MODIFICATION OF SYNAPSES IN ANDROGEN-SENSITIVE MUSCLE

I. Hormonal Regulation of Acetylcholine Receptor Number in the Songbird Syrinx¹

WILLIAM BLEISCH,² VICTORIA N. LUINE, AND FERNANDO NOTTEBOHM

Rockefeller University, New York, New York 10021

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Abstract

The songbird syrinx is sexually dimorphic and responds to changes in blood testosterone levels with changes in muscle size and in activity of cholinergic enzymes (Luine, V., F. Nottebohm, C. Harding, and B. S. McEwen (1980) *Brain Res.* 192: 89-107). Here, we demonstrate that there is a sex difference in the number of acetylcholine receptors (AChRs) in the syrinx and that alterations in the levels of circulating testosterone can cause changes in AChR number in syringeal muscles. These results suggest that the size or number of endplates in the syringeal muscles may be increased by circulating testosterone. The time course of these effects was examined after increases in blood testosterone in females and decreases in males. We also examined the effect of increased testosterone in syrinx isolated from neural influences by denervation and found that denervated muscle responded to testosterone with an increase in acetylcholinesterase activity but not with increases in protein content or AChR number.

The muscles of the songbird syrinx control frequency modulation during song. In species such as the canary, zebra finch, and European blackbird, in which only males sing, the muscles of the syrinx are strikingly larger in males than in females (Häcker, 1900; Arnold, 1974). This size difference is highly sensitive to fluctuations in levels of circulating testosterone. In adult zebra finches and canaries castration causes a decrease in the size of the syrinx in males, while testosterone treatment causes an increase in the size of the syrinx in females (Arnold, 1974; Luine et al., 1980). In the zebra finch, *Poephila guttata*, the muscles of the syrinx contain high levels of putative androgen receptors, comparable to levels present in classical androgen-sensitive tissues (Lieberburg and Nottebohm, 1979). The motoneurons which innervate these muscles also concentrate androgen (Arnold et al., 1976; Arnold and Saltiel, 1979). Thus, neuromuscular junctions in the syrinx are likely to be under regulation

by androgens. Consistent with this expectation, castration of adult male zebra finches is followed by a decrease in the specific activities of two enzymes involved in cholinergic transmission, acetylcholinesterase (AChE) and choline acetyltransferase (CAT), in both the syrinx and the nerve that innervates the syringeal muscles. Testosterone treatment of castrates prevents these decreases in activities, suggesting that the effect of castration is mediated by the decrease in circulating androgens (Luine et al., 1980).

We reasoned that the hormone-dependent changes in the levels of synaptic components might come about in either of two ways: (1) as an increase in the activity of cholinergic enzymes without a change in the number of acetylcholine receptors (AChRs), such as might occur in response to an increase in the amount of song-related neuromuscular transmission, or (2) as an increase in the size or number of neuromuscular synapses in the muscles, with an increase in total AChR number. To determine which of these alternatives is correct, we measured AChR number under conditions of hormonal change, using an assay for AChRs which is sensitive enough to measure accurately the low numbers of AChRs in a single syrinx. Then we further characterized the response of the syringeal muscles to changing hormone levels.

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² To whom correspondence should be addressed.

Materials and Methods

Materials. Zebra finches were obtained from Canary Bird Farms, Old Bridge, NJ. All birds were in full adult plumage and good health. Birds were castrated and implanted with hormone-filled Silastics as previously described (Luine et al., 1980; Bleisch et al., 1983). Syrinxes were denervated by removing a 1-cm portion of the tracheosyringealis nerve below the point of its branching from the main XIIth nerve. Purified AChR from *Torpedo* electroplaques was the generous gift of Dr. David Anderson, Rockefeller University. α -Bungarotoxin was obtained from Miami Serpentarium or from Boehringer-Mannheim. Monoiodinated [125 I]- α -bungarotoxin was prepared by iodination or was purchased from New England Nuclear (Boston, MA) and further purified as previously described (Bleisch et al., 1982).

Assays with muscle extracts. Birds were sacrificed by decapitation and syrinxes were removed as previously described (Bleisch et al., 1983).

For preparation of muscle extracts, tissues were weighed on ice, rinsed in ice-cold phosphate-buffered saline, and homogenized in 20 vol of 1.5% Triton X-100, 50 mM sodium phosphate, pH 7.2, on ice in a 2-ml ground glass-glass homogenizer (VWR Scientific, Philadelphia, PA). Homogenates were stored on ice until dissections were completed and an aliquot was then removed for determination of AChE activity. The remainder was then incubated at 30°C for 1 hr and centrifuged for 1 hr at 4°C in a Beckman 50 Ti rotor at 37,000 rpm or for 10 min at room temperature in a Dynac II clinical centrifuge with no. 271 rotor (Clay Adams) at 2,500 rpm. Pellets were discarded and supernatants were frozen over liquid nitrogen and stored at -40°C until assayed for toxin binding.

AChE activity was measured by a radiometric assay using [3 H]acetylcholine in the presence of an inhibitor of nonspecific cholinesterase as previously described (Luine et al., 1980). AChR number was measured as binding of [125 I]- α -bungarotoxin with a cation-exchange minicolumn assay (Kohanski et al., 1977; Bleisch et al., 1982). Briefly, Triton extracts of muscles were incubated with radiolabeled toxin for 3 hr, and then unbound toxin

was removed with carboxymethyl cellulose. Nonspecific binding was measured in parallel incubations in the presence of 0.6 to 1.0 mM gallamine. In all experiments except the binding curve, minicolumns were prewashed with 0.8 ml of 1.0% BSA before application of samples. This prewash increases the yield of AChRs from the columns to 79 to 88%. These estimates of yield were used in calculations of AChR number. Protein was determined by standard methods (Lowry et al., 1951) using BSA standards. Statistical tests were performed according to planned comparisons using the Student's *t* test, corrected for unequal variances when required, or by two-level analysis of variance (Winer, 1971). Unless noted, all variances are for interanimal variability.

Results

Sex difference in AChR number

The first experiment examined sex differences in the syrinx and revealed differences in the binding of α -bungarotoxin as well as in the weight and protein content of the syrinx. As in previous studies, there was a 2-fold difference in the weight and a 3-fold difference in the Triton-extractable protein content of the syrinxes of males and females (Luine et al., 1980; Fig. 1, A and B). In addition, extracts of male syrinxes bound 3.8 times as much α -bungarotoxin as extracts of female syrinxes (Fig. 1C). Expressed relative to total extractable protein, males had 24% more AChR than females (771 ± 61 versus 623 ± 29 fmol/mg, $p < 0.05$).

Further experiments were done to determine whether the sex difference in toxin binding to syringeal extracts was the result of a difference in the number of toxin-binding sites, or a difference in the kinetics of interaction of the toxin with receptors in male and female extracts. Extracts of male and female syrinxes were diluted to equal concentrations of binding sites. Binding curves were obtained for the two diluted extracts by incubating them with various concentrations of toxin and measuring bound toxin as described. Male and female binding curves are essentially superimposable (Fig. 2), indicating that there are no differences in the kinetics of toxin

Figure 1. Bar graphs demonstrating the sex difference in the weight (A), protein content (B), and AChR number (C) of the syrinx of the zebra finch. SEM of 10 males and 10 females are demonstrated by error bars.

Figure 2. Non-equilibrium binding of $[^{125}\text{I}]\text{-}\alpha\text{-bungarotoxin}$ ($[^{125}\text{I}]\text{-}\alpha\text{-BGTx}$) to AChRs from male and female zebra finches. Extracts of several syrinxes from males and females were separately pooled and diluted to 390 pM toxin-binding sites. These pooled extracts were then incubated at a 1/10 dilution with various concentrations of $[^{125}\text{I}]\text{-}\alpha\text{-bungarotoxin}$ in the presence (lower curve) or absence (upper curve) of 112 μM gallamine. After 3 hr, bound and free toxin were separated as described under "Materials and Methods" and bound toxin was counted and converted to concentration of bound toxin. Note that the same concentration of toxin-binding sites was included in all incubations. Error bars represent SEM for three assays for each condition.

Figure 3. The effect of testosterone treatment on the protein (A), AChR number (B), and AChE activity (C) in female zebra finches. Females received Silastic implants of testosterone (open bars) or cholesterol (stippled bars) as a control. Nine days later they were sacrificed and the syrinxes were divided into a muscle fraction (above, open and stippled bars) and a connective tissue fraction (below, solid bars), and each fraction was assayed for protein, AChE, and AChR as described under "Materials and Methods." SEM of five cholesterol- and four testosterone-treated birds are shown by error bars. Muscle fraction protein ($t = 6.73$, $df = 4.2$, $p < 0.005$), AChR ($t = 4.39$, $df = 3.6$, $p < 0.02$) and AChE ($t = 5.29$, $df = 3.3$, $p < 0.02$) differ significantly in the two groups.

interaction with male and female receptors. We conclude that the differences in toxin binding reflect differences in the number of toxin-binding sites; i.e., AChR number. In other experiments, syrinx muscle extracts were found to have no effect on the binding of $\alpha\text{-bungarotoxin}$ to purified AChRs from *Torpedo* electroplaques, suggesting that syrinx extracts do not contain endogenous inhibitors

of toxin binding which might affect measurement of AChR number (data not shown).

Specificity of testosterone effect

No effect of testosterone was observed on the larynx of females after 9 days of testosterone treatment (data not shown). This result and the lack of a castration effect

on muscles of the hyoid complex (Luine et al., 1980) indicate that the syringeal muscles are specialized in their extreme responsiveness to testosterone.

In the same experiment, the effect of testosterone on syringeal muscle was compared with the effect on syringeal connective tissue. The muscle was dissected away from underlying cartilaginous rings, and the muscle fraction and cartilage fraction were assayed separately for protein, AChE, and AChR. Much of the cartilage fraction was insoluble in the extraction buffer used here. The soluble portion of the cartilage fraction contained little AChR (3.4% of total for control females) or AChE (9.9% of total for control females), suggesting that this fraction contained little contaminating muscle (Fig. 3, B and C). Testosterone treatment for 9 days did not affect the cartilage fraction protein content although a significant 2.3-fold increase of muscle fraction protein was observed. Treatment of females with testosterone increased total AChR number 2.1-fold and AChE activity 5.0-fold (Fig. 3). When expressed relative to muscle fraction protein, AChE activity per protein was significantly elevated by 2.2-fold (11.17 ± 1.69 versus 5.19 ± 0.80 $\mu\text{mol/hr/mg}$, $p < 0.05$). In contrast, the increase in AChR number was equivalent to the increase in muscle fraction protein (2.74 ± 0.15 versus 3.04 ± 0.19 nmol/mg in controls).

Time course of hormone effects

In an effort to elucidate more about the mechanism of testosterone effects on AChR number, we examined the time course of changes in AChR number after changes in circulating androgen levels. In the first series of experiments, females were given testosterone implants at various times before sacrifice. Protein and AChR number increased more than 2-fold within 5 days of testosterone implantation, while total AChE increased more than 5-fold in the same time period (Fig. 4). One day after females were implanted with testosterone, total syringeal protein and AChR number were significantly increased over control levels ($p < 0.05$ for both). In contrast, AChE apparently was slower to respond to testosterone treatment, although the eventual increase was larger. AChE activity was not different from that of controls until 3 days after implantation, when both total and specific AChE increased significantly over control levels ($p < 0.01$ for total activity, $p < 0.05$ for specific activity).

Whereas administration of testosterone to females resulted in relatively rapid changes, changes in syringeal protein, AChR, and AChE occurred slowly after castration of males. Male zebra finches were castrated at 59, 23, and 11 days before sacrifice and then were sacrificed simultaneously along with intact birds ("0 days of castration") to determine the time course of the effect of castration. The loss of syringeal protein was quite gradual, continuing until well after the 11th day after castration (Fig. 5A). If the loss of protein is modeled by a first-order exponential loss (Berlin and Schimke, 1965), the apparent half-time of loss is 6.6 days, such that about 50% of the protein loss has occurred by 6.6 days after castration, and 75% has occurred by 13 days. AChE activity and AChR number decreased even more slowly than total protein (Fig. 5B), and this is reflected in the

Figure 4. Effect of testosterone on the protein (A), AChR number (B), and total AChE activity (C) in the syrinxes of female zebra finches. Females received testosterone in the form of Silastic implants, as described under "Materials and Methods." In each experiment, birds were implanted sequentially and sacrificed simultaneously. Assays are described under "Materials and Methods." The curve was fitted by eye. Each point represents means and SEM for three to four birds. Data are presented from three experiments, and each experiment is represented by a different symbol. Protein and AChR number are significantly elevated after 1 day of treatment ($p < 0.05$, by planned comparison tests; Winer, 1971, pp. 41–44). AChE activity is significantly elevated after 3 days of treatment ($p < 0.01$, relative to untreated controls).

apparent half-times for their loss: 15.8 days for AChE and 12.5 days for AChR.

Effect of testosterone on denervated muscle

In order to explore the role of the nerve in the response of syringeal muscle to testosterone, female zebra finches were implanted with testosterone or cholesterol and their syrinxes were simultaneously denervated. No significant effect of testosterone was seen on protein content or AChR in denervated syringeal muscle (Table I). However, AChE activity did increase consistently, and this

Figure 5. Effect of castration on protein (A) and on AChR number and AChE activity (B) in the syrinxes of male zebra finches. In each experiment, males were castrated sequentially and sacrificed simultaneously. The "0" day males were left intact. Assays are described under "Materials and Methods." Each point represents means and SEM for six males from two experiments.

increase was significant when two experiments were considered together.

Discussion

The results presented here indicate that the activity of AChE and the number of AChRs in the syrinx of the zebra finch both decrease following castration of males and increase following testosterone treatment of females. The effects of testosterone on the syrinx include an increase in the specific activity of the presynaptic enzyme, CAT (Luine et al., 1980), an increase in the specific activity of the innervation-sensitive "endplate" form of

AChE (Bleisch et al., 1983), and an increase in the total number of AChRs, a muscle membrane protein which is highly concentrated at the neuromuscular junction (Fertuck and Salpeter, 1976). These effects are all specific to the syringeal muscles and do not occur in other muscles. Together, these results suggest that testosterone may have a general effect on either the size or the number of synapses in the syrinx.

The effect on syringeal AChE apparently is specific relative to the general effect on muscle protein, since, when females are treated with testosterone, the activity of AChE increases more than muscle protein. In contrast,

TABLE I

Effects of testosterone on denervated syrinx in female zebra finches

Females received Silastic implants of testosterone or cholesterol, and their syringeal muscles were simultaneously denervated by sectioning the tracheosyringealis nerve. Birds were denervated and received implants either 5 days or 12 days before sacrifice. Entries are means $\pm$ SE, with sample sizes below in parentheses. All entries are the combined results of two experiments, except for the AChE results. Two-way analysis of variance revealed that the total AChE activity is significantly greater in testosterone-treated birds than in cholesterol-treated controls (for the two experiments combined, $F(1/16) = 4.81$, $df = 16$, $p < 0.05$; Winer, 1971). Total AChR and AChR per protein are significantly greater 12 days after denervation than after 5 days ($p < 0.05$ for comparisons within cholesterol and within testosterone groups for both measures). No other effects are significant by two-way analysis of variance.

	5 Days after Treatment		12 Days after Treatment	
	Cholesterol Control	Testosterone Treated	Cholesterol Control	Testosterone Treated
Protein (μ g)	555 $\pm$ 28 (6)	579 $\pm$ 15 (7)	498 $\pm$ 29 (9)	547 $\pm$ 24 (8)
Total AChR (nmol)	0.87 $\pm$ 0.17 (6)	1.18 $\pm$ 0.13 (7)	2.70 $\pm$ 0.84 (9)	2.12 $\pm$ 0.43 (7)
AChR/Protein (nmol/mg)	1.55 $\pm$ 0.26 (6)	2.06 $\pm$ 0.23 (7)	5.13 $\pm$ 0.12 (9)	3.62 $\pm$ 0.59 (7)
Total AChE (μ mol/hr)	3.99 $\pm$ 0.93 (4)	5.94 $\pm$ 0.96 (4)	5.38 $\pm$ 0.72 (6)	7.04 $\pm$ 0.71 (6)
AChE/Protein (μ mol/hr/mg)	9.60 $\pm$ 1.52 (4)	12.18 $\pm$ 2.15 (4)	10.74 $\pm$ 0.89 (6)	11.87 $\pm$ 0.92 (5)

AChR number increases proportionately to the general increase in muscle protein.

The testosterone sensitivity of synaptic components might result from actions of testosterone at several levels. Testosterone, acting on higher brain centers involved in song control, may increase the amount of use of the syrinx. It seems unlikely that increased use is entirely responsible for the effects of testosterone, since females treated with testosterone exhibit increased AChE activity and AChR number, even though they do not sing (Arnold, 1975). In addition, increases in muscle activity generally cause decreases in AChR number rather than an increase as observed (Fambrough, 1979). Thus, testosterone may affect the levels of synaptic components by primary actions on the motoneurons and muscle cells of this system. Presumably, any such actions are mediated by the androgen receptors which these cells contain.

On a cellular level, the effect of changing testosterone levels on AChR number and AChE and CAT activity raises several questions about the pre- and postsynaptic events which occur during synaptic modification. All three of these synaptic components decrease after castration and increase after testosterone treatment, but the mechanisms responsible for these effects must be different. Whereas neuromuscular AChRs occur only on the muscle membrane (Jones and Salpeter, 1983), CAT is normally located only in the presynaptic terminal. AChE at the neuromuscular junction is probably derived from both the presynaptic nerve and the postsynaptic muscle cell. This raises the question of the extent to which the effect of testosterone on AChE activity might be mediated by actions on the motoneurons. After denervation, the syringeal muscles are still capable of responding to testosterone with an increase in the activity of AChE, suggesting that the syrinx itself may be directly responsible for at least some of this increase.

In contrast to the increase in syringeal AChE in denervated syrinx treated with testosterone, no increase was seen in syringeal weight or protein. This result

resembles results obtained with the androgen-sensitive levator ani muscle of the rat (Burešová et al., 1972). Innervation may have a permissive effect on the expression of the testosterone sensitivity of general protein synthesis in both of these muscles. However, testosterone apparently has a specific effect on the synthesis or degradation of AChE in denervated syrinx.

No significant increase in AChR number was observed after testosterone treatment in denervated syrinx, but this observation must be interpreted carefully. Innervation may be necessary for the testosterone sensitivity of junctional AChR number. However, after denervation, the number of AChRs on muscle fibers increases dramatically as a result of synthesis of new extrajunctional AChRs (Brookes and Hall, 1975; Devreotes and Fambrough, 1976), which differ from existing junctional receptors in several respects (see Fambrough, 1979, for a review). The increase in syringeal AChRs from 5 to 12 days after denervation presumably reflects this phenomenon. Testosterone might affect the synthesis of these two populations of AChRs differently. If only junctional AChRs increase in response to testosterone, this would be difficult to detect in denervated muscle because of the large excess of extrajunctional receptors.

The mechanism of the effect of testosterone on AChRs in the syrinx can be analyzed in more detail by examination of the time course of the effects of androgen withdrawal and testosterone increase. If the amount of a protein decreases after removal of a hormonal stimulus as a result of a decrease in the rate of synthesis of that protein, with no change in its rate of degradation, then that protein's concentration will decrease exponentially with a rate equal to its rate of degradation (Berlin and Schimke, 1965). Applying this model to the syrinx, castration causes a decrease in syringeal AChR such that the apparent exponential degradation rate is 5.5% per day. This rate is only slightly less than the steady-state degradation rates reported for junctional AChRs in a variety of other muscles (Fambrough, 1979; Linden and Fambrough, 1979; Levitt et al., 1980). This suggests that

AChR loss may be the result of a rapid decrease in AChR synthesis, after which existing AChRs turn over at the usual rate, until a new steady-state level is achieved. This would be consistent with the classical model of receptor-mediated effects of steroid hormones; that is, that they cause an increase in the rate of synthesis of target proteins (see Berlin and Schimke, 1965).

The alterations in the amounts of synaptic proteins in syringeal muscles after changes in blood testosterone levels might be the result of several different changes at the morphological level. The amount of AChR, AChE, and CAT at each synapse may change, either as a result of an increase in the size of the terminal or as a result of a change in the concentration and density of these components. The amount of AChE, CAT, and AChR in extrajunctional regions may change. In addition, the number of synapses in the muscle may also change, either as a result of an alteration in the number of endings on each muscle fiber or as a secondary result of a change in the number of muscle fibers. However, there is little or no difference in the number of muscle fibers in the syrinx of males and females, despite large differences in testosterone levels (Bleisch, 1982; W. V. Bleisch and F. Nottebohm, manuscript in preparation). This implies that testosterone has little or no effect on muscle fiber number, and it follows that testosterone must increase the AChE, CAT, and AChR number per muscle fiber. In another androgen-sensitive muscle, the "levator ani" of the rat, castration causes decreases in AChE activity and AChR number, and these changes correspond to changes at individual neuromuscular junctions (Tucek et al., 1976; Bleisch, 1982; Bleisch et al., 1982).

Complexities attending the interpretation of enzyme and receptor changes induced by hormones at the neuromuscular junction might be usefully considered when interpreting similar changes in the brain. The processes illustrated here may be general to many synapses and may provide a useful model of hormonally regulated synaptic plasticity.

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