Androgen Mitigates Axotomy-Induced Decreases in Calbindin Expression in Motor Neurons

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Androgens can rescue axotomized motor neurons from cell death. Here we examine a possible mechanism for this trophic action in juvenile Xenopus laevis: regulation of a calcium-binding protein, calbindin, after axotomy. Western analysis revealed that a monoclonal antibody to calbindin D specifically recognizes a single ~28 kDa band in X. laevis CNS and rat cerebellum. Retrograde transport of peroxidase combined with immunohistochemistry demonstrated that somata, axons, and synaptic terminals of laryngeal motor neurons in nucleus (N.) IX–X of X. laevis are calbindin-positive. The number of calbindin-positive cells was compared in the intact and axotomized sides of N.IX–X of gonadectomized males that were either hormonally untreated or DHT-treated for 1 month. Although axotomy decreased the number of calbindin-positive cells by 86% in hormonally untreated males, the decrease was only 56% in DHT-treated animals. Compared with hormonally untreated animals, the number of calbindin-labeled cells in N.IX–X of DHT-treated males was increased in both the intact (14%) and axotomized sides (75%). We conclude that axotomy decreases and that DHT enhances calbindin immunoreactivity in N.IX–X. Axotomy-induced decrease in calbindin immunoreactivity precedes cell loss in N.IX–X and may impair the capacity of motor neurons to regulate cytoplasmic calcium. Androgen-mediated maintenance of calbindin expression is thus a candidate cellular mechanism for trophic maintenance of hormone target neurons.

Key words: dihydrotestosterone; motor neuron death; immunohistochemistry; calcium buffer; androgens; calbindin

Materials and Methods

Animals, axotomy, and hormone treatment. Three-month-old postmetamorphic juvenile male frogs (11 animals, 3.0–7.6 gm) obtained from Nasco (Fort Atkinson, WI) were gonadectomized under anesthesia with 0.1% 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis, MO). The larynx was accessed through an incision in the body wall; the right IX–X nerve was separated from the laryngeal muscle and severed, and ~1 cm of the proximal segment was removed. Four males (n = 4) were implanted with a SILASTIC tube (VST 030065, Dow Corning, Midland, MI) containing 5 mg of DHT (5α-17β-ol-3-one androstan; dihydrotestosterone, Sigma) while four males (hormonally untreated) received an empty tube placed into the dorsal lymph sac. One month after gonadectomy, tube implantation and denervation animals were anesthetized deeply and transcardially perfused with 5 ml of 0.6% NaCl, followed by 10 ml of 4% paraformaldehyde in 1× PBS (2.6 mM KCl, 1.4 mM KH₂PO₄, 136 mM NaCl, and 8 mM Na₂HPO₄, pH 7.2). The brain and
spinal cord (CNS) and the larynx were removed and post-fixed for 2 hr in 4% paraformaldehyde, followed by immersion in 20% sucrose in 1X PBS for 4–12 hr. Sections of the CNS were mounted consecutively onto different slides: the first section was stained with cresyl violet, the second section subjected to immunohistochemistry, the third section was not processed, and so forth. Three additional gonadectomized and DHT-treated males were not axotomized; crystals of horseradish peroxidase (HRP) were inserted into the laryngeal muscle, and the animals were allowed a survival period of 3 d. Animals were perfused, and cryostat sections of the CNS were obtained as described above.

**Immunohistochemistry.** Cryostat sections of the CNS and larynx were air-dried, immersed in a methanol–0.5% H2O2 solution for 30 min, and then washed in 0.1 m Tris-HCl. After a 30 min incubation in a solution containing 2% BSA and 0.3% Triton X-100 in 0.1 m Tris-HCl, sections were incubated overnight with mouse monoclonal anti-calbindin D antibody (clone CL-300, C-8666; Sigma), which has been used previously to demonstrate calbindin immunoreactivity in the dorsal rhombencephalon of *X. laevis* (Muñoz et al., 1995). To determine the optimal antibody concentration, we first incubated sections of the Xenopus CNS with increasing concentrations; a 1:400 concentration of calbindin antibody saturated the immunohistochemical reaction and was used for the comparison of the experimental groups. On the next day, sections were washed, re-incubated with 1:200 biotinylated horse anti-mouse IgG for 30 min and then with avidin–biotin–peroxidase complex (ABC) according to the manufacturer’s specifications (Vectastain, Vector Laboratories, Burlingame, CA). After washing in Tris buffer, sections were covered with a 0.05% solution of 3,3’-diaminobenzidine in 0.05 m Tris-HCl plus 0.05% H2O2 for ∼5 min to give a brown cytoplasmic reaction. Finally, sections were dehydrated and coverslipped. As a control for comparison of the experimental groups. On the next day, sections were

**RESULTS**

Calbindin expression in motor neurons of NIX–X

N.IX–X is located immediately caudal to the fourth root of cranial nerve IIX–X (Fig. 1C). Neurons of N.IX–X are embedded in the white matter of the brainstem lateral to the inferior reticular formation (Simpson et al., 1986). In N.IX–X, cell perikarya and neurites were immunolabeled with the calbindin antibody (Fig. 1A). Calbindin expression elsewhere in the rhombencephalon was limited to the Purkinje cells of the cerebellum, cells of the dorsal tegmental area of the medulla, and sparse cells in the reticular formation and central gray as well as the dorsal rhombencephalic calbindin-expressing nuclei previously described by Muñoz et al. (1995). The dorsal tegmental area of the medulla and the inferior reticular formation are the major sources of afferent input to N.IX–X (Wetzel et al., 1985). In addition to laryngeal motor neurons, cells in the trigeminal and facial nuclei were labeled with the calbindin antibody; no motor neurons in the spinal cord displayed calbindin immunoreactivity (data not shown). Specificity of immunoreactivity was confirmed by the absence of any immunohistochemical reaction in control sections (data not shown).

In DHT-treated animals motor neurons retrogradely labeled with HRP were either calbindin-immunopositive or -negative and were intermingled within N.IX–X, indicating that a subpopulation of motor neurons expresses the protein (Fig. 1A). In the absence of specific markers we cannot determine whether interneurons or glial cells in N.IX–X also express calbindin. Most calbindin-positive cells of N.IX–X that were not labeled with HRP had a motor neuron-like morphology (Fig. 1A); because injection of HRP may not have reached all motor neuron terminals, these cells also may have been laryngeal motor neurons. In motor neurons, calbindin immunoreactivity was localized to both the cytoplasm and dendrites (Fig. 1B). In addition, calbindin was present in axons of the N.IX–X nerve as they left the brainstem (Fig. 1C). When the nerve was followed to its target laryngeal muscle, calbindin immunoreactivity was seen in unmyelinated
axons, in Schwann cells that form the myelin sheath (Fig. 1D), and in terminals of laryngeal axons at the neuromuscular junction (Fig. 1E). Muscle fibers were not immunoreactive for the calbindin antibody. Calbindin immunoreactivity was found in laryngeal axons in the innervated muscle, whereas no calbindin reaction was found in the denervated side of either hormonally untreated or DHT-treated animals; denervation was confirmed with neurofilament immunolabeling of axons, and denervated muscle was severely atrophied (data not shown). These results indicate that motor neurons do not reinnervate the muscle after 1 month of axotomy, DHT treatment, or both.

The specificity of the calbindin antibody was analyzed by Western blot of proteins from rat cerebellum and Xenopus brain (Fig. 2). One band at ~28 kDa was immunolabeled in protein samples from both species. Immunolabeling of this protein was absent in membranes that were not incubated with the calbindin antibody. Because the molecular weight of the labeled protein is that expected for calbindin and because Western analysis revealed a protein of similar size in rat cerebellum (where Purkinje cells express high levels of calbindin; Jande et al., 1981), it is most likely that the immunolabeling reported here represents calbindin-D28k expression in the CNS of X. laevis.

Effects of axotomy and DHT treatment on calbindin immunoreactivity of N.IX–X

Once it was established that motor neurons of N.IX–X contain calbindin, we next analyzed the effect of axotomy and DHT treatment on the expression of this protein.

Axotomy decreases calbindin immunoreactivity in N.IX–X. In hormonally untreated animals a marked decrease in the immunohistochemical signal is noted on the axotomized side, compared with the intact side (Fig. 3A vs B). On the axotomized side of N.IX–X, the decrease of immunoreactivity in the neuropil is accompanied by an apparent absence of immunoreactivity in the cytoplasm of cells. An examination of adjacent cresyl violet-stained sections reveals that the decrease in calbindin immunoreactivity in the axotomized side of N.IX–X is not attributable to an absence of cells (Fig. 3A'). In hormonally untreated animals, cells of the axotomized side display more intense cresyl violet staining of the cytoplasm than cells of the intact side (Fig.

**Figure 1.** A, Intact motor neurons in N.IX–X of a DHT-treated animal double-labeled with HRP (brown reaction) and calbindin (green reaction). N.IX–X is heterogeneous with respect to calbindin expression: some HRP-labeled motor neurons express calbindin (arrowheads); some motor neurons do not (stars). Some cells with motor neuron-like morphology were not labeled with HRP (v). B, Detail of a motor neuron in N.IX–X; calbindin immunoreactivity is localized to both cytoplasm and dendrites. C, Axons from motor neurons of N.IX–X were immunolabeled with calbindin in the laryngeal nerve (arrowhead). D, Within the larynx, unmyelinated axons and Schwann cells associated with myelinated axons (arrowhead) were immunolabeled with calbindin antibody. E, Synaptic terminals at the laryngeal neuromuscular junction were immunoreactive for calbindin antibody. Scale bars: A, 50 μm; B, 10 μm; C, 100 μm; D, E, 25 μm.
animals, the number of cells on the intact side was 209 untreated animals (compare Fig. 3). In hormonally untreated animals, less calbindin immunoreactivity was noted on the axotomized side of N.IX–X (Table 1). Thus, there are no differences in either cell or nuclear diameter of intact and axotomized cells of N.IX–X in animals treated with DHT.

Cell size did differ significantly for the intact sides of N.IX–X of DHT-treated and hormonally untreated animals (p < 0.001, Table 1); thus DHT increases the size of intact motor neurons. In contrast, no differences were found for the axotomized sides of these groups (p > 0.7). Together, the results indicate that both axotomy and DHT treatment increase the size of cell bodies in N.IX–X without significantly affecting nuclear diameter. The effect of axotomy and DHT treatment is neither additive nor synergistic, suggesting a limit to the increase of cell size.

Effects of axotomy and DHT treatment on the number of calbindin-immunopositive cells of N.IX–X

Androgen and axotomy alter calbindin immunoreactivity in N.IX–X. One month after treatment there was a significant main effect of axotomy on the number of calbindin-positive cells in N.IX–X (F(3,15) = 27.6; p < 0.001; Figure 4). Axotomy for 1 month was accompanied by a decrease in the number of calbindin-immunoreactive cells in hormonally untreated animals (86%) and in DHT-treated animals (56%). Thus, whether DHT-treated or untreated, axotomy decreased the number of calbindin-immunoreactive cells in N.IX–X.

There was a significant main effect of DHT treatment on the number of calbindin-immunopositive cells in N.IX–X (F(4,15) = 5.1; p < 0.05; Fig. 4) but no significant interaction between hormone treatment and axotomy (F(11,15) = 0.55; p > 0.5). For the intact side of N.IX–X, there were 14% more calbindin-positive cells in DHT-treated animals than in hormonally untreated animals. For the axotomized N.IX–X, there were 75% more calbindin-immunopositive cells in DHT-treated animals than in hormonally untreated animals (Fig. 4). We conclude that in both the intact and the axotomized N.IX–X, there are more calbindin-immunoreactive cells in DHT-treated than in hormonally untreated animals.

**DISCUSSION**

Androgenic steroids have been shown to preserve target motor neurons from cell death both during normal development and in response to axotomy (for review, see Breedlove, 1992; Kujawa and Jones, 1995). We have examined how androgens exert their protective effects in an experimental model system: axotomized laryngeal motor neurons of juvenile *Xenopus laevis*. Laryngeal motor neurons express high levels of androgen binding and AR mRNA (Kelley, 1981; Pérez et al., 1996) and thus are targets for...
Figure 3. Intact and axotomized sides of N.IX–X in hormonally untreated and DHT-treated animals. Sections were immunolabeled with calbindin antibody, which produced a brown cytoplasmic reaction (A–D), and adjacent sections were stained with cresyl violet (A’–D’). In N.IX–X of hormonally untreated animals, axotomy caused a striking decrease in calbindin immunoreactivity (compare A with B) and an increase in cresyl violet staining of cells (compare A’ with B’). In DHT-treated animals, axotomy caused a decrease in calbindin immunoreactivity (compare C with D) in cells of N.IX–X but had no effect on cresyl violet staining (compare C’ with D’). Scale bar, 200 μm.
animals (**) the intact side, DHT treatment increases cell diameter, as compared with untreated cell diameter in N.IX–X (*). Cell diameter 9.5 ± 0.5, 15.4 ± 2.6*, 16.2 ± 1.4**, 15.5 ± 3.4

Table 1. Size of cells in N.IX–X is affected by axotomy and androgen treatment

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Cell and nuclear diameters (µm) in the intact and axotomized sides of N.IX–X of hormonally untreated and DHT-treated animals. Data presented are means ± SD; n = 4 animals in all cases. In hormonally untreated animals, axotomy increases the cell diameter in N.IX–X (p < 0.05). In contrast, cell diameters of neurons from the intact and axotomized sides of DHT-treated animals do not differ significantly. On the intact side, DHT treatment increases cell diameter, as compared with untreated animals (*/p < 0.01). Neither axotomy nor hormonal treatment significantly affected diameters of cell nuclei in N.IX–X (ANOVA, p > 0.5).

Figure 4. Number of calbindin-immunopositive cells and number of cresyl violet-stained cells (mean ± SD; n = 4 animals per group) counted in the intact and axotomized N.IX–X of hormonally untreated (black bars) and DHT-treated (white bars) animals 1 month after axotomy. There were no significant differences in the number of cresyl violet-stained cells. There was a significant main effect of hormone treatment (p < 0.05) and axotomy (p < 0.01) on the number of calbindin-positive cells, but there was no significant interaction between treatments. For the axotomized side, the number of calbindin-immunopositive cells in the intact sides/number in the axotomized sides × 100 is displayed within the bars.

Calbindin, axotomy, and cell death

We show here that axotomy decreases the number of calbindin-immunoreactive cells in N.IX–X, suggesting that motor neurons deprived of access to target muscles downregulate this protein. Not all proteins, however, are decreased in expression by axotomy. In this study, axotomy increased Nissl staining, a change believed to reflect a general increase in protein synthesis in neurons (Barr and Hamilton, 1948; Lieberman, 1971). Axotomy also increased AR mRNA expression (Pérez and Kelley, 1996). We suggest that downregulation of calbindin expression is a specific response to axotomy that may contribute to the subsequent death of laryngeal motor neurons.
Increased levels of cytoplasmic calcium are associated with cell death in many systems. Changes in calbindin expression have been observed in the CNS after injury. For instance, kindling reduces calbindin content in granule cells of the dentate gyrus (Köhr and Mody, 1991); Renshaw interneurons decrease calbindin immunoreactivity after axotomy of the presynaptic motor neurons (Sanna et al., 1993), and the number of calbindin-immunopositive cells increases after axotomy of sympathetic ganglion cells (Sánchez-Vives et al., 1994). In patients with Huntington’s and Alzheimer’s diseases, calbindin expression is downregulated in the forebrain and striatum (Iacopino and Christakos, 1990a).

In motor neurons, analysis of electrophysiological properties after axotomy suggests that these cells experience elevated intracellular calcium; axotomized motor neurons have a larger after-hyperpolarization potential and faster inactivation of calcium currents than intact neurons (Unemiy a et al., 1993). Interestingly, these characteristics are reminiscent of the physiology of injured granule cells of the hippocampus that lose calbindin expression (Köhr and Mody, 1991). In addition, axonal degeneration in axotomized motor neurons is mediated via calcium influx (George et al., 1995). In Xenopus laryngeal motor neurons, a decrease in calbindin immunoreactivity is an early response of motor neurons to axotomy that anticipates cell loss. After axotomy, an impaired buffering capacity resulting from decreased calbindin expression may contribute to the elevation of free calcium leading to cell death.

Does the axotomy-induced decrease in calbindin immunoreactivity reflect a decrease in calbindin expression? Calbindin immunoreactivity can be influenced by calcium levels. In biochemical experiments the immunoreactive signals for calbindin in blots from cytosolic extracts of rat cerebellum were directly proportional to calcium concentration in the incubation buffer; formalin fixation reduced, but did not eliminate, calcium effects (Winsky and Kuznicki, 1996). In hippocampal slices opposite results have been obtained: slices preincubated in low calcium showed enhanced immunoreactivity, whereas those in high calcium showed decreased immunoreactivity (Dutar et al., 1991). Both of these studies used the same mouse monoclonal anti-calbindin D antibody used here. Changes in calbindin immunoreactivity induced by axotomy thus may reflect changes in the state of the protein because of altered intracellular calcium levels rather than an actual decrease in protein synthesis or stability. The resolution of these issues awaits the cloning of Xenopus calbindin so that regulation of its mRNA can be studied.

**Androgens, calbindin, and cell survival**

In N.IX–X androgen treatment increases cell survival after axotomy (Pérez and Kelley, 1996) and maintains calbindin immunoreactivity (this study). Expression of calbindin also can be regulated by other steroid hormones. For instance, corticosterone induces calbindin expression in the hippocampus (Iacopino and Christakos, 1990b), and calbindin mRNA expression is under the control of estrogen in uterine myometrium (Romagnolo et al., 1996).

In both the intact and axotomized sides of N.IX–X, more calbindin-immunoreactive cells were found in DHT-treated animals than in hormonally untreated animals. Although protective effects of calbindin are controversial (Baimbridge et al., 1992), calbindin is thought to protect injured neurons and glial cells from excitotoxicity via reduction in intracellular calcium levels (Sloviter, 1989; Mattson et al., 1991; Bao-Kuan et al., 1996; Peterson et al., 1996). Calbindin-immunoreactive cells are spared in *in vitro* assays of neuronal survival, and calbindin expression is induced by brain-derived neurotrophic factor, tumor necrosis factor, and neurotrophin-3 (NT-3), all of which exert trophic effects on target neurons (Collazo et al., 1992; Mattson et al., 1995; Ventimiglia et al., 1995; Marty et al., 1996). In a model system for amyotrophic lateral sclerosis (IgG-mediated toxicity of hybrid motor neurons), calbindin transfection protects cells from death (Ho et al., 1996). Although the increased expression of calbindin evoked by androgens is not specific to axotomized cells, it might contribute specifically to survival by sustaining cell physiology in a state closer to that of intact motor neurons.

Axotomy induces changes in gene expression: some changes are associated with cell survival and others with neurodegeneration. In laryngeal motor neurons of N.IX–X, axotomy for 1 month induces upregulation of AR mRNA expression. By facilitating androgen binding and thus enhancing the expression of androgen target genes, increased receptor expression may represent the first step in androgen rescue of motor neurons (Pérez and Kelley, 1996). Another such example is the induction of tubulin expression by testosterone in axotomized facial motor neurons of hamsters (Jones and Oblinger, 1994), which may facilitate axonal regeneration (Yu, 1982; Kujawa et al., 1991). In contrast, axotomy decreases calbindin expression, a change that may favor calcium-mediated cell death. We conclude that one explanation for androgen-mediated cell rescue is increased transcription of “survival” genes and that calbindin may be such a gene.

**REFERENCES**


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