ANABOLIC-ANDROGENIC STEROID INDUCED ALTERATIONS IN CHOLINE ACETYLTRANSFERASE MESSENGER RNA LEVELS OF SPINAL CORD MOTONEURONS IN THE MALE RAT

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Abstract — The effect of chronic supraphysiological doses of anabolic-androgenic steroids, such as those illegally used by recreational, amateur and professional athletes to increase muscle mass and strength, on motoneurons has not been established. The choline acetyltransferase activity levels of perineal muscles in the male rat are modulated by plasma testosterone levels. These muscles are innervated by the sexually dimorphic motoneurons of the spinal nucleus of the bulbarvocavemosus. Since the primary source of choline acetyltransferase in muscle is motoneuronal, testosterone may modulate perineal muscle choline acetyltransferase activity by regulating choline acetyltransferase messenger RNA levels in motoneurons. The purpose of this study was to determine if choline acetyltransferase messenger RNA levels in cervical and lumbar spinal motoneurons are affected by chronic (four weeks) changes of plasma testosterone levels in the adult male rat. Using in situ hybridization, choline acetyltransferase messenger RNA levels were analysed in four motor columns: the spinal nucleus of the bulbarvocavemosus, the retrodorsal lateral nucleus of the lumbar spinal cord, and the lateral motor columns of the cervical and lumbar spinal cords. Chronic exposure to supraphysiological levels of testosterone (five- to ten-times physiologic levels) significantly increased choline acetyltransferase messenger RNA in all four motor columns. Subsequent to castration, choline acetyltransferase messenger RNA levels decreased in motoneurons of the spinal nucleus of the bulbarvocavemosus and the retrodorsal lateral nucleus. This observation suggests that the decrease in choline acetyltransferase activity levels of muscles innervated by spinal nucleus of the bulbarvocavemosus moto- neurons may be due to changes in choline acetyltransferase protein levels. Indeed, testosterone replacement therapy of castrated males prevented the decline of choline acetyltransferase messenger RNA levels in motoneurons.

The results of this study demonstrate that anabolic-androgenic steroids can affect the levels of specific messenger RNAs in motoneuron populations throughout the spinal cord suggesting that motoneuronal characteristics are modulated by circulating anabolic-androgenic steroid levels regardless of the purported "androgen sensitivity" of the specific neuromuscular system. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: testosterone, gene expression, in situ hybridization.

Administration of supraphysiological doses of exogenous anabolic-androgenic steroids (AAS), such as testosterone, are commonly believed to assist in increasing muscle strength and mass during exercise training.11,74,79 Both skeletal muscle fibres and somatic motoneurons express the androgen receptor, suggesting that AAS may directly regulate the transcription of specific genes in both cellular components of the neuromuscular system (transcriptional regulation).6,58,96 skeletal muscle fibres;29,45,59 motoneurons62,65). Previous studies on the effect of supraphysiological AAS levels on neuromuscular systems have focused on the AAS-induced changes of skeletal muscle fibre properties.2,14,16,24,60 The effect of chronically elevating plasma testosterone levels above physiological on motoneuron gene expression has not been investigated.

Castration of adult male rats decreases circulating levels of testosterone and induces profound changes in the morphology, connections, and gene expression of motoneurons in the sexually dimorphic spinal nucleus of the bulbarvocavemosus (SNB).4,43 In the male rat, SNB motoneurons innervate the sexually dimorphic bulbocavemosus/levator ani (BC/LA) muscle complex and the external anal sphincter.
muscle. Castration alters the steady state levels of a number of mRNAs coding for molecules or enzymes involved in intercellular communication in SNB motoneurons. These include: the neuroeffector peptides, calcitonin gene-related peptide and cholecystokinin, and the gap junction forming protein connexin.

Castration also decreases the activity levels of choline acetyltransferase (ChAT), the enzyme that synthesizes the neurotransmitter acetylcholine, of the LA muscle. The castration-induced decrease in ChAT activity is prevented by testosterone replacement therapy. In skeletal muscle, the primary source of ChAT is the presynaptic terminal of the neuromuscular junction. Modulation of LA muscle ChAT activity by circulating testosterone levels suggests that the transcriptional and/or translational rates of ChAT in SNB motoneurons may be altered by circulating testosterone levels. The purpose of the present study was to determine whether changes in plasma testosterone levels of adult male rats alters the steady state levels of ChAT mRNA of motoneurons located in lumbar and cervical spinal cord segments. Portions of this research have been presented in preliminary form.

**EXPERIMENTAL PROCEDURES**

**Animals**

Plasma testosterone levels were altered for a 28 day period in age-matched, adult male Long Evans rats (45-50 days-old). All animals were purchased from Charles Rivers (Wilmington, MA). Animal care and killing were consistent with the established guidelines of the American Veterinary Medical Association. Experimental protocols were approved by the University of California at Los Angeles Chancellor's Animal Research Committee. In Experiment I, plasma testosterone levels were chronically increased to supraphysiologic levels; and, in Experiment II, plasma testosterone levels were decreased below normal physiologic levels.

**Experiment I: Chronic elevation of plasma testosterone levels above physiologic levels.** Gonadally intact adult male rats were assigned to one of three groups: i) males that did not receive any exogenous testosterone supplement, ii) males subcutaneously implanted with a single Silastic capsule packed with crystalline testosterone propionate (TP capsule: 45 mm long, 1.57 mm i.d., 3.17 mm o.d.; 1-TP) or, iii) males subcutaneously implanted with two TP capsules (2-TP). The TP capsules were subcutaneously implanted under ether anesthesia. After 28 days, the animals were deeply anaesthetized with sodium pentobarbital (150 mg/kg) and killed by transcardial perfusion with saline followed by 4% paraformaldehyde in 0.1 M Sorenson's phosphate buffer. The cerebral and lumbarosacral segments from each spinal cord were removed, cryoprotected, sectioned longitudinally at 15 μm in a cryostat and mounted on Superfrost Plus® microscope slides (Fisher Scientific). The slides were stored desiccated at -70°C until processed for radioimmunoassay of plasma testosterone levels. The testosterone in each plasma sample was extracted into ether and chromatographically separated from other steroids prior to radioimmunoassay.

**Experiment II: Chronic decrease of plasma testosterone levels below physiologic levels.** Age-matched adult male rats were assigned to three groups (4-5 rats/group): castrated males, gonadally-intact males, or testosterone-treated castrated males. Castrations were performed under ether anaesthesia. Five male rats were subcutaneously implanted with a single 45 mm long TP capsule at the time of castration thereby maintaining circulating testosterone levels in the high physiologic range. Four weeks later, the animals were deeply anaesthetized with sodium pentobarbital (150 mg/kg) and killed by transcardial perfusion with saline followed by 4% paraformaldehyde in 0.1 M Sorenson's phosphate buffer. The lumbarosacral spinal cords were removed, cryoprotected, sectioned in the transverse plane at 20 μm in a cryostat, mounted on Superfrost Plus® microscope slides (Fisher Scientific), and stored desiccated at -70°C until processed for in situ hybridization histochemistry.

**In situ hybridization histochemistry**

In situ hybridization on spinal cord sections was performed as described by Popper and Micevych. A radiolabeled antisense cRNA probe was prepared by in vitro transcription with T7 RNA polymerase using linearized plasmid containing a 2329 base pair cDNA clone encoding rat ChAT (provided by Dr. S. Bernard, Laboratoire des Neurobiologie Cellulaire et Moléculaire, Gif-sur-Yvette, France) as a template. The ChAT cRNA probe was radiolabeled with [35S]UTP (specific activity: 1000-1500 Ci/mmol; NEN-Dupont). The ratio of radiolabeled UTP to unlabelled UTP used during the transcription reaction was 1:3. The plasmid was cut at an HpaII site within the cDNA insert so that the resulting 78 base pair radiolabeled probe is complementary to the 3'end of ChAT mRNA. This radiolabeled probe can hybridize to all the known alternate splice ChAT mRNA species currently known to exist in the rat spinal cord. Slide mounted sections were pretreated sequentially with proteinase K (1 μg/ml; 10 min at 37°C), 0.25% acetic anhydride in 0.1 M triethylenamine (10 min at room temperature), 2 μg/ml standard saline citrate (SSC), dehydrated in ethanol and air-dried before an 1 h incubation at 60°C in prehybridization medium [250 μM denatured poly(A), 250 μM denatured sheared salmon testes DNA, 5 × hybridization buffer and 40% formamide]. The sections were hybridized with the 35S-labeled antisense cRNA probe (4 ng/slide) for 1 h at 60°C in prehybridization buffer containing 5% dextran sulfate. The sections were subsequently treated with RNase A (15 min; 37°C), washed in decreasing concentrations of SSC containing diethioctetrol, with a final wash in 0.1 × SSC for at 55°C for 30 min. The sections were dehydrated, air-dried, dipped in Kodak NTB-2 nuclear emulsion diluted 1:1 with distilled water, and exposed at 4°C for 14 days. The slides were developed using Kodak D-19 developer, dehydrated, and coverslipped with Permoun mounting medium (Fisher Scientific).

Accumulation of silver grains over motoneurons was not observed when rat spinal cord sections were pretreated with RNase A prior to hybridization, or when an [35S]UTP-labeled sense ChAT cRNA probe 2329 nucleotides long, containing the portion of ChAT mRNA complementary to the antisense ChAT cRNA probe was used, confirming the specificity of the 35S-labeled antisense ChAT cRNA probe.

**Data analysis**

Silver grain densities were determined over motoneurons with the aid of a computer-assisted image analysis system.
Fig. 1. Emulsion autoradiographs of specific in situ hybridization to ChAT mRNA in somatic motoneurons in a transverse spinal cord hemisection taken at segmental level L₅. A) High-power magnification showing specific silver grain deposition over four motoneurons; Scale bar=50 μm. B) Low-power magnification showing the location of the four motor columns observed at this segmental level: (1) RDLN; (2) Dorsal Lateral Nuclei; (3) Ventral Motor Pool; and, (4) SNB; Scale bar=100 μm.

(Optimas Imaging Software, Bioscan Inc., Edmonds, WA) from digitized video images under dark-field illumination. The autofluorescence of the cell body, due possibly to lipofuscin granules, was used to determine and demarcate cell boundaries from a second digital image of the same visual field using green excitation. Analysis was restricted to those motoneurons in which the cell nucleus was clearly visible. Motoneuron cross-sectional area measurements were not included in this report due to the disruption of cellular morphology and tissue integrity caused by requisite treatment of the tissue with proteinase K. The background levels of silver grain density were also determined over adjacent areas of neuropil. For Experiment I, data was collected for motoneurons located in the SNB, the retro-dorsal lateral nucleus (RDLN; L₅-₆ₓ), and the lateral motor columns of spinal cord segments L₅-₆ₓ (L₅₋₆₅) and C₁₋₅₅, (C₅₋₅₅), which provide innervation to the hindlimb (including the Sol and EDL muscles) and the forelimb, respectively. For Experiment II, silver grain density determinations were done on SNB (L₅₋₆ₓ) and RDLN motoneurons. RDLN motoneurons specifically innervate the intrinsic muscles of the hindpaw. Autoradiographic silver grain densities were normalized, across slides and animals, by dividing the silver grain density over a labelled cell by the background silver grain density. The resulting value was designated the labelling ratio and is a measure of specific hybridization over an individual cell. Statistical analyses were performed using ANOVA (SigmaStat for Windows v1.01, Jandel Scientific). Differences in the mean labelling ratio for ChAT mRNA between motor columns were statistically analysed in the gonadally-intact males from Experiment I. Testosterone treatment effects were evaluated for each motor column separately. Planned post hoc comparisons were evaluated using the Student Newman–Keuls test (SigmaStat for Windows v1.01, Jandel Scientific). Differences were considered statistically significant if P<0.05. All values are reported as means ± S.E.M.

RESULTS

Gonadally-intact male rats

As illustrated for the SNB, RDLN, dorsal lateral nuclei and the ventral motor pool of spinal cord segment L₅, all motoneurons in spinal cord segment levels L₄₋₆ₓ and C₁₋₅₅ express ChAT mRNA (Fig. 1). In addition, cell bodies in area X also express ChAT mRNA. These results are consistent with the results of previous in situ hybridization histochemical studies. However, previous immunohistochemical studies have shown that there are some strongly ChAT immunoreactive cell bodies in lamina III. The lack of detectable ChAT mRNA levels in cell bodies located in lamina III suggests that the anti-ChAT antibody used by Phelps and her colleagues may recognize an epitope that ChAT shares with other proteins. Alternately, the ChAT mRNA levels of lamina III neurons may be substantially lower than that observed among area X neurons and ventral horn motoneurons and be below the level of detectability by in situ hybridization.

In the four gonadally intact, TP-untreated male rats from Experiment I, motoneuronal ChAT mRNA levels were determined in an average of 47 SNB (range: 44–50), 41 RDLN (range: 32–48), 42 L₅₋₆₅ (range: 38–46) and 44 C₁₋₅₅ (range: 36–48) motoneurons/animal. ChAT labelling ratios, a measure of ChAT mRNA levels, of individual motoneurons in the SNB, RDLN, L₅₋₆₅ and C₁₋₅₅...
motor columns ranged from 2.21 to 15.76 (Fig. 2). Significant differences in the mean ChAT labelling ratio between motor columns were observed ($P<0.007$). The mean ChAT labelling ratio of RDLN motoneurons was greatest ($7.64 \pm 0.62$), followed in descending rank order by $C_{Lat}$ ($6.05 \pm 0.77$), SNB ($5.23 \pm 0.59$) and $L_{Lat}$ motoneurons ($4.35 \pm 0.21$). Post hoc comparisons indicated that the mean ChAT labelling ratio of RDLN motoneurons was significantly greater than that of $C_{Lat}$, SNB, and $L_{Lat}$ motoneurons ($P<0.05$) and, the mean ChAT mRNA level of $C_{Lat}$ motoneurons was significantly greater than that of $L_{Lat}$ motoneurons ($P<0.05$).

**Experiment I: Chronic elevation of plasma testosterone levels above physiologic levels**

After a 28 day exposure to exogenous testosterone the mean body weight of the 1-TP and 2-TP animals were 10% lower than that of the age-matched untreated control male group ($n=4$ rats/treatment group; $P<0.05$; Table 1). In the TP-treated animals, plasma testosterone levels were $5 \times (1$-TP) and $10 \times (2$-TP) greater than in the control group ($P<0.05$; Table 1). Elevation of plasma testosterone levels resulted in a significant loss of testes weight ($P<0.05$; Table 1) and a significant increase in seminal vesicle weight ($P<0.05$; Table 1). Exogenous testosterone treatment significantly increased BC/LA muscle complex weight over control values ($P<0.05$; Table 1); and had no effect on Sol or EDL muscle weight (Table 1). There were no significant differences in body, testes, seminal vesicle, or BC/LA muscle complex weights (Table 1) between the two groups of animals receiving exogenous testosterone treatment. Even though testosterone treatment induced a slight decrease in body weight, BC/LA muscle weight (normalized to body weight) increased by approximately 35%.

Chronic exposure to supraphysiologic levels of circulating testosterone increased the steady state levels of ChAT mRNA in cervical and lumbar motoneurons (Fig. 3). ChAT labelling ratios were determined on an average of 43 SNB (range: 29–50), 42 RDLN (range: 32–48), 42 $L_{Lat}$ (range: 36–48) and 45 $C_{Lat}$ (range: 36–51) motoneurons/animal. The mean motoneuron ChAT mRNA level (i.e. labelling ratio) was increased significantly by TP treatment in all four motor columns (SNB, $P<0.001$; RDLN, $P<0.02$; $L_{Lat}$, $P<0.001$; $C_{Lat}$, $P<0.004$; Fig. 4). For the SNB and $L_{Lat}$ motor columns, the increase in the mean ChAT labelling ratio induced by plasma testosterone levels was dose-dependent ($P<0.05$; Fig. 4). Chronic elevation of plasma testosterone levels to 5- and 10-times physiological also significantly increased the mean ChAT labelling ratios of RDLN and $C_{Lat}$ motoneurons (Fig. 4). However, the increase in ChAT mRNA levels observed in RDLN and $C_{Lat}$ motoneurons from animals whose plasma testosterone levels were 10-times physiologic level was not significantly greater than that of the animals whose plasma testosterone levels were 5-times physiologic (Fig. 4). The TP-induced increase in motoneuronal ChAT labelling ratios over those reported in the intact untreated group was greatest among $L_{Lat}$ motoneurons (1-TP, 188%; 2-TP, 254%), intermediate among SNB motoneurons (1-TP, 156%; 2-TP, 215%), and lowest among $C_{Lat}$ (1-TP, 159%; 2-TP, 180%) and RDLN motoneurons (1-TP, 153%; 2-TP, 179%).

**Experiment II: Chronic decrease of plasma testosterone levels below physiologic levels**

ChAT mRNA levels were measured on an average of 40 SNB (range: 18–59) and 62 RDLN (range: 23–84) motoneurons in five gonadally intact, five castrated, and four TP-treated castrated male rats. The mean ChAT labelling ratio of SNB and RDLN motoneurons in castrates was significantly lower than that of gonadally-intact males and of TP-treated castrates (Fig. 5; $P<0.05$). In addition, the mean ChAT labelling ratios of SNB and RDLN motoneurons in gonadally-intact male rats were greater (1.42 and 1.23 times greater) in Experiment II than in Experiment I. The difference in labelling ratio between the two experiments was potentially due to the difference in section thickness (Experiment I: 15 \(\mu m\); Experiment II: 20 \(\mu m\)) rather than to penetration of tissue by the cRNA probe during in situ
Table 1. Systemic effects of supraphysiological testosterone levels in gonadally-intact adult male rats after a 28 day testosterone propionate treatment regimen*

<table>
<thead>
<tr>
<th></th>
<th>INTACT</th>
<th>INTACT + 1-TP CAPSULES</th>
<th>INTACT + 2-TP CAPSULES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)*</td>
<td>422 ± 3^*</td>
<td>382 ± 14</td>
<td>385 ± 17</td>
</tr>
<tr>
<td>Plasma T levels (ng/ml)*</td>
<td>1.8 ± 0.2^*</td>
<td>10.1 ± 0.9</td>
<td>20.0 ± 1.4</td>
</tr>
<tr>
<td>Testes (g)*</td>
<td>3.30 ± 0.10^*</td>
<td>2.92 ± 0.09</td>
<td>2.85 ± 0.13</td>
</tr>
<tr>
<td>Seminal vesicles (g)*</td>
<td>1.21 ± 0.15^*</td>
<td>2.48 ± 0.42</td>
<td>2.64 ± 0.08</td>
</tr>
<tr>
<td>Muscles (muscle weight/body weight) × 10^{-4}</td>
<td>34.6 ± 2.0^*</td>
<td>46.3 ± 3.6</td>
<td>47.2 ± 2.8</td>
</tr>
<tr>
<td>BCLA (bilateral)</td>
<td>2.93 ± 0.38</td>
<td>2.78 ± 0.27</td>
<td>2.94 ± 0.39</td>
</tr>
<tr>
<td>Sol (bilateral)</td>
<td>4.36 ± 0.16</td>
<td>4.36 ± 0.35</td>
<td>5.42 ± 0.39</td>
</tr>
</tbody>
</table>

*Silastic capsules 45 mm in length with a 1.57 mm i.d. and 3.17 mm o.d. filled with TP were subcutaneously implanted in animals receiving TP treatment (five animals/treatment group. All values are given as the mean ± S.E.M.

**Significant differences between groups (ANOVA: P<0.02).

**Post hoc comparisons revealed that there were significant differences between the intact and 1-TP capsule groups and the intact and 2-TP capsule groups (P<0.05).

**Post hoc comparisons revealed that there were significant differences between the 1-TP capsule and 2-TP capsules treatment groups (P<0.05).

hybridization histochemistry since the tissue sections were treated identically.

**DISCUSSION**

The present study demonstrates that in the adult male rat, circulating testosterone levels modulate the steady state levels of ChAT mRNA in somatic motoneurons of the lumbar and cervical spinal cord. Chronic elevation of plasma testosterone levels for 28 days, to approximately 5- and 10-times physiological levels, in gonadally-intact adult male rats significantly increased motoneuron ChAT mRNA levels in the SNB, RDLN, L4-6, and C1-4 motor columns. Decreasing plasma testosterone levels below physiological levels significantly reduced ChAT mRNA levels in SNB and RDLN motoneurons. These observations suggest that the castration-induced decrease in ChAT activity of the levator ani muscle may be a consequence of the decrease in ChAT mRNA levels of SNB motoneurons. Conversely, the AAS-induced increase in ChAT mRNA levels of motoneurons may result in an increase in muscle ChAT activity levels. Castration does not eliminate the expression of ChAT in motoneurons, implying that the basal expression of ChAT by motoneurons is not dependent on circulating testosterone levels. Testosterone replacement therapy of castrated male rats maintained ChAT mRNA levels among SNB and RDLN motoneurons within the range observed among age-matched gonadally-intact males. Previous studies have shown that castration alters a-calcitonin gene-related peptide, cholecystokinin, connexin, a-tubulin and beta-actin mRNA levels in SNB motoneurons without a significant effect on motoneurons innervating muscles that are not sexually dimorphic. The results of the present study indicate that circulating AAS levels, including supraphysiological levels, can modulate the levels of specific mRNAs in somatic motoneurons regardless of whether they are, or are not, sexually dimorphic or innervate "androgen-sensitive" muscles in male rats.

Alterations of circulating testosterone levels in gonadectomized female rats have been shown to alter the cross-sectional area of RDLN motoneurons. In contrast, castration of adult male rats does not significantly alter the cross-sectional area of RDLN motoneurons. RDLN motoneurons innervate hindlimb muscle including the flexor digitorum longus (FDB). Interestingly, though the FDB muscle was significantly larger in adult male rats compared to female rats, muscle weight was unaffected by fluctuations in serum testosterone levels in either sex. These results also suggest that circulating testosterone levels can affect motoneuron properties without an apparent effect on skeletal muscle weight. The apparent lack of a direct effect of supraphysiological testosterone levels on EDL and Sol muscle weight, and the significant increase in BCLA muscle weight may be due to differences in their androgen binding capacity.

Biochemical analyses have shown that androgen binding levels of rat hindlimb skeletal muscles are 60-times lower than in the prostate; whereas those of the BCLA muscle complex are only seven times lower than in the prostate. These observations suggest that "androgen-responsive" neuromuscular systems such as the SNB-BCLA system represent one extreme where both the neuronal and muscular components are able to respond to physiologic plasma testosterone levels. Indeed, the very notion of "androgen sensitivity" needs to be re-examined. Previous studies have defined androgen sensitivity solely on the basis of the endpoints used to assess the responsiveness of a particular system. The results of the present study suggest that all neuromuscular systems are capable of responding to changes in plasma testosterone levels. Thus, the androgen sensitivity of a specific neuromuscular system may be dependent on direct and indirect androgen receptor-mediated regulation of gene expression.
initiated through intercellular and intracellular signalling pathways and the initial androgen receptor levels of each component.

Testosterone-induced modulation of motoneuron ChAT mRNA levels may be dependent on: intracellular events initiated by activation of the motoneuronal androgen receptors, testosterone-mediated changes in descending inputs, and/or on muscle-derived trophic factors. Using both steroid autoradiography and androgen receptor immunoreactivity, Breedlove and his colleagues have shown the majority of motoneurons in the fifth and sixth lumbar spinal cord segments accumulate testosterone (or dihydroxytestosterone) and express the androgen receptor.\textsuperscript{5,9,20} Expression of the ChAT gene in motoneurons may be directly modulated by testosterone, through activation of the androgen receptors, if ChAT gene enhancer regions contain hormone androgen response elements (HRE/AREs).\textsuperscript{5,8,58} The consensus HRE/ARE is recognized by androgen receptors, glucocorticoid and progesterone receptors.\textsuperscript{5,8,58} Computer-assisted analyses of the published sequence of one ChAT enhancer region (nucleotides 1260–1757) in the mouse\textsuperscript{49} (Gene Bank accession no.: DI2486; 4060 base pairs DNA) suggests that there are two potential half-palindrome HRE/AREs (HRE/ARE no. 1: nucleotides 1535–1549, GGGAggcaTGTTCT; and HRE/ARE no. 2: nucleotides 1596–1610, ccGgAggcTTcGTc). Various studies have shown that homology with the right-half of the HRE/ARE palindrome is sufficient for AR dependent transcriptional regulation.\textsuperscript{1,25,36,57,70} Whether there is a similar enhancer region in the rat ChAT gene is unknown. However,
Fig. 4. The effect of chronically elevating plasma testosterone levels to supraphysiologic levels using subcutaneously-implanted TP capsules on the mean ChAT mRNA levels of motoneurons in SNB, RDLN, L\textsubscript{Lat}, and C\textsubscript{Lat} motor columns in gonadally-intact male rats. The mean plasma testosterone levels among the untreated intact males, those with a single TP capsule (1-TP), and those with two TP capsules (2-TP) were 1.8, 10.1, and 20.0 ng/mL, respectively. *Post hoc comparisons revealed that there were statistically significant differences between: the gonadally-intact and intact+1-TP capsule treatment groups; and the gonadally-intact and the intact+2-TP capsules treatment groups (P<0.05). **Post hoc comparisons revealed that there were statistically significant differences between the intact+1-TP capsule and the intact+2-TP capsules treatment groups (P<0.05).

Fig. 5. Effect of chronic subphysiological plasma testosterone levels on the mean ChAT mRNA levels in SNB and RDLN motoneurons. *Post hoc comparisons revealed that there were statistically significant differences between: the castrate and gonadally-intact groups; and, the castrate and castrate+TP groups.

Kengaku et al.\textsuperscript{28} have strong evidence suggesting that the organization and sequence of the exons are highly conserved in the mouse and rat ChAT genes.

AAS regulation of motoneuronal ChAT mRNA levels may also involve the regulation of motoneuronal gene expression by a transsynaptic mechanism. Neurons in the lateral vestibular nucleus, which project to motoneurons through the vestibulospinal tracts\textsuperscript{18,63} may express AR mRNA.\textsuperscript{65} However, thoracolumbar spinal cord transection of neonatal ovariectomized female pups receiving testosterone treatment does not affect SNB motoneuron morphology.\textsuperscript{17} This result would suggest that supraspinal inputs may not play a role in AAS-induced motoneuron plasticity. Additional mechanisms include muscle–nerve interactions through trophic factors produced and released by skeletal muscle fibres. A number of studies have shown that AAS action on the BC/LA muscle complex is an important determinant of AAS-induced secondary effects on SNB motoneurons.\textsuperscript{39,30,56} In vitro studies have shown that the ChAT activity of many types of cholinergic neurons is modulated by factors released and synthesized by target tissues including muscle.\textsuperscript{22,37,41,60,67} The production of these muscle-derived factors are modulated by muscle activity.\textsuperscript{21,66} Whether the expression of any of these muscle-derived factors are regulated by circulating AAS levels has not been established. However, previous studies from this laboratory have shown that the castration-induced increase in α-calcitonin gene-related peptide mRNA levels of SNB motoneurons is dependent on the release of inactivity-induced muscle factors\textsuperscript{50,52} suggesting that, at least for the SNB motor column, the AAS-induced increase in ChAT mRNA levels may be indirectly dependent on AAS-induced alterations in muscle activity which in turn modulate the expression of muscle derived neurotrophic factors.

A potential consequence of the testosterone-induced modulation of ChAT mRNA levels would be to alter the characteristics of neurotransmission at the neuromuscular junction. A TP-induced increase in muscle ChAT activity resulting in increased acetylcholine levels available for release at the neuromuscular junction may result in a decrement of the relative contribution of neurotransmission failure to peripheral muscle fatigue. Neurotransmission failure can be due to a failure in axon action potential propagation, reduced endplate excitability of the muscle fibre, and/or diminished neurotransmitter release at the neuromuscular junction.\textsuperscript{12,61,64,67} In the rat diaphragm, muscle neurotransmission failure occurs in all muscle fibres.\textsuperscript{27} Johnson and Sieck have suggested that motor unit type-dependent differences in acetylcholine release. re-uptake and synthesis may be associated with the susceptibility of a motor unit to neurotransmission failure.\textsuperscript{27} Thus, TP treatment may decrease neurotransmission failure potentially resulting in an increase in the fatigue resistance of muscles containing a high proportion of fast-twitch fatigable motor units. Employing direct muscle stimulation, Eggington has shown that AAS treatment increases the fatigue resistance of the fast-twitch EDL muscle.\textsuperscript{15} Interestingly, AAS treatment did not affect EDL muscle weight.

In the rat, castration causes a decrease in LA muscle ChAT activity as well as a decrease in ChAT
mRNA levels of SNB motoneurons. In addition, the ChAT activity of the LA muscle in castrated male rats receiving TP replacement therapy was similar to that observed in gonadally-intact males. Similar results, have been reported for the "androgen-sensitive" syringal muscles of male zebra finches. However, the ChAT activity of syringeal muscles in castrated male zebra finches receiving pharmacological TP dose for one week was unchanged from that observed in castrates. These data suggest that the increase in motoneuron ChAT mRNA levels induced by chronic TP treatment of gonadally-intact males may precede TP-induced increases in the ChAT activity of muscles like the rat LA and the zebra finch syringeal musculature that accumulate androgens at much greater levels than non-sexually dimorphic muscles. Indeed, alterations in plasma testosterone levels did not affect the ChAT activity of two zebra finch hyoid muscles, the geniohyoid and ceratoglossus muscles with low androgen binding capacities. Interestingly, Arnold et al. have shown that in the zebra finch the motoneurons innervating the syringeal muscles accumulate androgens and those innervating the hyoid muscles do not accumulate androgens. This would suggest that the TP-induced increase in motoneuron ChAT mRNA levels innervating rat hindlimb muscles, where the androgen binding capacity of the muscle is low, may not result in a TP-induced change in muscle ChAT activity.

Tucek et al. have previously shown that castration of immature male rats causes a diminution in the weight and ChAT activity of the Sol and LA muscles. Indeed, subsequent testosterone treatment of males castrated at one month of age for five weeks significantly increases ChAT muscle activity and muscle weight of both muscles. In contrast, testosterone treatment of mature (seven weeks of age) gonadally-intact male rats caused a progressive loss of muscle weight and ChAT activity in the Sol and EDL muscles; whereas, testosterone treatment of gonadally-intact male rats was associated with an increase in LA muscle weight without an apparent effect on muscle ChAT activity. These data would suggest that there may be a dissociation between motoneuron ChAT mRNA levels and muscle ChAT activity in TP-treated gonadally-intact adult male rats. Experiments are currently underway to determine if supraphysiological TP treatment alters the ChAT activity of the motor nerve and muscle, as well as contribution of neurotransmission failure to muscle fatigue resistance, in specific rat hindlimb muscles.

CONCLUSIONS

In summary, motoneuronal ChAT mRNA levels varied significantly between different motor columns of the cervical and lumbar spinal cord in adult male rats. Motoneuron ChAT mRNA levels in cervical and lumbar motor columns are dramatically modulated by circulating testosterone levels regardless of the purported "androgen sensitivity" of the muscles that they innervate. These data suggest that the cellular properties of the motoneuronal component of the neuromuscular system can be modulated by circulating AAS levels. Furthermore, the effects of AAS on athletic performance and muscle strength in males may involve changes in both components of the neuromuscular system.

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