Sex differences in the pattern of ste-
Croul S, Radziwsky A, Sverstuk A, Murray M (1998) NK1, NMDA,
5HT1a, and 5HT2 receptor binding sites in the rat lumbar spinal cord: analysis of the effects of androgen receptor knockout mice. J Neurobiol 32:1

dendritic length of mammalian motoneurons in adulthood. Science

232:395
Gazzaley AH, Weiland NG, McEwen BS, Morrison JH (1996) Differ-
ential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. J Neurosci 16:6830–6838.
Hart BL (1973) Effects of testosterone propionate and dihydroestro-

Kalb RG, Lidow MS, Hausted MJ, Huckfeld S (1992) N-methyl-D-
aspartate receptors are transiently expressed in the developing spinal cord ventral horn. Proc Natl Acad Sci USA 89:8502–8506.