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Neuromuscular Junctions in Androgen Sensitive Muscles: A Model for Synaptic Plasticity

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NEUROMUSCULAR JUNCTIONS IN ANDROGEN SENSITIVE MUSCLES: A MODEL FOR SYNAPTIC PLASTICITY

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by

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ABSTRACT

Androgen-sensitive muscles are specialized muscles in which both muscle and motoneurons are highly sensitive to androgens. Previous studies have shown that alterations in the levels of circulating androgens affect the activities of cholinergic enzymes in two androgen-sensitive muscles; the syringeal muscles of songbirds and the levator ani muscle of the rat. This study sought to characterize the nature of the effects of androgens on the neuromuscular junctions in these muscles.

I measured the relative abundance of the various molecular forms of acetylcholinesterase (AChE) in muscles of the zebra finch. Castration causes a large decrease in AChE activity, but has little or no effect on the relative abundance of AChE forms. This is in marked contrast to the effect of denervation, which causes changes in the relative abundance of the forms of AChE, as well as in total activity.

To quantify the postsynaptic effects of androgens, I measured acetylcholine receptor number (AChRs). AChR number was androgen-sensitive in both the syrinx and levator ani, decreasing after castration of males, and increasing in response to treatment with testosterone. I performed a detailed examination of the time course of the effect of alterations in androgens was examined in detail in the syrinx. I also examined the effect of increased androgens in syrinx isolated from neural influences by denervation, and found that isolated muscle could respond with a small increase in AChE activity.

Since the number of fibers in the levator ani does not change after

castration or testosterone treatment, and each fiber has a single endplate, changes in AChR number in this muscle are strong evidence for effects of androgens on endplates. In the syrinx, no evidence of sex differences in the number of muscle fibers was seen. However, some fibers have multiple endplates. Therefore, changing hormone levels may cause changes in the number of endplates per fiber, as well as in the number of AChRs per endplate. An examination of individual endplates in these muscles visualized histochemically indicates that androgens exert at least part of their effects on synaptic components by causing alterations at individual endplates. Androgen-sensitive neuromuscular junctions such as these may provide powerful systems in which to study the mechanism and adaptive significance of synaptic plasticity in vertebrates.

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TABLE OF CONTENTS

1. CHAPTER I: REVIEW

1.1. INTRODUCTION

The concept of plasticity of behavior and its relationship to plasticity in the brain of adult animals has been a central issue for investigators with diverse approaches to neurobiology. One popular hypothesis has been that behavioral plasticity involves modifications in the function or number of synapses. In the vertebrate brain, studies of synaptic plasticity have been hampered by the complexity of the vertebrate nervous system. This obstacle has led many investigators to turn to simpler systems in which to study synaptic plasticity.

Simple invertebrate nervous systems have provided one extremely productive approach to the study of the relationship between synaptic and behavioral plasticity. There is good evidence that short-term synaptic plasticity is the basis of behavioral plasticity in at least one well-understood invertebrate system; that of habituation and sensitization of the gill withdrawal reflex in Aplysia, a sea slug. In this system, the mechanism of short-term synaptic plasticity has been carefully studied, and a complete explanation at the level of cellular processes may indeed have been formulated (1; 2; 3).

In vertebrates, the most intensively studied example of long term neuronal plasticity in the adult may be the phenomenon of long term potentiation (LTP) in the hippocampus (see 4 for review). LTP may involve increases in postsynaptic neurotransmitter receptor sites, changes in presynaptic release of transmitter and morphological changes at synapses (5). Unfortunately, the physiological significance of LTP

remains unclear. Other examples of long-term synaptic plasticity measured by morphological or physiological criteria include responses to destruction of presynaptic elements (6; 7; 8), neurotoxins (9) pharmacological manipulations (10) and the like. The possibility that these responses are pathological rather than adaptive raises some doubt about whether these systems will prove to be appropriate models of synaptic plasticity in physiological contexts.

In vertebrates, perhaps the most promising systems in which to study synaptic plasticity are neuronal pathways which respond to hormones. In these systems, the environmental variable which regulates synaptic modification is the level of circulating hormones, a parameter which is manipulated relatively easily by removing the source of the hormone and treating with exogenous hormones. Another advantage of studying the effects of steroid hormones on the nervous system is that much is already known about the mechanism of steroid action in other systems (11; 12; 13). In these systems, steroid hormones enter the target cell where they bind to specific protein receptors. Complexes of receptor and steroid hormone then migrate to the cell nucleus, where they interact with the chromatin to cause the synthesis of specific mRNAs. The increase in the amounts of these mRNAs causes increases in the synthesis of specific cell proteins. Other mechanisms for steroid hormone action have been identified (14; 15) and it is possible that they are involved in the systems to be considered here. To date, no conclusive evidence for primary effects of gonadal steroids on neurons exists (15). However, steroid hormone action on the nervous system may be as simple as described above.

The profound behavioral effects of sex steroids has provided a powerful system in which to study the biochemical and physiological basis of behavioral plasticity. For example, sex steroids are accumulated by cells in regions of the brain which are involved in control of sexual behavior in several species (16). Estrogen and progesterone together increase sexual receptivity in female rats. Estrogen has effects on the activity of enzymes involved in neurotransmitter synthesis and degradation, as well as neurotransmitter receptor number (17; 15; 18). In the canary, androgens induce birds to sing, and increase the volume of song control nuclei (19; 20) as well as the length of dendrites in these nuclei (21). In these examples, study of effects of steroid hormones in the brain have led to discoveries of changes in the number or function of synapses which may be responsible for behavioral effects.

Despite progress made in elucidating the basis of hormone-induced behavioral plasticity, there are numerous obstacles to using these systems to study the mechanism of synaptic plasticity at a cellular level. In particular, the heterogeneity and complexity of the brain makes it difficult to study one system in isolation. In addition, the primitive level of understanding of the neurochemistry and cell biology of synaptic transmission at most synapses has hindered study of synaptic modification, especially in response to manipulations which remain within the physiological range. For these reasons, I chose to study the effect of a steroid hormone, testosterone, on the best characterized vertebrate synapse; the neuromuscular junction.

Although the postsynaptic cell at the neuromuscular junction is a

muscle fiber and not a neuron, the junction is none the less a synapse, equipped with all the machinery of any other chemical synapse. The junction is also relatively easy to study because of the homogeneity and isolation of the nerve-muscle connection, and because of the fortuitous availability of a powerful arsenal of reagents and techniques which allow manipulation and study of components of junctional machinery. Because of these advantages, the neuromuscular junction has been under intensive investigation for several decades. The enzymes responsible for synthesis and degradation of the neurotransmitter, acetylcholine, have been isolated and characterized in functional form, as has the receptor for the transmitter; the nicotinic acetylcholine receptor (22). The physiology of transmission is also well-characterized. In fact, the neuromuscular junction is the source of most of our knowledge about the mechanisms of synaptic information transfer (23).

Because of the powerful techniques available for studying the junction, and since so much is known about synaptic transmission at the neuromuscular junction, we might expect that the neuromuscular junction would provide a powerful model for studying synaptic plasticity. Synaptic plasticity, as suggested above, may be the key to the cellular basis of behavioral plasticity. With this motivation, I chose to study the effects of androgens on androgen-sensitive neuromuscular junctions.

We would expect to find androgen-sensitive neuromuscular junctions in androgen-sensitive muscles. Most mammalian muscles are sensitive to androgens to some degree; a fact reflected in the larger muscle mass of males relative to females (24). Here we will be considering muscles in which the androgen-sensitivity is profound; in which the muscle is more

than twice as large in males than in females. There are several examples of androgen-sensitive muscles in vertebrates. Among these are the dewlap muscles of some lizards, the muscles of the frog larynx, and the two muscles which are the subject of this dissertation, the muscles of the syrinx of songbirds and the penile muscles of the rat. All of these muscles are involved in sexually dimorphic behaviors; courtship displays in lizards, mate-calling in frogs, singing in songbirds and copulation in the rat.

1.2. THE LEVATOR ANI

The mis-named "levator ani" of the rat (m. dorsalis bulbocavernosus (25), m. sphincter ani (26)) exists only in males. It is a perineal muscle which attaches to the urethral bulb and may be involved in penile motility. The marked androgen-sensitivity of the levator ani was first recognized in 1941 by Wainman and Shiponoff (27) who noted that this muscle dwindled following castration. The effect of treatment with androgen derivatives after castration soon became part of a standard test for the myotrophic effects of steroids (28), although this test has been criticized by those who believe that the response of the levator ani is not generalizable to other muscles (29). Effects of alterations of androgen levels are assumed to be due to changes at the level of single muscle fibers, since the number of fibers in the levator ani of the mouse and rat remain constant after castration and T treatment (30; 31).

The levator ani contains high affinity binding sites for androgens, with affinity and specificity similar to androgen receptors from other androgen-sensitive tissues (32; 33; 34). These receptors are present in concentrations variously estimated as 4 to 15 fmoles/mg of protein, much

higher than levels found in other rat skeletal muscles, (1 to 3 fmoles/mg; 35; 36). The levator ani may also contain receptors for estrogen (34; 37; 38). It has recently been shown that the motoneurons which innervate the levator ani and other penile muscles accumulate androgens, but not estrogen (39).

The laboratory of Ernest Gutmann pioneered the use of androgen-sensitive muscles for the study of neuromuscular interaction. Initially, this group suggested that the response of the levator ani to castration might serve as a useful model for studying changes in neuromuscular transmission during "disuse" atrophy. Other models of disuse, such as studies on the effects of denervation, had been criticized on the basis that these manipulations interfere with neural function (40). The assumption was made that castration resulted in a cessation of neuromuscular activity, and that muscle atrophy was the result of disuse (41; 42). Later, Gutmann and coworkers accepted the alternative hypothesis that testosterone had direct effects on the levator ani muscle, but they continued to work on the levator ani, examining the effects of castration and testosterone replacement with a detailed study of levels of acetylcholinesterase (AChE) and choline-acetyltransferase (CAT) (43).

Gutmann's group demonstrated that alterations in androgen levels could have profound effects on the levels of cholinergic enzymes in the levator ani. When rats were castrated at one month of age and sacrificed after 2 months, AChE had decreased by 60% from levels of intact controls (41). Later work demonstrated that treatment by injections of testosterone propionate could partially reverse this effect (43).

Choline-acetyltransferase was also apparently sensitive to alterations in androgen levels. Unfortunately, these CAT measurements were almost certainly inaccurate as a result of contamination with carnitine acetyltransferase activity (44). Therefore, effects of testosterone on neuromuscular junctions in the LA was never demonstrated unequivocally.

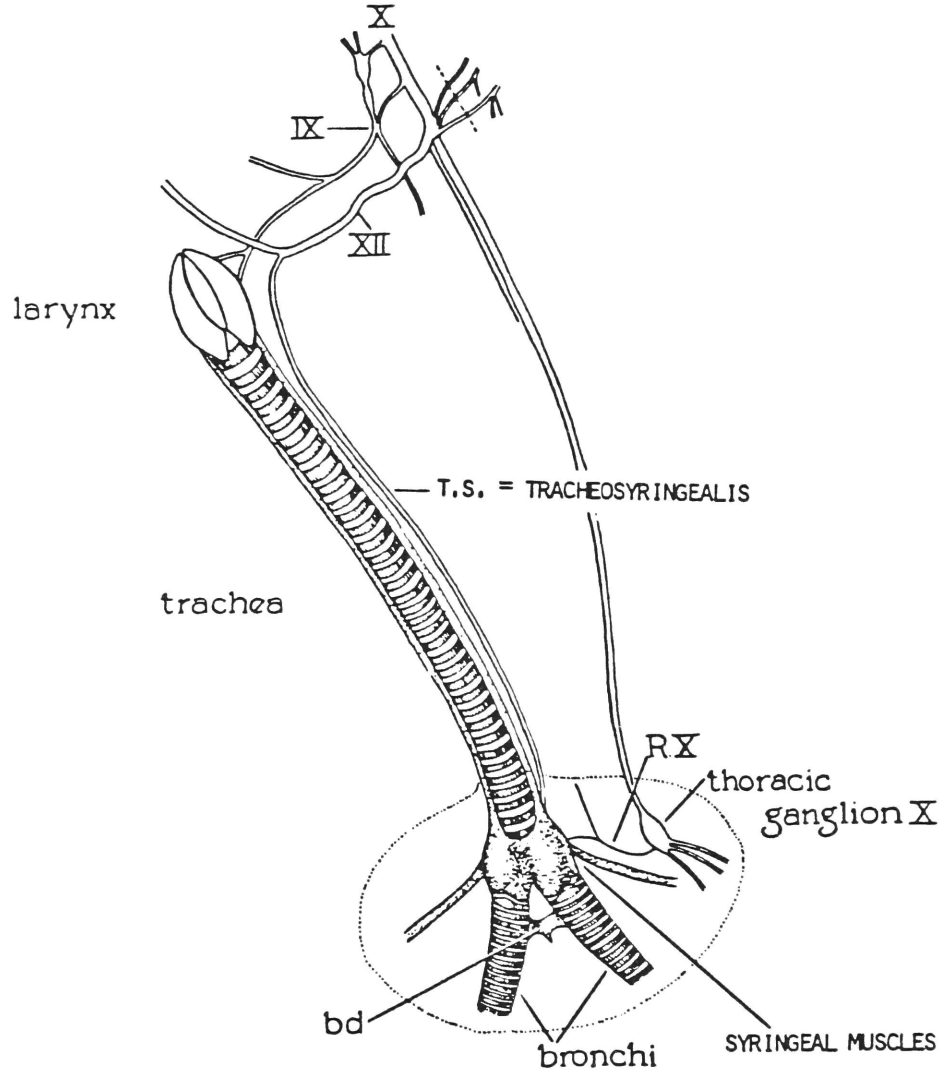
1.3. THE SYRINX

Another source of androgen-sensitive muscle which has received intensive study is the syrinx, the vocal organ of birds. The syrinx is located at the bifurcation of the bronchi and is a specialization of tracheal and bronchial structures (Fig. 1). Sound is produced by the vibration of two membranes which are located on the inner surfaces of the bronchi. The muscles of the syrinx connect the ossified, cartilaginous rings of the trachea and bronchi (see 45 for a review). These muscles control the tension on the membranes and the bore of the airway, and are thus responsible for the frequency modulation of sounds produced during song (46). The muscles of the syrinx are innervated by the tracheosyringealis branch of the hypoglossus nerve (47).

The syrinx is sexually dimorphic and androgen-sensitive. The marked difference in size of the syrinxes of male and female songbirds was noted by Häcker (48 as reviewed in 49) and re-discovered by Arnold (49), who also found that castration caused a decrease in the size of the syrinx of male zebra finches, while testosterone therapy after castration restored the size to intact levels.

The syrinx is involved in a well-characterized, androgen-sensitive behavior: the production of song. When males are actively courting

Figure 1: A schematic diagram of the syrinx, showing the position of the syringeal muscles in relation to the trachea and bronchi and the innervation of these muscles by the trachesyringealis branch of the hypoglossal nerve. Adapted from 134.



females, they may spend hours each day in song. Singing in zebra finches is normally a male specific behavior; females do not sing, unless treated neonatally with hormone (50). In the adult male, song is also androgen-dependent; castration decreases the amount of singing, and testosterone therapy following castration restores it to intact levels (49; 51). Song has provided a sensitive behavioral assay which has been a powerful tool in the description of the entire efferent pathway of song production (52; 53). The brain nucleus HVC, the nucleus Robustus Archistriatalis and the syringeal portion of the hypoglossus nucleus, which together make up the main song control pathway, all show nuclear accumulation of testosterone (54). These three brain regions are also all sexually dimorphic in size (55). Thus the syrinx is the final step in a pathway which shows striking sexual dimorphism and hormonal-sensitivity, and which controls a behavior which is both markedly sexually dimorphic and hormone dependent. A response of the synapses in the syringeal muscle to alterations in androgen levels would be part of an overall adaptive response of the entire song control pathway in preparation for the increased use required by the breeding season.

Arnold et al. (54), in an autoradiographic study of steroid hormone accumulation in the brain, found that the motoneurons in the hypoglossus nucleus accumulated testosterone in their cell nuclei. This was the first demonstration of concentration of gonadal steroids in motoneurons. This accumulation is restricted to androgens; estrogen is not accumulated (56). The motoneurons which have androgen receptors are restricted to the caudal portion of the hypoglossus, which innervates the syrinx exclusively (52). The rostral portion, which contains cells innervating the muscles of the hyoid complex, does not accumulate steroids.

Lieberburg and Nottebohm (57) demonstrated that the muscles of the syrinx also have high concentrations of specific proteinacious binding sites for androgen. This binding is in extremely high capacity for muscle; 40 to 50 fmoles/mg protein. These levels are 13 to 50 fold higher than levels reported for rat skeletal muscles (35; 36) and are comparable to the levels found in classical androgen-sensitive tissues, such as the rat prostate and mouse kidney. The syrinx levels are also 4 to 9 times higher than levels of receptor reported for the levator ani (34). Muscles of the hyoid complex and other zebra finch muscles have no detectable androgen receptors.

The fact that the muscles of the syrinx and the motoneurons which innervate them both accumulate androgens and presumably contain androgen receptors, raised the possibility that androgens might prepare the neurons and muscle to sustain the higher levels of use required by song. Preparation might involve induction of the components required for synaptic transmission. To explore this possibility, Luine et al. (58) examined the effect of alterations in circulating androgen levels on cholinergic enzymes in the syrinx and the nerve innervating it. They found that castration of males caused a decrease in the specific activity of AChE and CAT in the syrinx and in the nerve which innervates it. Specifically, in the zebra finch, two weeks of castration caused syringeal weight to decrease to 76% of intact levels. Total CAT and AChE activity decreased by 50%. In the tracheosyringealis nerve, castration for 4 to 5 weeks caused a 60% decrease in AChE and a 40% decrease in CAT. Treatment of castrates with silastic implants filled with testosterone raised their serum testosterone to levels comparable to those of courting males. These treatments also reversed some of the

effects of castration, restoring syrinx weight and the activity of AChE in muscle and nerve to intact levels and increasing the activity of CAT to a point between castrate and intact. These results indicate that testosterone acts on the syrinx and its innervation to increase the amounts of two enzymes important in transmission at the neuromuscular junction.

Thus, the syrinx provides a source of synapses for which both the presynaptic motoneurons and the postsynaptic muscle fiber are sensitive to androgen. This suggests that the neuromuscular junctions in the syrinx will provide an ideal model for the investigation of synaptic plasticity, in which synaptic modification can be easily studied in an adaptive context.

1.4. SUMMARY

Strategies for the study of synaptic plasticity have been briefly reviewed, with particular emphasis on those systems which are known to be physiologically relevant. The advantages of the study of synaptic plasticity in hormone-sensitive neural systems have been considered. In particular, the neuromuscular junctions in androgen-sensitive muscles provide the advantages of a well-characterized synapse and a readily modifiable environmental stimulus. Previous work on the mechanisms underlying the androgen-sensitivity of the levator ani muscle of the rat and the syrinx of songbirds has been described, and the effects of alterations in androgen levels on cholinergic enzymes in these muscles have been reviewed in detail.

2. CHAPTER II: EFFECT OF ANDROGENS ON AChE FORMS IN THE SYRINX

2.1. INTRODUCTION

In a continuing effort to characterize the mechanism of testosterone action on neuromuscular junctions in the syrinx, I undertook a study of the effects of castration and testosterone therapy on the relative and absolute abundance of the various molecular forms of AChE in the syrinx of the zebra finch.

The enzyme acetylcholinesterase (AChE, acetylcholine acetylhydrolase E.C. 3.1.1.7) exists in a variety of molecular species in all vertebrates (59; 60; 61; 62; 63; 64). In electric organ, muscle and motor nerve, a consistent pattern has emerged of up to six forms with a variety of molecular weights and structures, including globular and asymmetric forms (65; 64; 66). The relative abundance of these forms is not constant but varies in different muscles (67; 68; 69), across subcellular fractions (64; 70) and during the course of embryogenesis (61). In addition, the pattern changes dramatically after denervation and reinnervation. In general, large, asymmetric forms have been found to be specific to innervated muscle and motor nerve (60; 62; 61; however, see 69).

2.2. METHODS

Male zebra finches were obtained from Novak Aviaries, Deer Park, N.J. or from Canary Bird Farm, Old Bridge, N.J. All birds were in full adult plumage and good health when sacrificed.

Birds were castrated by surgical removal of testes and cautery of

the site of attachment to destroy any testicular remnants. Steroid treatments involved implantation of silastic capsules subdermally above the pectoral muscle mass. Silastic capsules were prepared in Silastic Tubing (Medical-Grade, Dow Corning, Midland MI), internal diameter 0.030 in., external diameter 0.065 in., 7 mm in length, and were sealed at each end with Silastic Medical Adhesive (Silicone Type A, Dow Corning). They contained 5mm of packed testosterone (4-androsten-17β-ol-3-one, Steraloids, Wilton, NH) or cholesterol (5-cholesten-3β-ol, Steraloids). Such silastics have been shown to raise plasma levels of testosterone to approximately those found in courting males (58). The syrinx was denervated by removing approximately 1 cm of the descending branch of the XIIth nerve from just below the point at which the nerve branches into ascending and descending portions.

Birds were sacrificed by ether overdose and exsanguination by heart puncture, or by decapitation. The syrinx and hyoid muscles were removed as previously described, except that the sternotrachealis muscle was sectioned at its point of attachment to the sternum (58). The larynx was removed by sectioning the trachea directly below the point of tracheal enlargement.

Tissue was immediately frozen on dry ice after removal and stored at -40° C until homogenized. Unless stated otherwise, muscles were homogenized in a ground glass-glass 1.6 ml. homogenizer (VWR) in ice-cold buffer containing 0.5% Triton X-100, 0.2 mM EDTA (Ethylene-diaminetetraacetate), 1 M NaCl and 10 mM N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (Calbiochem), pH 7.2. The pH of buffer solutions was adjusted before addition of NaCl. Proteolysis and other reactions

occurring in crude muscle homogenates may modify the distribution of molecular forms of AChE (68). In these experiments, homogenates were never allowed to warm to room temperature and were kept thawed on ice for no more than one hour before application to the gradients. In some cases, the homogenizing buffer contained a cocktail of protease inhibitors, consisting of 10 mM EGTA (Ethyleneglycol bis(aminoethylether)tetraacetate), 2 mM benzamidine, 40 µg/ml leupeptin, 20 ug/ml pepstatin, 1 mg/ml bacitracin and 5 µM N-ethylmaleimide (68). Homogenates were centrifuged in a cold clinical centrifuge for 10 minutes, and the supernatant was then centrifuged for 30 min. at 19,000 g. The supernatant was stored at -40° C until applied to the gradients.

Homogenates were diluted to constant protein with homogenizing buffer and applied to the top of a 4.9 ml, 5-20% sucrose gradient with a 0.2 ml pillow of 60% sucrose. All sucrose solutions were made up in the buffer used for homogenization, but without the addition of protease inhibitors. Gradients with homogenates were centrifuged for nine hours in a Beckman SW 50.1 rotor at 45,000 rpm (189,000 g average) at 4° C. After centrifugation, 54 to 59 twelve drop fractions were collected from the bottom of the tubes. Collection was performed at 4° C. Fractions were stored at -40° C until assayed for AChE. Recovery of AChE activity from the gradients was 75 to 123%. Beta-galactosidase (15.9S), bovine catalase (11.3S) and yeast alcohol-dehydrogenase (7.6S) were used as external gradient markers, or beta-galactosidase was added to homogenates as an internal marker and assayed enzymatically.

AChE was assayed by the radiometric assay of Johnson and Russell (71; see also 58) with 230 µM tetraisopropylpyrophosphoramide included

in the reaction mixture to inhibit nonspecific cholinesterase. Protein was determined by the method of Lowry (72). Beta-galactosidase was determined by standard procedures (Boehinger-Mannheim Biochemica Catalogue, Revised edition, 1970).

2.3. RESULTS

2.3.1. Distribution of AChE Forms and Verification of Methods

As in other species, sucrose density gradient sedimentation of zebra finch muscle homogenate revealed four main peaks of AChE activity. These peaks were found at 16.4S (H peak), approximately 10.5S (M peak), 6.1S (L2 peak) and approximately 4.0S (L1 peak, Fig. 2 and Table 1). Little difference was seen between homogenates made in the presence and absence of protease inhibitors, either in the sedimentation coefficients or the relative proportion of the heaviest molecular forms. The two most slowly sedimenting forms (L1 and L2) together accounted for most of the AChE activity. Combined, these two forms made up a relatively constant proportion of the total activity. However, the distribution of activity between these two peaks varied considerably from one experiment to the next, suggesting that one form was converted to the other in varying degrees. The 6.1S form is generally less abundant in those homogenates made without NEM and protease inhibitors. More subtle differences in distribution may also occur, but are obscured by the large variations in distribution between individual birds. In the presence of protease inhibitor, the distribution of molecular forms was relatively stable. When an aliquot of a homogenate with protease inhibitors was incubated for one hour at room temperature before sucrose density gradient centrifugation, the distribution of forms changed

Figure 2: Sucrose density analysis of AChE from the syrinx of an intact male zebra finch. AChE is expressed as μmoles of [^{3}H]-acetylcholine hydrolysed per hour per syrinx. The syrinx was homogenized in 1 M NaCl, 0.5% Triton X-100, 0.2 mM EDTA, 10 mM HEPES, pH 7.2 and a cocktail of protease inhibitors described in the text (Section 2.2). The arrows indicate the positions of β-galactosidase (15.9S), bovine catalase (11.3S) and yeast alcohol-dehydrogenase (7.6S) in parallel gradients.

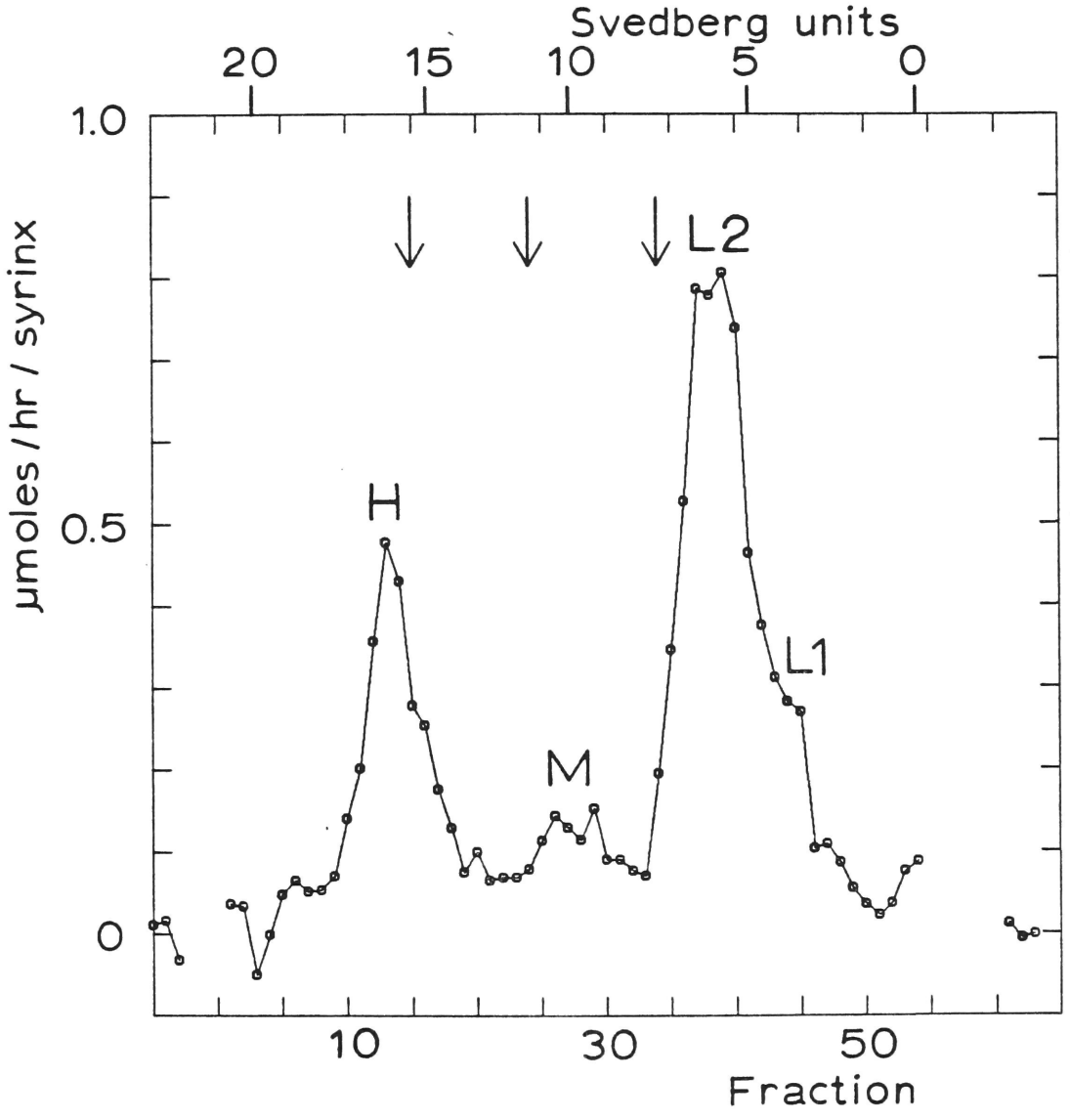


TABLE 1

MOLECULAR FORMS OF ACHE IN THE SYRINX

BUFFER SYSTEM	LOWEST PEAK (H FORM)	MIDDLE PEAK	
TRIS HCl	16.5	10.8	
"	15.5	9.9	
"	16.0	10.2	
HEPES	16.8	11.4	
"	16.4	10.8	
"	16.4	11.8	
HEPES	16.2	10.5	(N=2)
with	16.6	10.9	(N=6)
protease inhibitors	16.8	10.6	(N=3)
MEDIAN	16.4	10.8	

Table 1: Molecular forms of AChE in the syrinx of the zebra finch. A summary of S values computed for heavy syringeal AChE forms. Markers were included in the muscle homogenate. Beta-galactosidase (S=15.9) was the marker in all cases.

little, except that the 6.1S peak decreased, and the 4.0S peak increased proportionately (Fig. 3).

2.3.2. Distribution of Forms in Various Muscles

Despite the high reproducibility of the AChE distribution from a single homogenate (Fig. 3), a large amount of variability in the proportion of forms was found among intact males (Fig. 4). In syrinx of intact males, the H peak comprised from 8.1 to 27.7% of the total AChE activity recovered (15.7 $\pm$ 4.4%, mean and standard deviation) while the 6.1S and 4.0S peaks combined comprised most of the remainder.

The distribution of AChE forms varied markedly in different muscle types from the same animal (Fig. 5). Both larynx and hyoid had relatively more of the H forms than did syrinx (30.3% for one larynx and 13.8, 28.0 and 50.6% for three hyoids).

2.3.3. Effect of Denervation

After denervation, total syringeal protein fell 20% (from 1.98 mg in sham operated control, to 1.59 by 17 days, and 1.51 by 83 days). Total AChE activity rose 23% by 17 days, and 104% by 83 days (from 10.7 µmoles/hour/syrinx to 13.9 by 17 days, and 21.8 by 83 days). In contrast to the increase in total AChE activity, the proportion of AChE in the H peak fell after denervation, from 27.7% of total AChE to 1.5% by 17 days and 2.0% by 83 days. Expressed as absolute activity, the H peak fell from 2.96 µmoles/hr/syrinx to 0.21 by 17 days and 0.44 by 83 days (Fig. 6).

Figure 3: Analysis of AChE from the syrinx of and intact male zebra finch. AChE is expressed as μmoles/hr/syrinx. A homogenate was prepared in the presence of protease inhibitors as described in Fig. 2 legend. The homogenate was divided into aliquots that were subjected to a one hour incubation at room temperature (*) of on ice (o). The arrow indicates the position of β-galactosidase activity (15.9S), which was included with the homogenate as a internal marker.

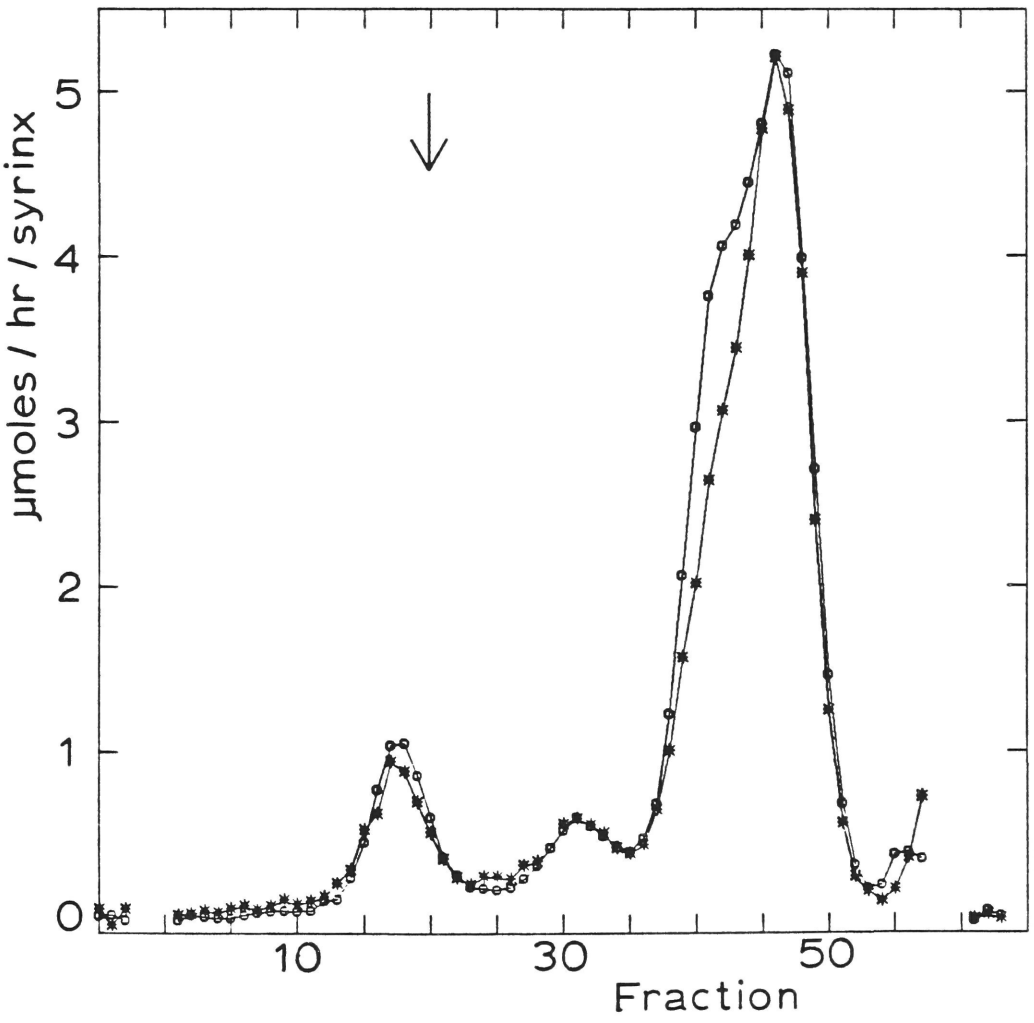


Fig 4: Sucrose density gradient analysis of syringeal AChE from three intact zebra finches, illustrating the variability between individual birds. AChE is expressed as µmoles/hr/mg protein. Procedures as in the legend to Fig. 2.

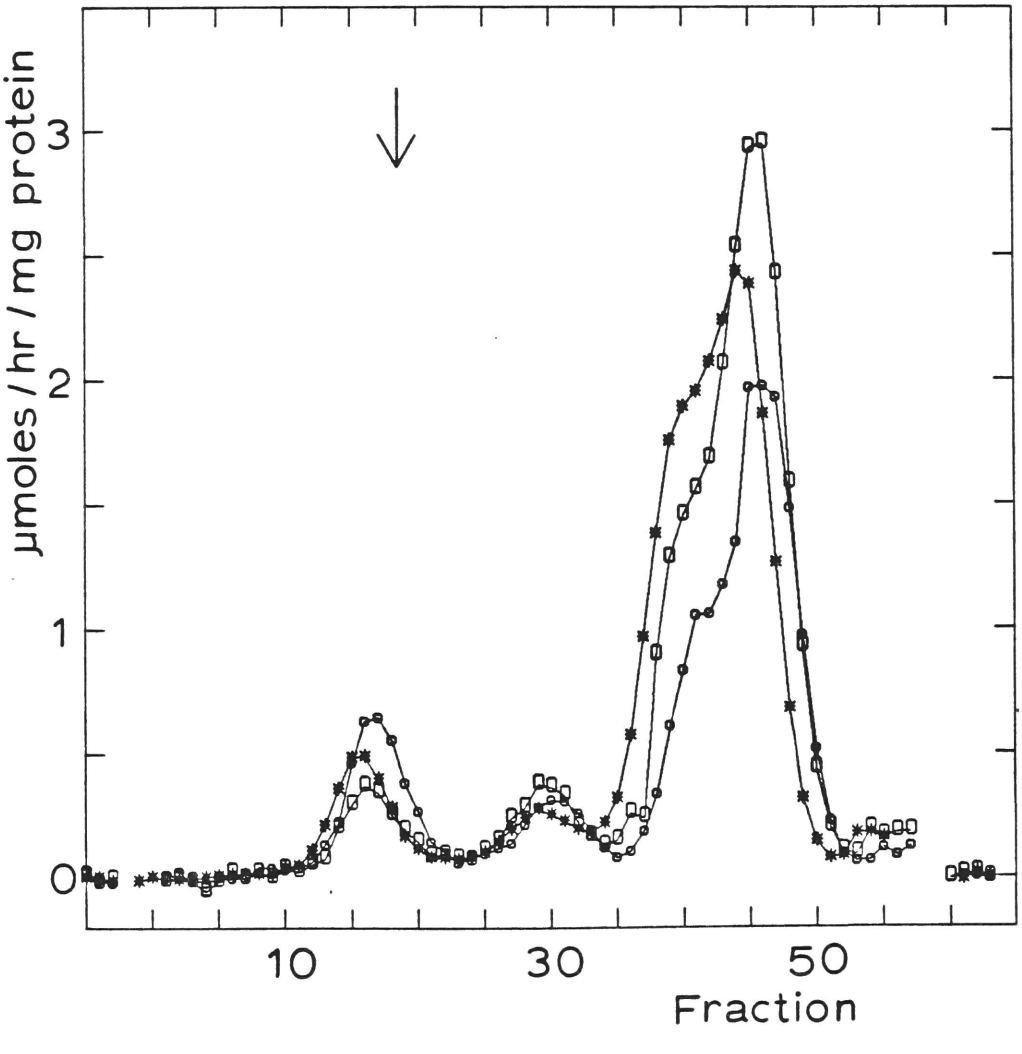


Figure 5: A comparison of AChE forms in syrinx (O), hyoid (X) and larynx (*) from one intact male. Activity is expressed as proportion of total activity in per cent. In this case, tissues were homogenized in buffer without protease inhibitors, containing 1 M NaCl, 1.0% Triton X-100, 50 mM $MgCl_2$, 10 mM TrisHCl, pH 7.0.

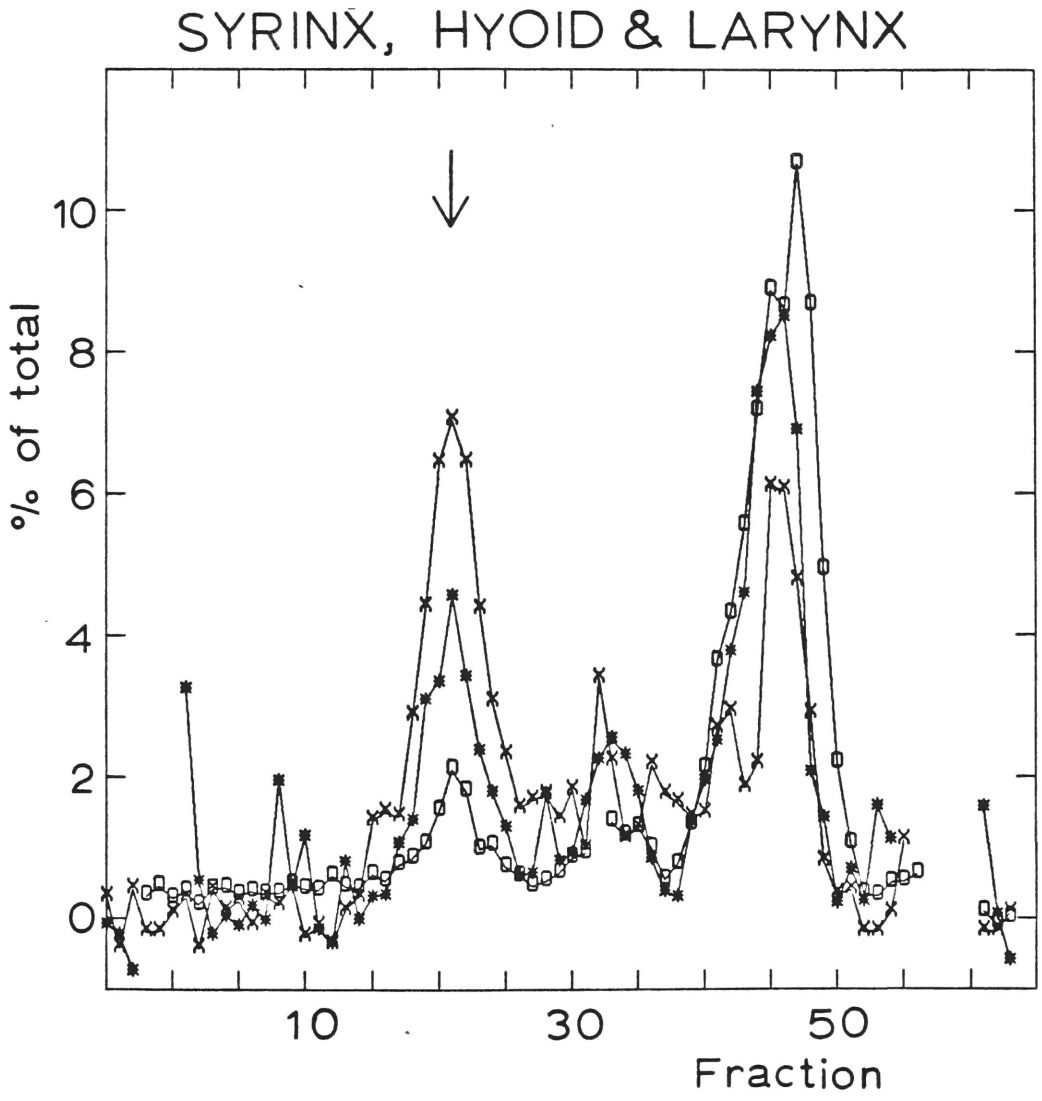
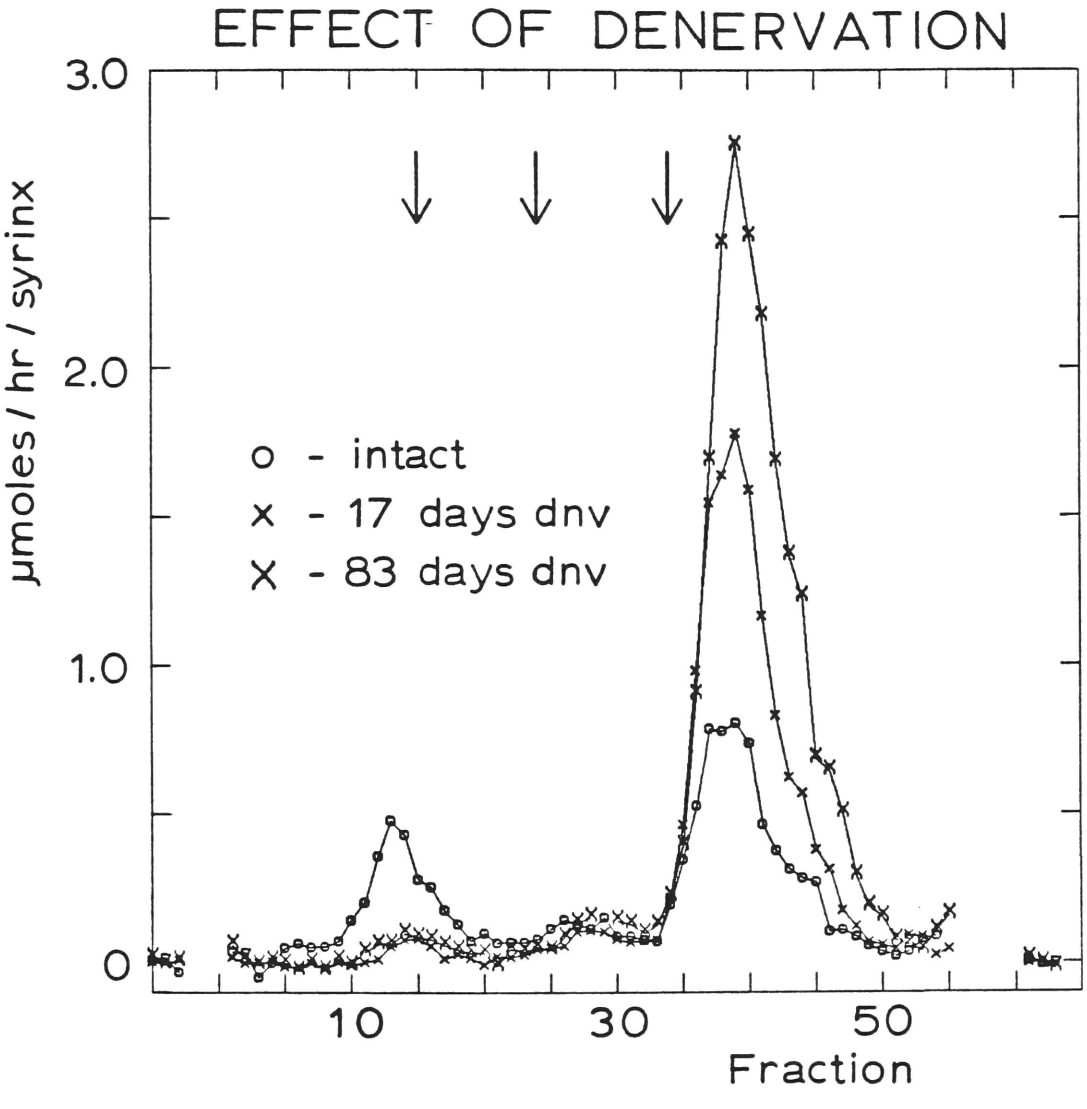


Figure 6: Sucrose density gradient analysis of syringeal AChE from an intact male zebra finch (o), a male denervated for 17 days (x) and a male denervated for 83 days (X). Activity is expressed in µmoles of substrate degraded per hour per syrinx. Denervation involved removing a segment from the tracheosyringealis nerve, which innervates the syringeal muscles. Homogenization as in the legend to Fig. 2.



2.3.4. Effects of Castration and Testosterone Therapy

As in previous studies (58), castration decreased total and specific AChE activity in the syrinx. In this case, total AChE decreased by 44% from intact levels (from 22.4 ± 4.9 to 12.7 ± 3.8 μmoles/hr/syrinx, mean and standard deviation for four birds from three experiments), while specific AChE activity decreased by 25%, (from 9.32 ± 3.36 to 7.00 ± 1.54 μmoles/hr/mg protein). In all cases, castrated birds treated with testosterone (T) had total and specific levels of AChE at or near those of intact birds (26.3 ± 3.0 μmoles/hr and 9.79 ± 2.32 μmoles/hr/mg respectively). As previously shown, none of these treatments changed AChE levels in larynx or hyoid.

Despite the large changes in total and specific AChE activity in the syrinx after castration, no obvious change in the distribution of molecular forms of AChE was observed after 32 days of castration (Fig. 7). This is most easily seen when activity on the gradients is expressed as proportion of total activity recovered (Fig. 8). In other experiments, no change was seen after 19 days and 55 days of castration (Figs. 9 and 10). Differences between the distributions in these experiments are small relative to the amount of variability between individual intact males (Fig. 4).

The proportion of activity in the H peak was examined in detail (Table 2). Intact males had 11.6% of AChE activity in the H peak (range of 8.1 to 17.2% means and ranges for four birds), while castrates had 13.6% (8.9 to 21.0%) and T-treated castrates had 11.6% (5.9 to 14.3%).

Figure 7: Sucrose density gradient analysis of syringeal AChE from an intact male zebra finch (o), two castrated males (x and X) and a castrated male treated with testosterone (*). Activity is expressed as specific activity in μmoles/hr/mg of protein. Homogenization as in the legend to Fig. 2. Castrated 32 days before sacrifice, implanted with testosterone 25 days after castration. This data corresponds to Experiment 2 of Table 2.

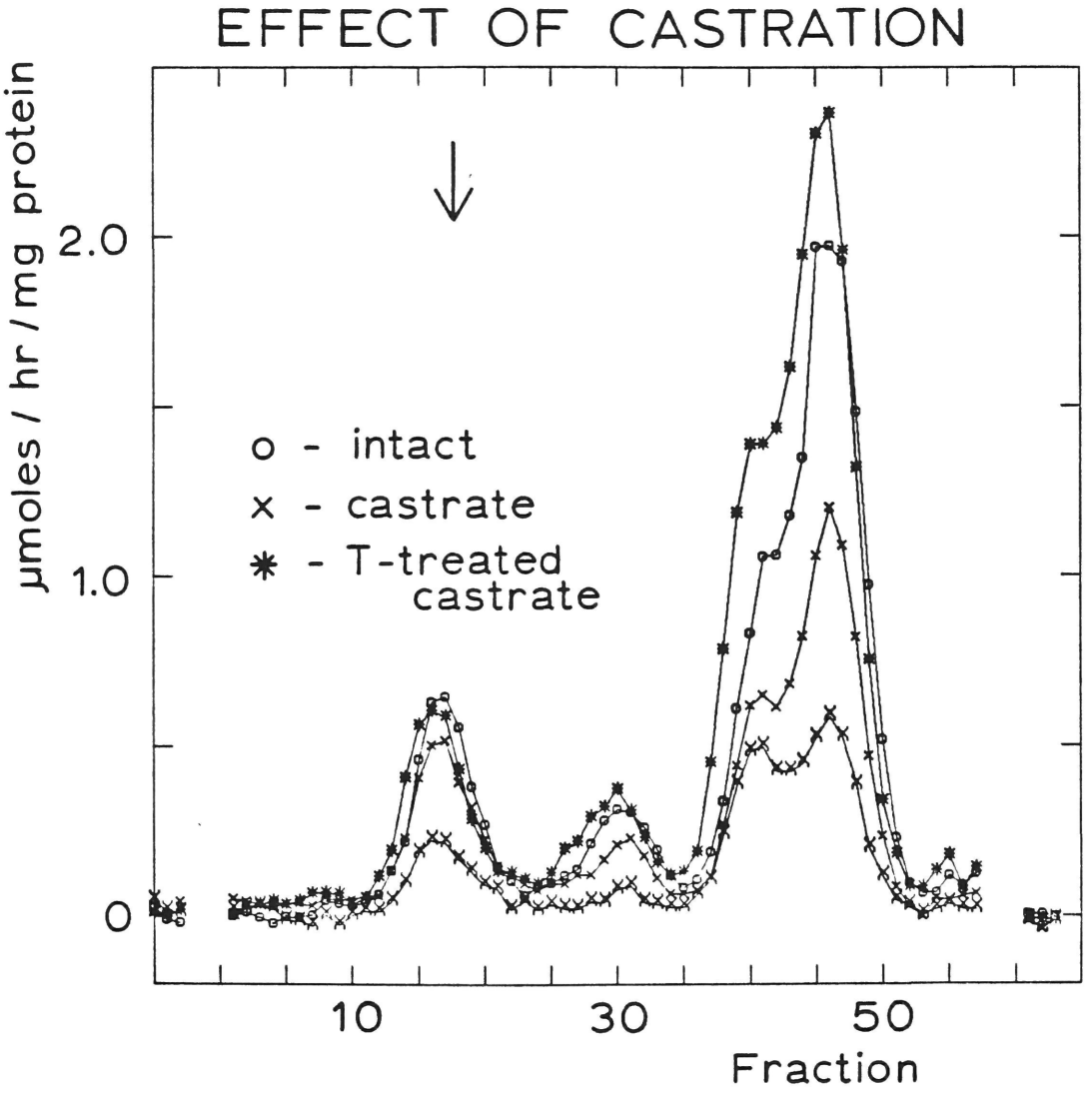


Figure 8: As in Fig. 7, but here activity is expressed as proportion of total activity recovered from the gradient in per cent.

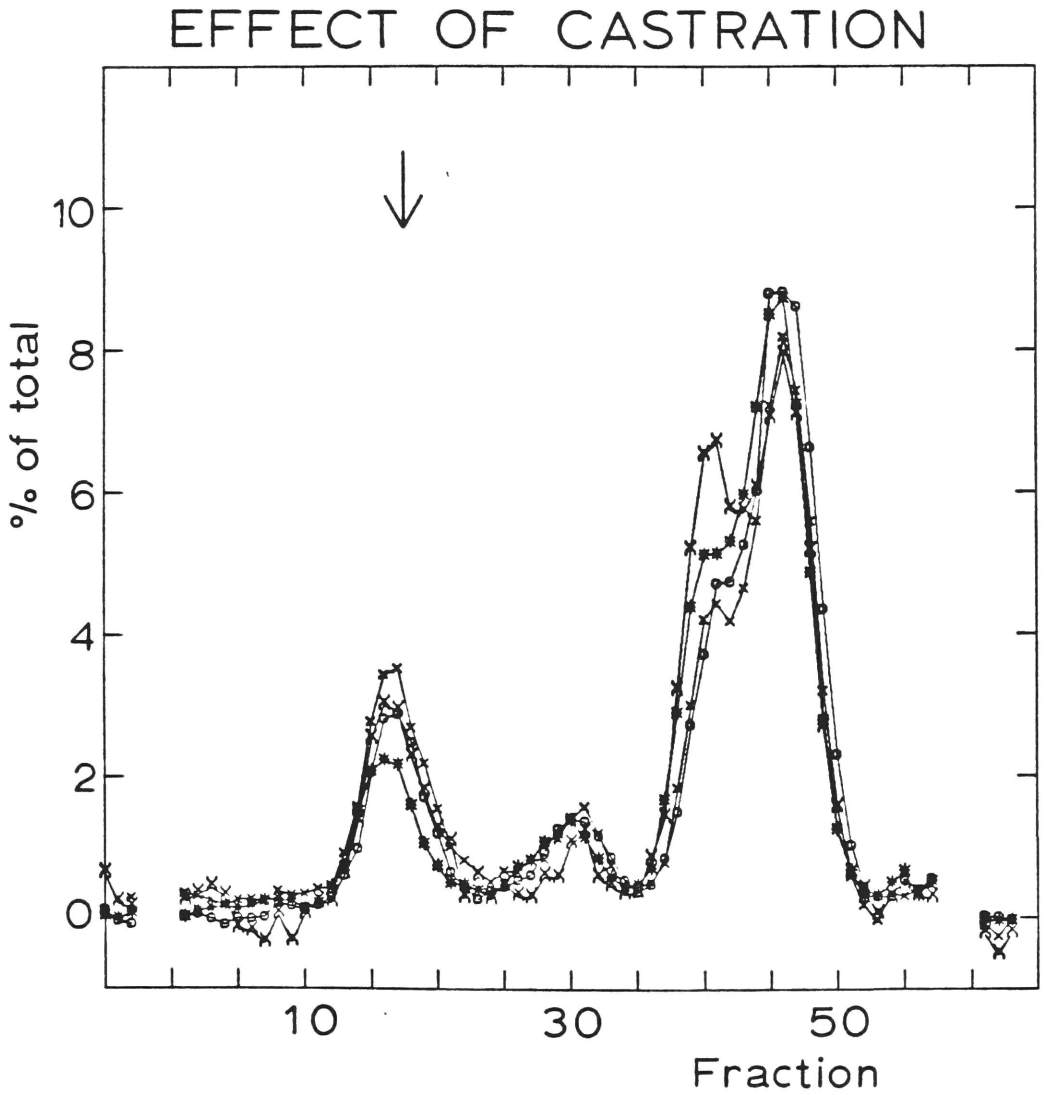


Figure 9: Another comparison of AChE forms in intact (o), castrate (X) and testosterone-treated castrate (*) males. Syringeal AChE is expressed as proportion of total activity in per cent. In this experiment, the homogenizing buffer did not contain protease inhibitors. Birds were castrated 19 days before sacrifice, implanted with testosterone 1 day after castration. This data corresponds to Experiment 1 of Table 2.

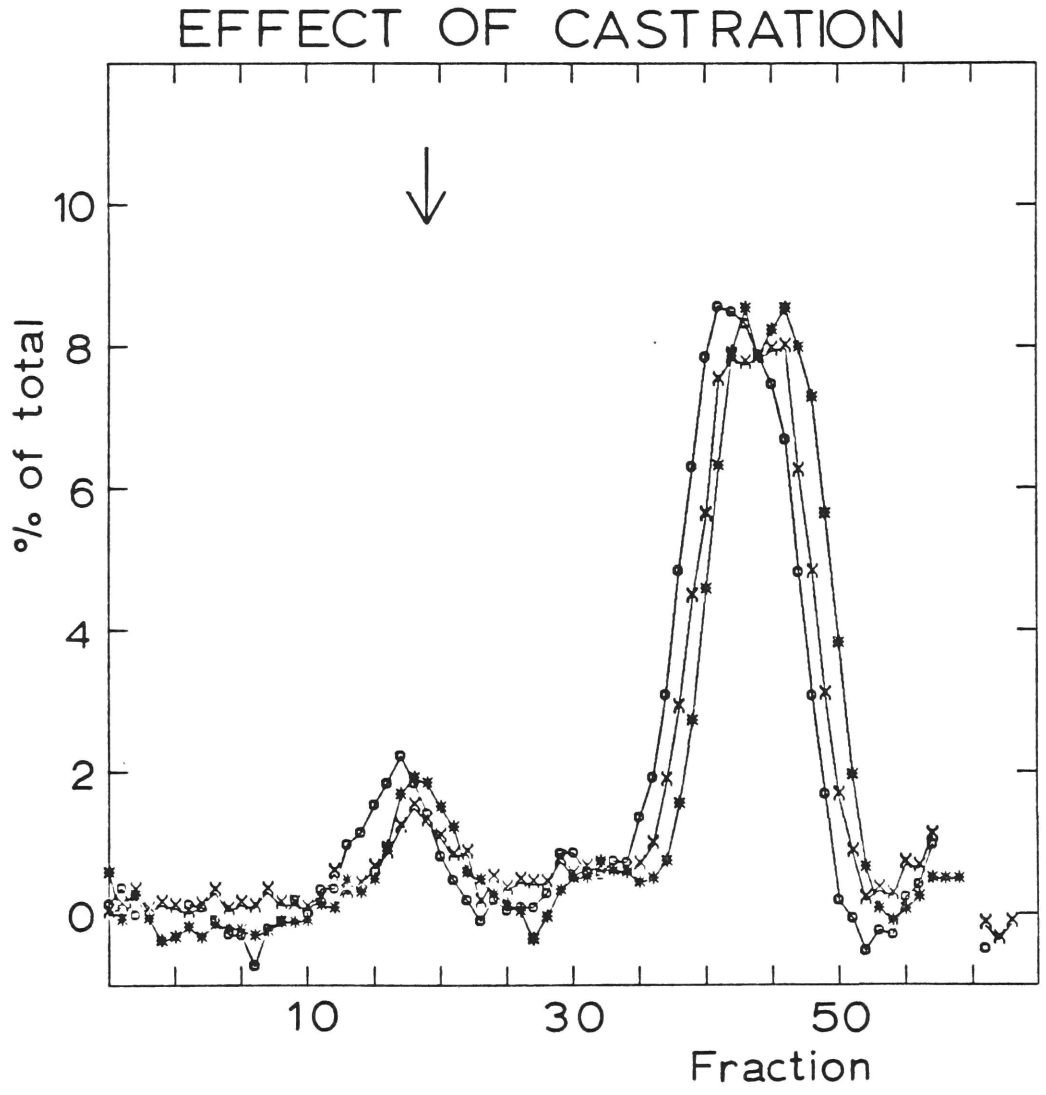


Figure 10: As in Fig. 9, but birds were castrated 55 days before sacrifice, implanted with testosterone 12 days after castration. This data corresponds to Experiment 3 of Table 2.

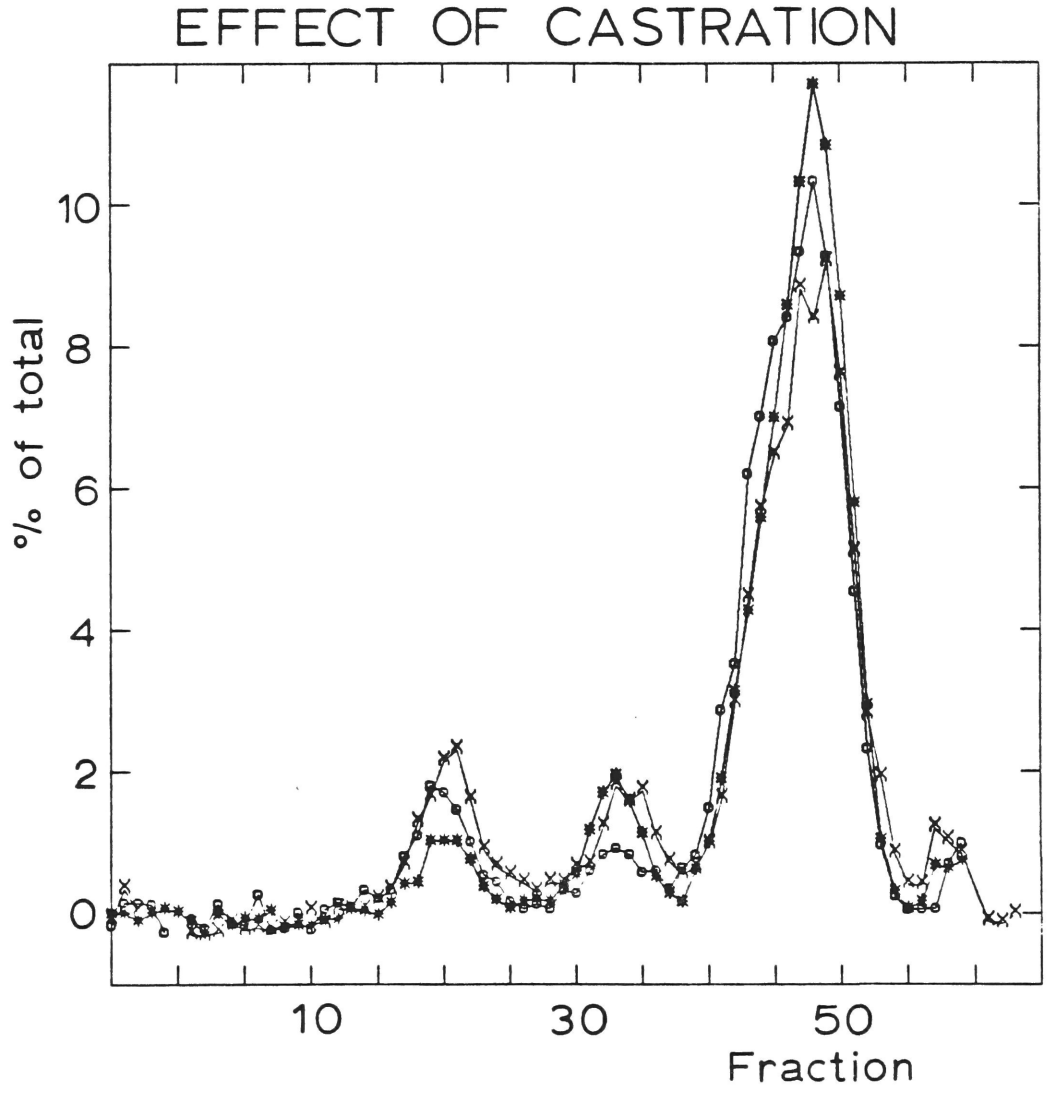


TABLE 2

EFFECT OF CASTRATION ON ACHE FORMS

EXPERIMENT	INTACT	CASTRATE	CASTRATE + T
	(Proportion of AChE in H peak, as per cent of total activity)		
1	10.79	8.92	12.02
2	17.19	20.95	14.28
	8.09	12.74	14.16
3	10.35	11.67	5.86
MEAN	11.61	13.57	11.58
(S.D.)	(3.91)	(5.18)	(3.95)

Table 2: Effect of castration on distribution of AChE molecular forms Effect of castration and testosterone therapy on the proportion of H form in the syrinx of adult male zebra finches. Values are the per cent of total AChE activity recovered from the the gradients in the H peak. Each entry represents one gradient run on one syrinx. Various buffers were used. In Experiment 2, protease inhibitors were included in the homogenizing buffer. Mean and standard deviations for the four birds are presented in the last row. In experiment 1, birds were castrated 19 days before sacrifice and received a silastic implant of testosterone 1 day after castration. In experiment 2, birds were castrated 32 days before sacrifice and received implants 7 days after castration. Two birds were included in each group in this experiment. In experiment 3, birds were castrated 55 days before sacrifice and received implants 12 days after castration.

2.4. DISCUSSION

The distribution of molecular forms of AChE in the zebra finch is similar to those reported for other vertebrates (59; 60; 62; 64; 70). At least 4 forms of the enzyme are clearly resolved. Curiously, the sedimentation coefficients of these forms are more similar to those reported for mammals (60; 64; 63; 69) than for gallinaceous birds (chicken 64; 70; quail 73).

In the rat, the most rapidly sedimenting form of AChE is specifically associated with the region of the motor endplates (60) however, it does not have this restricted distribution in chicken (67) and human muscle (63). I have not determined if the H form is restricted to the endplate region in zebra finch muscle. As in other species (60; 61), the heavy form of AChE is markedly reduced following denervation, and thus appears to be specific for innervated muscle.

As in other species, the distribution of AChE forms in the zebra finch varies between individual birds, and more dramatically, between muscles (67; 68). It may be that these differences reflect differences in the muscle fiber types and structure of endplates in these muscles. Silman et al. (68) suggested that the H form of AChE was most common in twitch muscles and relatively less abundant in slow-tonic muscles such as the anterior latisimus dorsi. Cholinesterase staining and autoradiographic visualization of acetylcholine receptors suggest that the syrinx contains both multi-terminal fibers and single terminal fibers (see below, Section 6.2.3.2). Since multi-terminal fibers are also slow-tonic fibers, while single-terminal fibers are generally twitch fibers, this may be the reason for the higher proportion of the H peak in the

hyoid and larynx than in the syrinx.

In contrast to the changes in distribution of AChE forms which follow denervation, variations in testosterone levels have no apparent effects on the distribution of AChE forms in the syrinx, despite large changes in total AChE activity. After castration, specific activity of AChE in the syrinx decreases on average by 40% of intact levels (58), but this decrease affects all forms of the enzyme to the same degree. This is true at three, four and seven weeks after castration. I cannot rule out a temporary change in the distribution of AChE forms at times earlier than three weeks, such as would be caused by differences in the turnover rates of the different forms.

There are many potential mechanisms for the action of testosterone on AChE in the syringeal muscles. All forms of AChE can be produced by both muscle (73; 74) and motoneurons (75; 76), and both syringeal muscles and their motoneurons have receptors for androgens (57; 56). Therefore, testosterone may act on either muscle or motoneurons to increase the activity of the various forms of AChE. In other experiments, testosterone caused a small increase in AChE activity in denervated syrinx, suggesting that at least some of its actions are not mediated by the nerve (see below, Section 4.3.4).

The contrast between the effect of denervation and reinnervation with the effect of castration and testosterone treatment may have important implications for the mechanism of regulation of the distribution of AChE activity among the molecular forms. The structures of AChE forms has been examined in detail in Torpedo (77) and Electrophorus (65). These include three globular forms; monomers, dimers and tetramers of

catalytic subunits; and various asymmetric or tailed forms, with globular tetramers attached to a collagen-like tail. Kinetic studies have shown that the catalytic activity and pharmacology of each of the forms is identical, suggesting that the catalytic sites are identical (60; 78; 70). The structures proposed for the forms in electric fish are consistent with what is known about the properties of the forms found in other vertebrate tissues (64; 66). In muscle, the form which sediments the most rapidly upon rate-zonal sedimentation (the H form here) probably corresponds to the largest of the asymmetric forms of Electrophorus (64; 70). This form consists of 3 tetramers of catalytic units linked to a collagen-like tail (65). It has been suggested that the asymmetric forms are anchored in the muscle basal lamina by their collagen-like tails (79; 77; 80). The mechanism of assembly of the tail with the catalytic subunits is unknown.

Knowledge of the structure of AChE naturally leads to hypotheses on the manner of assembly of the molecular forms. One possible mechanism for the appearance of several molecular forms is that these forms are the result of transcription of separate gene products. Alternatively, large, rapidly sedimenting forms might be the result of post-transcriptional modifications, and the process of assembly of these forms might be independent of the synthesis of catalytic subunits. This hypothesis is supported by the demonstration that the distribution of forms of AChE and pseudocholinesterase change in a similar manner after denervation and in the dystrophic condition in chickens (81). The catalytic sites of these two enzymes are immunologically distinct (82), so parallel regulation suggests that these different catalytic sites may be subject to a common assembly process. If this were the case,

innervation and muscle activity would presumably regulate this assembly process as well as changing the total number of catalytic sites (83).

If the molecular forms arise by post-transcriptional assembly, then in the syringeal muscles, testosterone must increase the number of AChE catalytic subunits available for assembly into all of the molecular forms, without affecting the assembly process itself, while denervation must cause an increase in synthesis of catalytic subunits, but prevent assembly of these subunits into heavy forms of AChE. Alternatively, if the various molecular forms of AChE are the products of distinct genes, then testosterone must affect transcription of all of these genes equivalently, while denervation must affect transcription of each gene differently. The final choice between these alternatives must await further studies on the mechanism of synthesis and assembly of the forms of AChE.

2.5. SUMMARY

I measured the distribution of molecular forms of acetylcholinesterase (AChE) in muscles of the zebra finch and found a pattern similar to those reported from other species. As in other species, the most rapidly sedimenting form of the enzyme decreases to barely detectable levels following denervation. Castration causes a large decrease in AChE activity, but has little or no effect on the relative abundance of AChE forms in the syrinx. This suggests that the number of AChE catalytic sites is changing without affecting the distribution of catalytic sites among the molecular forms. This is in marked contrast to the effect of denervation in the syrinx, which causes changes in the distribution of activity, as well as in the total activity of AChE.

It had been hoped that a study of the effect of alterations in androgen levels on the distribution of AChE forms in the syrinx would help to characterize the effect of androgens on the neuromuscular junctions in the syrinx. However, because of the variability between species in the restriction of the heavy form of AChE to the endplate, and the lack of any difference in the response of the heavy form and the lighter forms, it is unclear what these results imply about effects of androgens on synapses in the syrinx.

3. CHAPTER III: ASSAYS FOR ACETYLCHOLINE RECEPTORS

3.1. INTRODUCTION AND REVIEW

In a further effort to characterize the effect of androgens on the neuromuscular junctions in syringeal muscles, I chose to examine the effect of castration and testosterone treatment on the number of acetylcholine receptors (AChRs) in the syrinx. For this purpose, I perfected an assay for AChR number which was sensitive enough to measure the low number of AChRs in the syrinx.

Methods for the quantification of specific receptors using high-affinity radiolabelled ligands have received increased attention in recent years as a result of the availability of many new ligands with high affinities for novel receptor species. An extensive literature has appeared on the theory and methods of receptor-binding assays (see 84). All of these techniques rely on the same basic principle. A preparation containing an unknown quantity of the receptor of interest, along with other material, is incubated with a high concentration of a radiolabelled ligand with predetermined specific activity. After equilibrium has been achieved, the "bound" ligand is rapidly separated from the unbound or "free" ligand by one of a variety of techniques. The radioactivity of the bound ligand is then determined. As a control for the presence of other binding-sites which are not of interest, binding is also measured in the presence of competitors which specifically block the binding of the ligand to the binding-site of interest, while having little or no effect on interactions of the ligand with other binding sites. The difference in binding of ligand in the presence and absence of the competitor is the "specific" binding; a measure of the amount of

binding of ligand to the receptor site of interest. For most ligands and receptors, the assay must be repeated at a variety of concentrations of ligand, in order to determine the affinity of ligand and receptor and the absolute number of binding sites present. In the case of irreversible binding, such an analysis is not necessary, since "equilibrium" is achieved only when all of the binding sites are occupied with ligand. However, in this case it must be shown that assay conditions insure saturation of the binding sites; that is, that all of the sites are occupied by ligand under the conditions of the assay.

Alpha-bungarotoxin binds to the nicotinic AChR of vertebrate muscles and of the electroplaques of electric fish (85) with high specificity, that is, the toxin interacts with much lower affinity with other proteins. The binding of α-bungarotoxin to the AChR is also of extremely high affinity, and has such a slow rate of release that it can be considered in most applications to be irreversible (86; 87). Alpha-bungarotoxin is a small peptide of 8,000 dalton molecular weight with a single tyrosine. It can be radiolabelled with either iodine-125 (88) or tritium (89). After these labelling procedures, the toxin retains its receptor binding activity, making it an ideal reagent for receptor binding assays for nicotinic AChR.

Several assays using radiolabelled bungarotoxin have been published in the last decade, and several of these were explored in theory or in practice in the search for an optimal assay for measuring AChR in muscle. The existing assays fall roughly into two groups, depending on whether the source of the AChRs are membrane vesicles or detergent solubilized preparations. Membranes containing AChRs which have bound

radiolabelled toxin can be separated from unbound toxin by filtration (90; 91), by centrifugation (92; 93) or by separation on the basis of charge (94). Solubilized AChRs can be separated from unbound toxin on the basis of the difference in the size of these molecules (95; 96), the difference in their solubility in ammonium sulfate (97; 98), or the difference in their charge (99; 100; 101; 94).

Preliminary experiments with the syrinx indicated that substantial loss of α-bungarotoxin binding occurred when membrane preparations were prepared by homogenization and sieved through 2 layers of fine nylon mesh, as compared with the binding in detergent extracts of minced syrinx which were strained after extraction (approximately a 45% loss). This suggested that homogenates of syrinx prepared without detergents retained substantial amounts of large particulate material. Therefore, I concentrated on assays designed for detergent solubilized AChRs.

The difference in charge between the toxin and the receptor molecules has proved to be the most generally useful basis for assays of solubilized AChRs. Such assays rely on the fact that the iso-electric point of α-bungarotoxin is relatively high (9.2 pH units), while the iso-electric point for the complex of receptor and toxin is relatively low (4.9 for Torpedo AChR; 99). This fact is the basis for a simple, rapid assay used to quantify AChRs solubilized from fish electric organ (99; 100). This assay utilizes anion-exchange paper discs, which tightly bind the receptor-toxin complex, while allowing unbound toxin to be removed by extensive washing. The paper discs themselves are then counted to measure the amount of toxin bound to receptor. Unfortunately, this assay requires relatively large concentrations of recep-

tors for a high ratio of specific to nonspecific binding (the signal to noise measure of receptor binding assays), since substantial toxin binds to the paper in the absence of AChRs. Preliminary studies indicated that the high blank in this assay made it unreliable for determination of AChR number in syrinx extracts. A simple modification of this assay greatly reduces the blank, making it possible to use the assay for the quantification of AChR number in muscle extracts (101). Unfortunately, the recovery of toxin-receptor complexes is low; only 55% are retained on the anion-exchange paper.

An assay which relies on a cation-exchange resin proved to be reliable and relatively easy, and was chosen as the standard assay for the studies which follow. This assay was proposed by Kohanski et al. (94) and utilizes mini-columns of carboxymethyl-cellulose resin. The incubation mixture of toxin and toxin-receptor complexes is applied to these mini-columns, which bind the free toxin but allow the anionic toxin-receptor complexes to flow through. The complexes are eluted directly into vials for counting. While construction of disposable mini-columns is time consuming, this assay offers the advantage of excellent yields (up to 83%, see below) and low blanks (less than 0.2% of the applied toxin). In addition, regeneration of ion-exchange resin allows mini-columns to be re-used, making this assay simple and convenient. This assay was used for all of the AChR measurements described below.

3.2. CHARACTERIZATION OF THE ACETYLCHOLINE RECEPTOR ASSAY

3.2.1. Preparation of Radiolabelled α-Bungarotoxin

Alpha-bungarotoxin (Boehringer-Mannheim) was routinely iodinated by

a modification of standard procedures (88). 0.1 ml of 1 mg/ml toxin was mixed with 2-3 mCuries of $Na^{125}I$ (Amersham) in about 5 ul. Iodination was begun with 0.1 ml of 0.05% Chloramine T and terminated after 60 sec by addition of 0.1 ml of 0.1% Na metabisulfite, followed by 0.2 ml of 1% KI. Free iodide and toxin were then separated on a Sephadex G-25 column. The first peak was collected, mixed with phenol red as a marker and applied to a column of CM-52 cellulose for further purification as described below.

The amount of free radiolabel eluted from mini-columns in the absence of AChR (the "blank") was unacceptably high when commercial [^{125}I]-α-bungarotoxin (New England Nuclear) was used, amounting to 10.5 to 13.1% of applied counts. Molecular sieve chromatography of the radiolabelled toxin on Sephadex G-10 did not remove these contaminants, but purification on a CM-52 column with step-gradient elution reduced the blank substantially, to 0.65-0.75% of applied counts. Since gradient elution provided the added advantage over step elution of allowing separation of mono- and di-iodinated toxin and improving specific activity by removing some un-iodinated toxin, gradient elution of iodinated toxin from CM-52 columns was used routinely to further purify both freshly radiolabelled toxin and radiolabelled toxin purchased from New England Nuclear (102). This procedure reliably produced toxin of high purity, with blanks of 0.1-0.2% of the applied counts. The radiolabelled toxin was made 0.1% in bovine serum albumin (BSA), aliquoted into plastic tubes, frozen over liquid nitrogen and stored at -40° C until needed.

Specific activity of the toxin, used to convert counts to moles of

toxin-binding sites, was determined by a radio-receptor assay using crude Torpedo electroplaque membrane extract as a source of receptor (see below). Radiolabelled toxin was mixed with various known concentrations of unlabelled α-bungarotoxin (Miami Serpentarium) and after mixing, AChR was added. When the concentration of radiolabelled toxin exactly equals the known concentration of unlabelled toxin, the binding of radiolabelled toxin will be half-maximal. It should be noted that this assay, while more accurate than measuring protein concentration of labelled toxin, still relies on the assumption that all label is on active toxin. 82-97% of radiolabelled toxin can be recovered from CM-52 mini-columns in the presence of excess AChR, suggesting that this is not a large source of inaccuracy. Despite this fact, absolute measurement of receptor number in a standard of AChR from Torpedo electroplaque extract varied over 5 fold when different preparations of radiolabelled toxin were used, and it is likely that inaccurate determination of toxin specific activity is one source of this variability.

Purified toxin was generally used within one half-life and always within two half-lives of iodination. The amount of radiolabel in these preparations which reacted with AChRs decreased by approximately 17% in two half-lifes, while specific activity changed negligibly, relative to that predicted from radioactive decay.

Torpedo electroplaque membrane extract was the generous gift of Dr. Lee Rubin (Rockefeller University, N.Y., N.Y.). It was prepared by homogenization of diced electroplaque in 1.0% Triton, 5mM Tris HCl, pH 7.4 and a cocktail of protease inhibitors. This homogenate was then extracted for 30 minutes at 4^{o} C and spun for 60 minutes at 18,000 rpm

in a Beckman SS34 rotor (average 39,000 X g). The supernatant was made 0.1% in Thimerosol and stored at 4° C.

3.2.2. Extraction of AChR

A variety of procedures have been used to extract AChRs from vertebrate muscle (96; 103; 104). Chiu et al. (105) compared the extraction of [^{3}H]-α-bungarotoxin bound to mouse diaphragm under a variety of conditions. They found maximal solubilization (85% of bound α-bungarotoxin) after extraction in 1.5% Triton at 27° C for 3 hours.

I chose to extract AChRs in 1.5% Triton X-100. The effect of extraction time and temperature (4° or 30° C) was examined. Incubation of homogenates at 30° C gave maximal specific binding after between 30 and 100 min. No additional AChR was extracted from the high-speed pellet remaining after extraction by an additional 60 min at 30° C. In the experiments described below, muscle homogenate was extracted in Extraction Buffer (1.5% Triton X-100, 50 mM sodium phosphate, pH 7.4) at 30° C for 1 hour, after which insoluble material was removed by centrifugation.

3.2.3. Incubation Time

Using syrinx extracts as a source of AChR, I optimized the parameters of the bungarotoxin binding assay. The optimal incubation time was determined by examining the total specific binding and the ratio of specific to non-specific binding. Extracts with low concentrations of AChR were incubated with 680 pM [^{125}I]-α-bungarotoxin for various times. Nonspecific binding was determined in the presence of 2.2 uM gallamine. Under these conditions, specific binding rose for 3 hours

and was unchanged between 3 hours and 5 hours. In subsequent experiments, 3 hour incubations were used as standard condition, with toxin concentrations of 400 pM to 1 nM. There was negligible increase in specific binding with increasing toxin concentrations, as long as free toxin concentration (total minus bound) remained above 100 pM.

3.2.4. Competition

Because of concern about the possible differences between saturable binding and specific binding, I chose not to use unlabelled α-bungarotoxin as a competitor, but rather to use a nicotinic antagonist of another class. Preliminary experiments suggested that curare caused unusual effects at concentrations of 100uM and above, lowering the blank in membrane filter assays considerably, perhaps by precipitating α-bungarotoxin. Instead, the synthetic curarine, gallamine was used. Concentration dependence of inhibition of binding indicated that there was no difference in inhibition in the presence of 100 uM to 1 mM gallamine after up to 5 hours of incubation in the presence of 650 pM [^{125}I]-α-bungarotoxin. Nonspecific binding (defined as binding occurring in the presence of saturating concentrations of gallamine) amounted to 14% of the total binding. Binding was also measured in the presence of 100 nM unlabelled α-bungarotoxin. Unsatuarable binding (defined as binding occurring in the presence of excess unlabelled α-bungarotoxin) was 34% lower than nonspecific binding, amounting to 9% of total binding. This suggests that some small number of saturable sites for α-bungarotoxin may not be competable with gallamine under these conditions. Since this nonspecific, saturable binding amounted to only 11% of the specific binding, this phenomenon was not pursued.

3.2.5. Preparation of Mini-columns

The preparation of mini-columns was based on the protocol of Kohanski et al. (94) with modifications. Carboxy-methyl cellulose resin (Whatman CM-52) was equilibrated in Column Buffer (0.01% Triton, 1 mM Na phosphate, pH 7.2, 0.01% Thimerosol) by repeated washing. Conductivity was measured to determine equilibration. Mini-columns were poured from a slurry of approximately 1 g wet resin per 2 ml slurry volume.

Disposable columns were made from Fisher Pasteur pipets with small glass-wool plugs. These were filled with settled resin to a height of 6.0 cm and rinsed with 1 ml of Column Buffer. Mini-columns were prepared within two hours before application of samples. In some assays, reusable mini-columns were used. These were constructed from micro-tulip tubes (8 mm internal diameter, 10 cm long, Radnoti Glass) plugged with a 4 mm glass bead and filled to a height of 3.0 cm with 1.8 ml of settled resin. After use in an assay, free toxin was eluted from the resin with 5 ml of 100 mM sodium phosphate, pH 7.2, after which columns were washed with 10 ml of Column Buffer and stored in Column Buffer.

3.2.6. Estimate of Yield of AChR from Mini-Columns

The yield of toxin-receptor complex from the mini-columns was estimated by determining the recovery of known quantities of toxin-receptor complexes applied to the columns under standard conditions. Complexes were formed by incubation of Torpedo electroplaque extracts with [^{125}I]-α-bungarotoxin and removing free toxin on CM-52 mini-columns. The composition of the solution containing these purified com-

plexes was adjusted to match the composition of the standard incubation mixture, and this mixture was counted, then applied to a second mini-column and eluted under standard conditions, and the eluate counted. In the absence of a pre-wash, recovery from columns varied between 58% and 76%. It was found that yield could be improved by pre-washing the mini-columns with 1 ml of 1% BSA. This increased yield to 79-88%. These estimates of yield were employed in calculations of the absolute quantities of AChR in muscles.

3.2.7. The Acetylcholine Receptor Assay Protocol

Extracts of muscle were thawed and 25 µl of each extract in Extraction Buffer was added to 125 µl of 0.1% bovine serum albumin (BSA) and, in some tubes, 0.56 mM or 1 mM gallamine triethiodide as a control for nonspecific binding, all in 10 mM sodium phosphate, pH 7.2. Tubes were incubated for 45 minutes at room temperature after which a solution containing radiolabelled α-bungarotoxin was added to the reaction mixture for a total volume of 250 ul containing 0.15% Triton X-100, 0.07% BSA, 400 pM [^{125}I]-α-bungarotoxin and 280 or 500 uM gallamine (when present), in 14 mM sodium phosphate, pH 7.2. Reaction mixtures were incubated for three hours at room temperature to permit saturation of the receptors with toxin, and the bound and free toxin were then separated by ion-exchange chromatography as follows; 0.2 ml of the reaction mixture was applied to a mini-column of cation exchange resin (described above) and the reaction mixture was washed in with 0.2 ml and eluted with 2.4 ml of Column Buffer. The entire 2.8 ml of eluate was collected in glass scintilation vials and counted in a Packard Auto-Gamma counter at 48% efficiency. Nonspecific binding, determined in the

presence of gallamine, was subtracted from total binding to calculate specific binding. The recovery of Torpedo electroplaque receptor was used to estimate yield of AChR from the columns and this correction was used in all calculations.

3.2.8. Linearity

In the presence of 610 pM [^{125}I]-α-bungarotoxin and with extract of syrinx as a source of AChR, the AChR assay proved to be linear with increasing protein concentration in the incubation mixture, from 0 to 92 µg/ml protein (23 µg) and above, corresponding to 0 to 140 pM AChR (35 fmoles). Subsequent experiments demonstrated that, in the presence of 500 pM labelled toxin and with electroplaque extract as the source of AChR, binding was linear to 160 pM (40 fmoles), but then gradually flattened to a plateau above 1 nM AChR. Data was not used if the concentration of AChRs exceeded 30% of the concentration of total labelled toxin in the reaction mixture.

3.3. OTHER ASSAYS

Acetylcholinesterase was measured according to the method of Johnson and Russell (71; 58), with 230 µM tetraisopropylpyrophosphoramide included in the reaction mixture to inhibit nonspecific cholinesterase. The source of homogenates for measurement of acetylcholinesterase (AChE) varied from one experiment to another, as noted in the text. In most experiments, an aliquot of each homogenate was taken for AChE measurement before incubation to extract AChR. These homogenates were spun for 2 min at maximum speed in a Beckman Model 152 microfuge. In some experiments, AChE activity was measured in the same extracts used for meas-

urement of AChR. Incubation of the extracts for one hour at 30° C did not decrease AChE activity. Protein was determined by the method of Lowry (72).

3.4. SUMMARY

Conditions for extraction of AChR from muscle and for measurement of the binding of [^{125}I]-α-bungarotoxin in these extracts were studied. For further investigations on AChR number in muscle, an assay was chosen which involves solubilization of muscle with non-ionic detergent, incubation of the resulting extract with [^{125}I]-α-bungarotoxin and separation of bound and free toxin on mini-columns of cation-exchange resin. This assay is sensitive enough to measure the small amounts of AChR in a single zebra finch syrinx. Therefore, it was possible to investigate the effect of alterations in the levels of circulating androgens on the number of acetylcholine receptors in the syrinx, in an effort to further characterize the effects of androgens on synapses in syringeal muscles.

4. CHAPTER IV: ANDROGEN EFFECTS ON SYNAPTIC COMPONENTS IN THE SYRINX

4.1. INTRODUCTION

In innervated muscles, acetylcholine receptors (AChRs) are generally localized in the muscle membrane in the junctional region (106; 92; 107; 108; 109) and are absent from motor nerve terminals (110). Thus, measurement of changes in total AChR number provides an assay for postsynaptic effects of alterations in androgens.

Since both the synthetic and degradative enzymes responsible for metabolism of the transmitter acetylcholine decrease in the syrinx after castration (58), I reasoned that the number or size of synapses might decrease after castration, and therefore that the number of AChRs might also decrease. Alternately, if castration exerted its effect on the syrinx by decreasing neuromuscular activity, then castration might cause supersensitivity of the muscles, and the number of AChRs might increase (106; 111). To determine which of these alternatives is correct, I measured AChR number under conditions of hormonal change. I then went on to further characterize the response of the syringeal muscles to changing hormonal levels.

4.2. METHODS

4.2.1. Animals

Zebra finches were obtained from Canary Bird Farms, Old Bridge, New Jersey. All birds were in full adult plumage and good health. Birds were castrated, implanted and denervated as previously described (Section 2.2). Purified AChR from Torpedo electroplaques was the generous

gift of David Anderson, Rockefeller University.

4.2.2. Assays with Muscle Extracts

Birds were sacrificed by decapitation and syrinxes removed as previously described (Section 2.2).

For preparation of muscle extracts, tissues were weighed on ice, rinsed in ice cold phosphate-buffered saline and homogenized in 20 volumes of Extraction Buffer (1.5 % Triton X-100, 50 mM sodium phosphate, pH 7.2) on ice in a 2 ml ground glass-glass homogenizer (VWR, Philadelphia). Homogenates were stored on ice until dissections were completed and an aliquot was then removed for determination of acetylcholinesterase (AChE) activity. The remainder was then incubated at 30°C for one hour and centrifuged for one hour at 4°C in a Beckman 50 Ti rotor at 37,000 rpm or for 10 minutes at room temperature in a Dynac II clinical centrifuge with #271 rotor (Clay Adams) at 2,500 rpm (888 X g). Pellets were discarded and supernatants were frozen over liquid nitrogen and stored at - 40°C until assayed for toxin binding.

AChE activity was measured as previously described (Section 3.3. I would like to thank Eveline Schmidt, Joseph Rhodes and Dr. Victoria Luine for performing the AChE assays presented in this chapter). AChR number was measured as binding of [^{125}I]-α-bungarotoxin with the cation-exchange mini-column assay described above (Section 3.2.7). Non-specific binding was measured in parallel incubations in the presence of 0.6 to 1.0 mM gallamine. In all experiments except the binding curve, mini-columns were prewashed with 0.8 ml of 1.0% bovine serum albumin before application of samples to increase the yield of AChR from the

columns. Estimates of yield were used in calculations of AChR number (Section 3.2.6). Protein was determined by standard methods (72). Statistical tests were performed according to planned comparisons using the Student's t-test, corrected for unequal variances when required, or by two-level ANOVA (112).

4.3. RESULTS

4.3.1. Sex Difference in AChR Number

The first experiment examined sex difference 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 two-fold difference in the weight and a three-fold difference in the Triton-extractable protein content of the syrinxes of males and females (58, Fig. 11). In addition, extracts of male syrinxes bound 3.8 times as much α-bungarotoxin as extracts of female syrinxes (Fig. 11).

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 toxin-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. 12), indicating that there are no differences in the kinetics of toxin interaction with male

Figure 11: Bar graphs demonstrating the sex difference in the weight (A), protein content (B) and AChR number of the syrinx of the zebra finch. Means and standard error of the mean (SEM) of 10 males and 10 females.

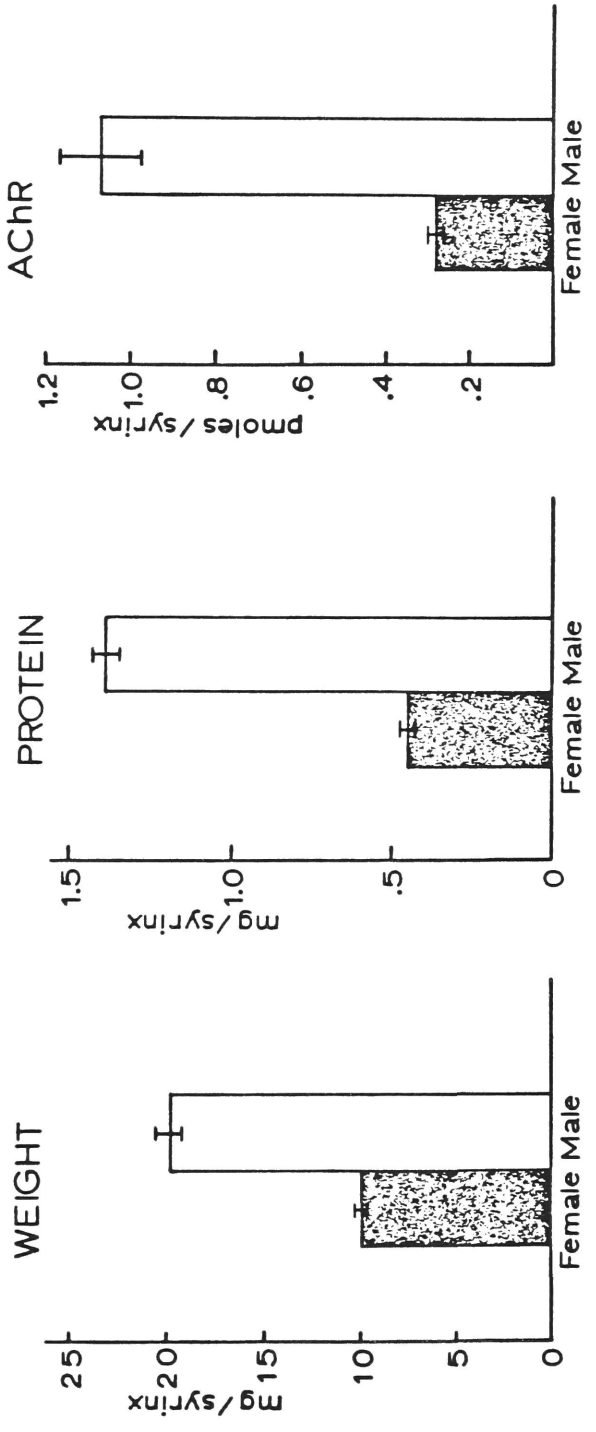
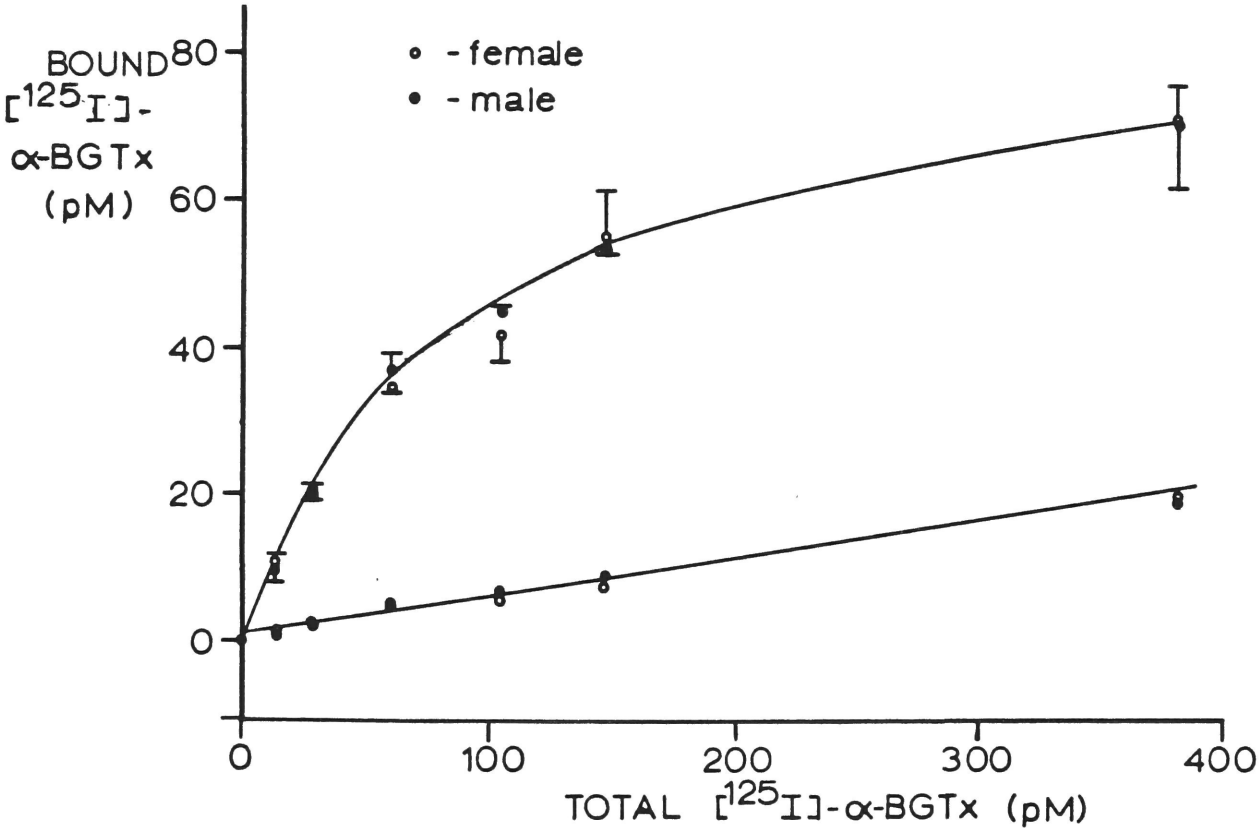


Figure 12: Binding of [^{125}I]-α-bungarotoxin to AChRs from male and female zebra finches. Extracts of several syrinxes from males and females were separately pooled and diluted to 390 pM of toxin binding sites. These pooled extracts were then incubated at a 1/10 dilution with various concentrations of [^{125}I]-α-bungarotoxin in the presence (lower curve) or absence (top curve) of 112 uM gallamine. After three hours, bound and free toxin were separated as described in the text (Section 3.2.8) 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.



and female receptors. This indicates that the difference in toxin binding between male and female reflects a difference in the number of toxin-binding sites; that is, AChR number. In other experiments, syrinx muscle extracts were found to have no effect on the binding of α-bungarotoxin to purified AChR from Torpedo electroplaques, suggesting that syrinx extracts do not contain endogenous inhibitors of toxin binding which might affect measurement of AChR number.

4.3.2. Specificity of Testosterone Effect

In order to examine the specificity of the testosterone effect on the muscles of the syrinx, testosterone's effect on the syrinx was compared to its effect on the muscles of the larynx. No effect of testosterone was observed on these muscles. After 9 days of testosterone treatment, no increase in protein content or AChR number in the larynx was seen (Table 3). This result resembles the lack of a castration effect on muscles of the hyoid complex (58).

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. The cartilage-fraction contained little AChR (3.4% of total for control females) or AChE (9.9% of total for control females), suggesting that it contained little contaminating muscle (Fig. 13). 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 ($p < 0.025$). Treatment of females with testosterone increased total AChR number 2.1 fold ($p < 0.02$) and

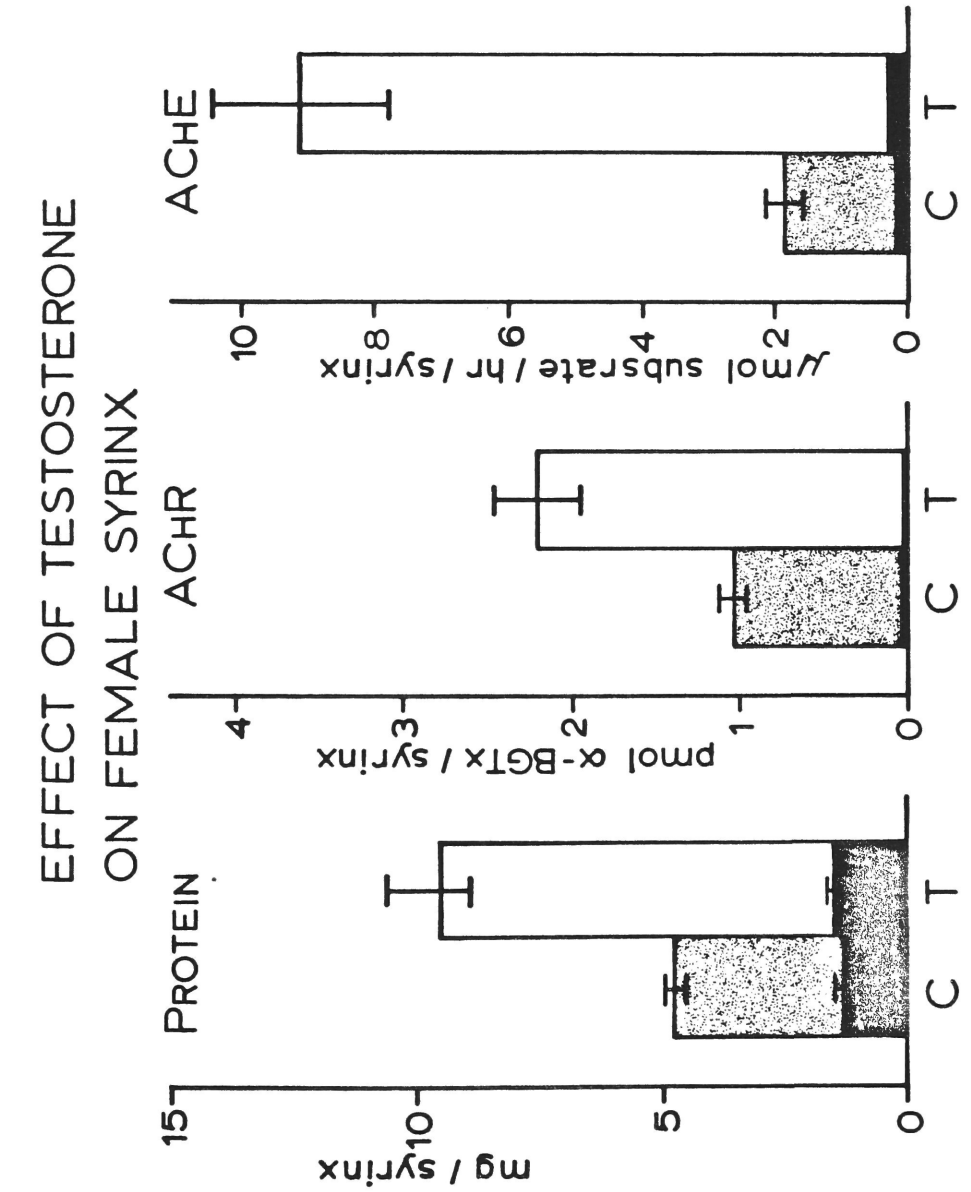
TABLE 3

EFFECT OF TESTOSTERONE ON THE LARYNX
IN THE FEMALE ZEBRA FINCH

	CHOLESTEROL CONTROLS	TESTOSTERONE TREATED
WEIGHT (mg)	15.8 ± 1.5	17.2 ± 0.7
PROTEIN (μg)	983 ± 121	978 ± 44
TOTAL AChR (nmoles)	1.31 ± 0.18	1.46 ± 0.14
AChR / PROTEIN (nmoles/mg)	1.37 ± 0.17	1.49 ± 0.08

Table 3: Effect of testosterone on the larynx in female zebra finches. Females received silastic implants packed with testosterone or cholesterol (as a control) 9 days before they were sacrificed. Each entry is the mean ± standard error of four cholesterol treated birds or three testosterone treated birds. No significant effect of testosterone treatment was seen on any of the parameters shown here. Data on the syrinxes of these birds are presented in Fig. 13.

Figure 13: 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 (clear bars) or cholesterol (stippled bars) as a control. Nine days later they were sacrificed and the syrinxes were divided into a muscle fraction (above, clear and stippled) and a connective tissue fraction (below, dark) and each fraction was assayed for protein, AChE and AChR as described under methods. Means and SEM of 5 cholesterol- and 4 testosterone-treated birds. Muscle fraction protein ($t = 6.73$, $df = 4.2$, $p < .005$), AChR ($t = 4.39$, $df = 3.6$, $p < .02$) and AChE ($t = 5.29$, $df = 3.3$, $p < .02$) differ significantly in the two groups.



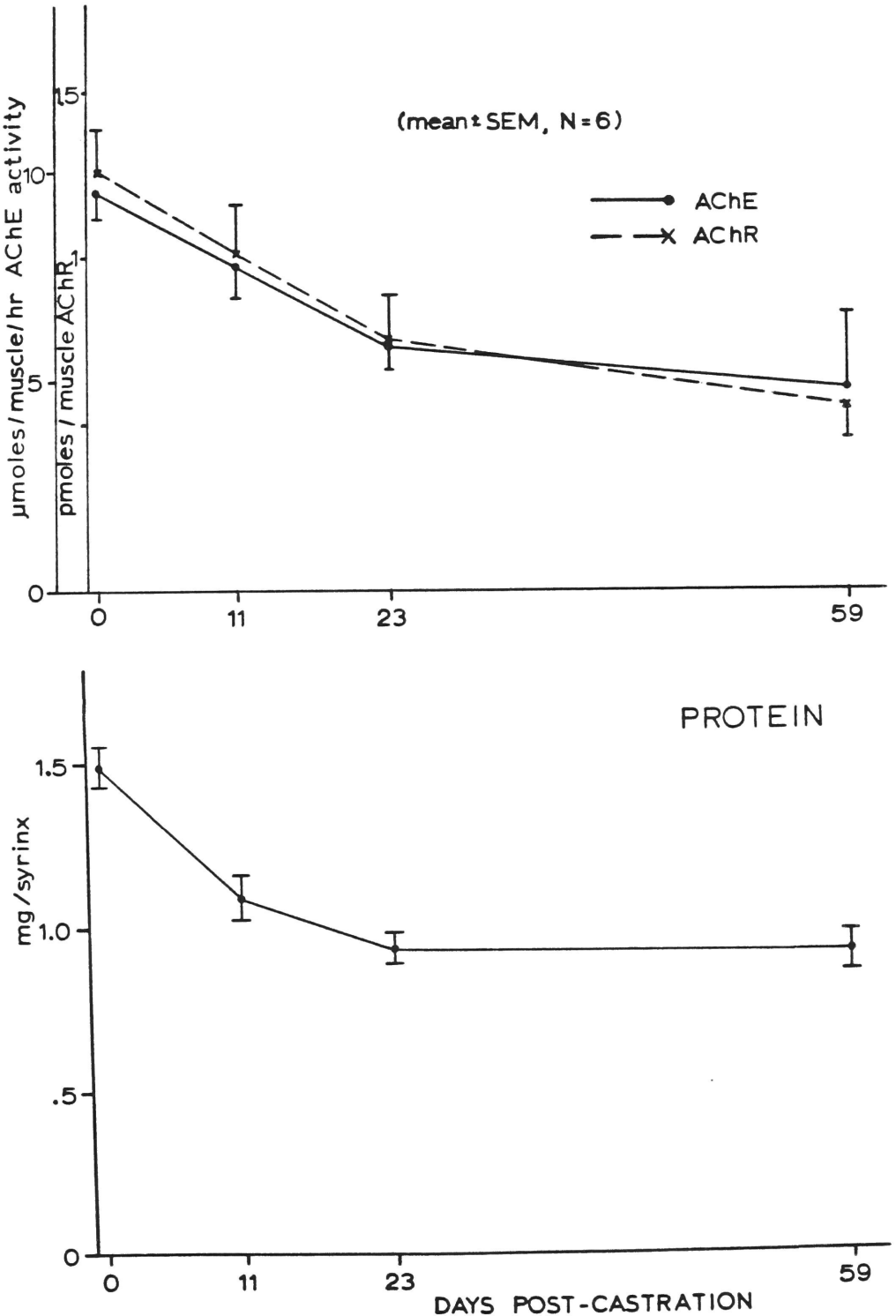
AChE activity 5.0 fold ($p < 0.02$, Fig. 13). When expressed relative to muscle-fraction protein, AChE activity per protein was significantly elevated by 2.2 fold ($p < 0.05$). In contrast, the increase in AChR number was equivalent to the increase in muscle-fraction protein, so that there was no significant change in AChR number per muscle-fraction protein ($p \cong 0.3$, Fig. 13).

4.3.3. Time Course of Hormone Effects

In an effort to elucidate more about the mechanism of testosterone effects on AChR number, I examined the time course of changes in AChR number after changes in circulating androgen levels. In the first series of experiments, male zebra finches were castrated at 59, 23 and 11 days before sacrifice and then sacrificed simultaneously along with intacts ("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. 14). If the loss of protein is modeled by a first-order exponential loss (113), 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 13.2 days. AChE activity and AChR number decreased even more slowly than total protein (Fig. 14), and this is reflected in the apparent half-times for their loss: 15.8 days for AChE and 12.5 days for AChR.

While changes in protein, AChR and AChE occurred slowly after castration, administration of testosterone to females resulted in relatively rapid changes. Protein and AChR number increased more than two fold within five days of testosterone implantation, while total AChE

Figure 14: Effect of castration on protein (A), total AChE activity (B) and AChR number (B) in the syrinxes of male zebra finches. In each experiment, males were castrated sequentially and sacrificed simultaneously. "0" day males were left intact. Assays are described under methods. Each point represents the mean and SEM for six males from two experiments, with three males in each experiment.



increased more than five fold in the same time period (Fig. 15). 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 was much slower to respond to testosterone treatment. AChE activity was not different from controls until three days after implantation, when both total and specific AChE had increased significantly over control levels ($p < 0.01$ for total, $p < 0.05$ for specific).

4.3.4. 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 4). However, AChE activity did increase consistently, and this increase was significant when two experiments were considered together (2-level ANOVA, $F(1,16) = 4.81$, $p < 0.05$, two-tailed test, Fig. 16). The specific activity of AChE did not increase significantly (2-level ANOVA, $F(1,15) = 2.92$, $p \cong 0.11$, Table 4).

4.4. DISCUSSION

The results presented here indicate that the activity of AChE and the number of AChRs both decrease following castration of males and increase following testosterone treatment of females. From this and previous studies, we know that testosterone affects the concentration or activity of several proteins which are involved in the synthesis, bind-

Figure 15: Effect of testosterone on the protein (A), total AChE activity (B) and AChR number (C) in the syrinxes of female zebra finches. Females received testosterone in the form of silastic implants. In each experiment, birds were implanted sequentially and sacrificed simultaneously. Results represent means and SEM from three experiments. Protein and AChR number are significantly elevated after 1 day of treatment ($p < 0.05$, by planned comparison tests, 112, pp. 41-44). AChE activity is significantly elevated after three days of treatment ($p < 0.01$, relative to untreated contols).

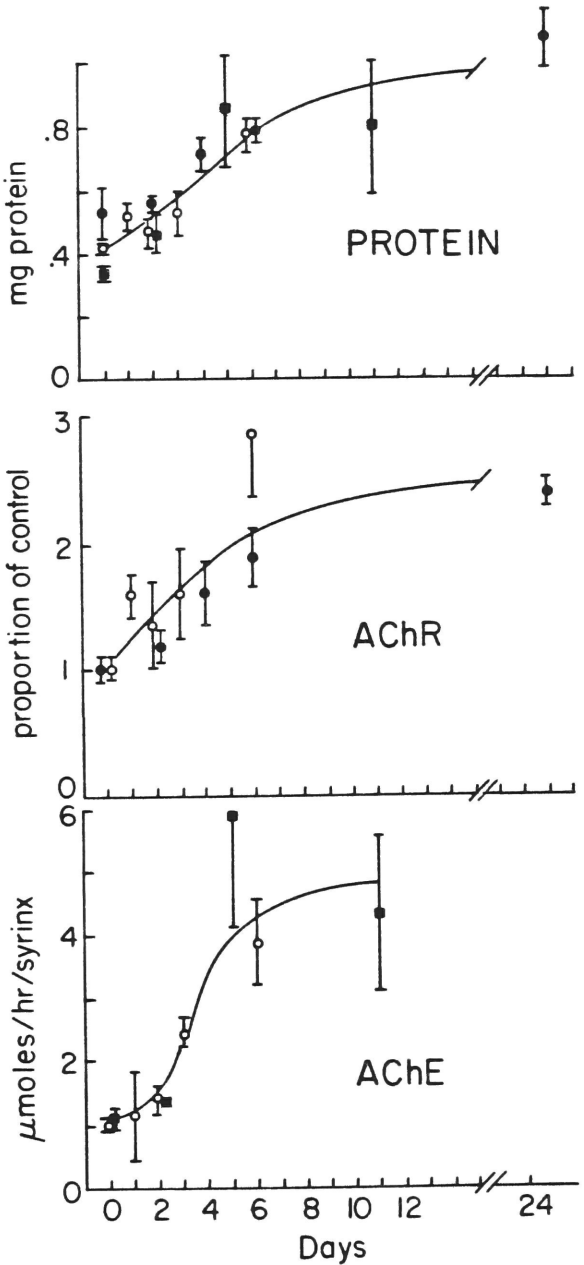


TABLE 4

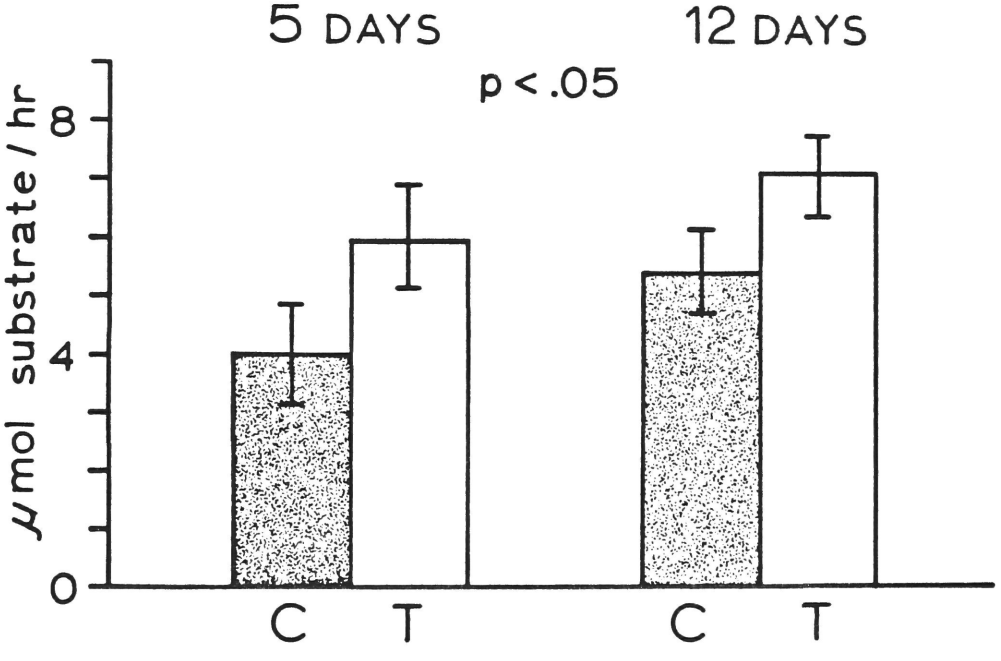
EFFECT OF TESTOSTERONE ON DENERVATED SYRINX
IN THE FEMALE ZEBRA FINCH

	5 DAYS AFTER TREATMENT		12 DAYS AFTER TREATMENT	
	CHOLESTEROL CONTROL	TESTOSTERONE TREATED	CHOLESTEROL CONTROL	TESTOSTERONE TREATED
PROTEIN (μg)	555 ± 28 (6)	579 ± 15 (7)	498 ± 29 (9)	547 ± 24 (8)
TOTAL AChR (nmoles)	0.87 ± 0.17 (6)	1.18 ± 0.13 (7)	2.70 ± 0.84 (9)	2.12 ± 0.43 (7)
AChR / PROTEIN (nmoles/mg)	1.55 ± 0.26 (6)	2.06 ± 0.23 (7)	5.13 ± 0.12 (9)	3.62 ± 0.59 (7)
AChE / PROTEIN (μmoles/hr/mg)	9.60 ± 1.52 (4)	12.18 ± 2.15 (4)	10.74 ± 0.92 (5)	11.87 ± 0.92 (5)

Table 4: 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 five days or twelve days before sacrifice. Entries are means ± standard errors, with sample sizes below in parentheses. All entries are the combined results of two experiments, except for the AChE results, which are from the same experiment as shown in Fig. 16. Total AChR and AChR per protein are significantly greater 12 days after treatment than 5 days ($p < 0.05$ and $p < 0.005$ respectively). No other effects oare significant by 2-way ANOVA (112).

Figure 16: The effect of testosterone treatment on total AChE activity in the syrinxes of denervated females. Females were denervated and simultaneously received silastic implants filled with testosterone (clear bars) or cholesterol (stippled bars) as a control. Five or 12 days later, they were sacrificed and total AChE was measured. Results are means and SEM of 4, 4, 6, and 6 birds each, from left to right. For 5 days, $t = 1.45$, $df = 6$, $p \cong 0.20$, ns. For 12 days, $t = 1.65$, $df = 10$, $p \cong .15$, ns. For the two experiments combined, $F(1/16) = 4.81$, $df = 16$, $p < .05$.



ing and degradation of the transmitter, acetylcholine in the muscles of the syrinx. The effects of testosterone on the syrinx include an increase in the specific activity of the presynaptic enzyme, choline-acetyltransferase (CAT) (58), an increase in the specific activity of the innervation-sensitive "endplate" form of AChE (Section 2.3.4) and an increase in the total number of AChRs, a muscle membrane protein which is highly concentrated at the neuromuscular junction (110). These results suggest that testosterone has a general effect on either the size or number of synapses in the syrinx.

The effect of testosterone is specific to syringeal muscles, since no effect has been observed on muscles of the hyoid complex (58) or larynx. The effect of testosterone on syringeal AChE is apparently 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, 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 may increase the amount of use of the syrinx by its behavioral effects, which are the result of hormone actions on higher brain centers. Therefore, the effects of testosterone may be the result of changes in the activity of the neuromuscular junctions in the syrinx. However, it seems unlikely that this mechanism is entirely responsible for the effects of testosterone on the syrinx, since females treated with testosterone exhibit increased AChE activity and AChR number, even though they do not begin to sing (51). In addition, if testosterone caused an

increase in synaptic activity, this would be expected to cause a decrease in AChR number rather than an increase as observed (111). Thus, testosterone may affect the levels of synaptic components by their 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 activity raises several questions about the pre- and postsynaptic events which occur during synaptic modification. While junctional AChRs are produced only by muscle cells, AChE at the neuromuscular junction is probably derived from both the presynaptic nerve and the postsynaptic muscle cell. CAT is normally only synthesized by the motoneuron. All three of these synaptic components decrease after castration and increase after testosterone treatment, but the mechanisms responsible for these effects must be different. This may be reflected in the differences in the responses of syringeal AChE activity and AChR number to testosterone treatment of females. Both the amount and time course of the changes in these components differ. These differences raise the question of the extent to which the effect of testosterone on AChE activity might be mediated by actions on the motoneurons. To address this question, I examined the response of AChE and AChR to testosterone in the denervated syrinx. After denervation, the syringeal muscles are still capable of responding to testosterone with an increase in AChE, suggesting that the syrinx itself may be directly responsible for at least come of the increase in AChE.

In contrast to the increase in syringeal AChE in females which were

denervated and 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. In the levator ani, three days of denervation prevents the *in vitro* stimulation of amino acid incorporation by testosterone (114). We may conclude that innervation has at least a permissive effect on the expression of the testosterone-sensitivity of protein synthesis in both of these muscles. The testosterone-induced increase in AChE activity in denervated syrinx implies that there may be a specific effect on the synthesis or degradation of AChE under these conditions.

No significant increase in AChR number was observed after testosterone treatment in denervated syrinx, but this observation must be interpreted carefully. After denervation, the number of AChRs on muscle fibers increases dramatically as a result of synthesis of new AChRs (103; 115). These new AChRs differ from existing junctional AChRs in several respects (see 111 for a review). They are not localized at the neuromuscular junction, but occur over the entire muscle surface. In addition, the rate of metabolic turnover of these extrajunctional receptors is much higher than that of the adult junctional receptors. After denervation, existing junctional receptors remain localized at the junctional region and continue to turn over more slowly than extrajunctional receptors (116). The fact that junctional and extrajunctional receptors differ in their metabolic characteristics suggests that 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 syringeal muscle because of the large excess of extrajunctional AChRs. Alternately,

innervation may be necessary for the testosterone-sensitivity of junctional AChR number. Other techniques which allow discrimination of junctional and extrajunctional AChRs may make it possible to determine if there are effects of testosterone on junctional receptors in denervated syrinx.

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 testosterone 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 half-time of loss equal to the rate of degradation (113). Applying this model to the syrinx, castration causes a decrease in syringeal AChRs such that the apparent exponential half-time of loss of AChRs is 12.5 days, for a degradation rate of 5.5% per day. This rate is only slightly less than degradation rates reported for junctional AChRs in innervated muscle (111; 116; 117). This suggests that AChR loss may be the result of a rapid decrease in AChR synthesis, after which existing AChRs are degraded 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 specific protein products (see 113).

In contrast to the gradual decrease of AChR number and AChE activity after castration, AChR and AChE increase rapidly after testosterone-treatment of females. AChR number increases with an

apparent half-time of 3 to 4 days or an exponential rate of increase of 19.8 % per day. In non-stimulated muscle, the exponential rates of appearance and degradation are equal (113; 115), and both would be expected to be on the order of 10 days. The change in the rate of appearance of AChRs after increases in testosterone levels may reflect an increase in both the rate of their synthesis and the rate of degradation, such as would occur if newly synthesized AChRs were rapidly metabolized and had a metabolic turnover-time more like embryonic AChRs than like adult junctional AChRs (see 111). Alternately, it may be that the rate of degradation of AChRs is not altered after testosterone-treatment, but rather that the rate of synthesis changes dynamically, and after an initial burst of rapid synthesis, synthesis decreases to a level slightly higher than before treatment.

The alterations in the amounts of synaptic proteins in syringeal muscles after castration of males and testosterone treatment of females 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. 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.

4.5. SUMMARY

I examined the effect of alterations in the levels of circulating androgens on the number of AChRs in the syrinx. These experiments

demonstrated that there is a sex difference in the number of AChRs in syringeal muscle and that alterations in the levels of circulating androgens can cause changes in the number of AChRs in the syrinx. The time course of these effects was examined after increases in androgens in females and decreases in androgens in males. I also examined the effect of increased androgens in syrinx isolated from neural influences by denervation, and found that isolated muscle did respond with an increase in AChE activity, but not with increased protein content or AChR number.

5. CHAPTER V: ANDROGEN EFFECTS ON AChR NUMBER IN THE LEVATOR ANI

5.1. INTRODUCTION

The effect of alterations of plasma androgen levels on the number of AChRs in the syrinx led me to expect that similar effects would occur in other androgen-sensitive muscles. As outlined above (Section 1.2), the levator ani muscle (LA) of male rats responds to castration with marked atrophy, and this effect can be largely reversed by treatment with exogenous testosterone (27). These changes are accompanied by changes in the amounts of the cholinergic enzymes, acetylcholinesterase (AChE) and choline-acetyltransferase (CAT) (41; 43). Since the number of muscle fibers does not change after these treatments (30), it seems likely that changes occur at individual endplates. Such changes have been described morphologically (118; 43; 119).

On this basis, I expected that the quantity of acetylcholine receptors (AChRs) in the LA might decrease after castration and increase with subsequent testosterone therapy. I used the AChR assay described above to explore this possibility.

5.2. METHODS

CD strain intact male rats of equivalent age were purchased from Charles River. Rats weighed between 250 and 270 grams and were 9 to 10 weeks old when castrated by the abdominal route. Intact rats were sham-operated as a control. In the first series of experiments, after one week of castration, some castrates received daily subcutaneous injections of testosterone propionate (TP, 127 µg/kg body weight) in sesame oil. In these experiments animals were sacrificed two weeks

after castration. Three experiments make up this first series; one comparing intact and castrate males, one comparing intact, castrate and TP-treated castrate males and one comparing castrates and TP-treated castrates. In this final experiment, untreated castrates received injections of sesame oil. In another experiment, after one week of castration, castrates received silastic capsule implants packed with crystaline 5-α-dihydro-testosterone (DHT) or cholesterol. These implants were constructed from silastic tubing (Dow Corning) and measured 1.57 mm in internal diameter, 3.18 mm in outer diameter and 12 mm in length. Implants such as these should produce levels of serum androgens in the range of 1 ng/ml (120). In this experiment, rats were sacrificed 12 days after the initiation of treatment, 19 days after castration.

Rats were sacrificed by decapitation and the levator ani (LA, dorsal bulbocavernosus 25) was blunt-dissected from surrounding connective tissue and sectioned at the point where it enters the mass of the ventral bulbocavernosus. In one experiment, the extensor digitorum longus muscle (EDL) was also removed. Muscles were rinsed in ice cold phosphate buffered saline, weighed, minced and homogenized in ice-cold Extraction Buffer (1.5% Triton X-100, 50 mM sodium phosphate, pH 7.2). In most experiments, muscles were homogenized in a 2 mL Wheaton ground glass-glass homogenizer. In the experiment involving DHT treatment and the EDL muscle, muscles were homogenized with a Brinkman Polytron Model # PT10/35 with a PT10ST probe generator with two pulses of 15 seconds each at setting 7. The Polytron rapidly minces tissue with small shearing blades.

Extracts were incubated for one hour at 30° C before measurement of

AChR number and AChE activity. This incubation did not decrease AChE activity (A. Harrelson, unpublished observation). Approximately 25% of the total AChE activity in homogenates of LA muscle was lost after centrifugation and is presumably insoluble in low-salt solutions (64). AChR number was determined as described above, either with disposable mini-columns without BSA wash, or, in the experiment involving DHT treatment and the EDL muscles, with permanent mini-columns washed with 1 ml of 1.0% BSA (Section 3.2.7). Protein was determined by standard methods (72).

Data from single experiments were analyzed by analysis of variance (ANOVA) with one-level design using the Newman-Keuls test or by planned comparison tests using the Student's t-test (112). Because of differences in baseline values between experiments, when results from two experiments were combined, data was analyzed by ANOVA with two-level design, with treatment as one level and experiment as the other (112). $p < 0.05$ was considered significant. All standard deviations are N-1 weighted.

5.3. RESULTS

5.3.1. Muscle Weight and Protein

Two weeks of castration caused a 50.6% decrease in the weight ($p < 0.005$, Table 5A) and a 51.1% decrease in the protein content of the LA ($p < 0.005$, Table 5A). Castration also decreased the variance of muscle weight and protein ($p < 0.001$, Table 5A), but not the coefficient of variation. In rats castrated for one week, one week of treatment with TP prevented some of this decrease in weight and protein. The effect of

TP treatment was not significant in any single experiment, but was significant when results from two experiments were combined (Table 5B). TP treatment increased muscle weight by 20.4% relative to untreated castrate levels ($p < 0.025$, Table 5B) to 58.4% of intact levels (Fig. 17A). TP treatment increased muscle protein by 20.9% over untreated castrate levels ($p < 0.05$, Table 5B) to 55.1% of intact levels (Fig. 17B). TP treatment also significantly increased the variance of weight and protein from those of castrates ($p < 0.05$, Table 5B).

19 days of castration caused a 59% drop in muscle weight ($p < 0.005$, Fig. 18A). and a 63% drop in muscle proteins ($p < 0.01$, Fig. 18B). DHT treatment initiated one week after castration increased muscle weight 50% over untreated castrate levels ($p < 0.01$, Fig. 18A) to 62% of intact levels. Similarly, DHT treatment increased muscle protein 44% over untreated castrate levels ($p < 0.001$, Fig. 18B) to 54% of intact levels.

5.3.2. Acetylcholine Receptors

The LA of 260 gram intact male rats contained 11.8±2.4 fmoles AChR/mg wet weight or 153±33 fmoles AChR/mg protein (mean and SD). These values correspond to Triton-extractable AChRs. Some proportion of AChRs may not be detergent extractable under these conditions (see Section 3.2.2). This corresponds to 2.79±0.48 pmoles of AChR per muscle. Two weeks of castration caused a 47.8% decrease in total AChR number ($p < 0.001$, Table 5A). In castrated male rats, one week of TP treatment partially prevented this decrease. As was the case with weight and protein, this effect of TP was not significant in any single experiment, but was significant when the results from two experiments were analyzed

TABLE 5

RESPONSE OF THE LEVATOR ANI
A: EFFECT OF CASTRATION

PARAMETER	INTACT (Means and SD)	CASTRATE (Means and SD)	DIFFERENCE (%)	TREATMENT EFFECT
Weight (mg)	242 (29)	119 (12)[a]	-50.6	$F(1,13) = 181$ $p < 0.005$
Protein (mg)	18.5 (2.1)	9.0 (1.0)[b]	-51.1	$F(1,14) = 195$ $p < 0.005$
Total AChR (pmoles)	2.79 (0.48)	1.51 (0.19)[c]	-47.8	$F(1,13) = 74$ $p < 0.005$
AChR/Protein (fmoles/mg)	153 (33)	168 (21)	+10.0	$F(1,20) = 1.85$ n.s., $p = 0.21$

Table 5: Effects of castration and TP treatment on the levator ani.

A. The effect of two weeks of castration on weight, protein, AChR number and AChR number/protein. Results from 2 experiments were combined, each with 6 rats in each treatment. Hypothesis testing was done by 2-level ANOVA, with experiment as one level and treatment as the other. Only treatment effects are reported here. The F test was performed, with correction of degrees of freedom to account for inhomogeneity of variances (112, pp. 205-206).

a-e Variances significantly different according to the F test with degrees of freedom 10 and 10.

a $F=5.84$, $p < 0.01$.

b $F=4.41$, $p < 0.01$.

c $F=6.38$, $p < 0.01$.

d $F=4.00$, $p < 0.05$.

e $F=3.06$, $p < 0.05$.

TABLE 5

B: EFFECT OF TP TREATMENT ON CASTRATES

PARAMETER	CASTRATE (Means and SD)	CASTRATE + TP (Means and SD)	DIFFERENCE (%)	TREATMENT EFFECT
Weight (mg)	125 (15)	151 (30)[d]	+20.4	$F(1,14) = 6.94$ $p < 0.025$
Protein (mg)	10.4 (1.6)	12.6 (2.8)[e]	+20.9	$F(1,15) = 5.43$ $p < 0.05$
Total AChR (pmoles)	1.44 (0.32)	1.83 (0.38)	+27.2	$F(1,20) = 7.62$ $p < 0.025$
AChR/Protein (fmoles/mg)	142 (22)	153 (22)	+7.4	$F(1,20) = 1.39$ n.s., $p = 0.25$

Table 5: Effects of castration and TP treatment on the levator ani.
B. The effect of one week of treatment with testosterone propionate (TP) after one week of castration on weight, protein, AChR number and AChR number/protein. Combined results from 2 experiments, each with 6 rats in each treatment. Hypothesis testing as in the legend to Table 5(A).

Figure 17: Results from a single experiment comparing wet weight (A) or Triton-extractable protein (B) from the levator ani of intact male rats, castrates and castrates treated with testosterone propionate (TP). Rats were castrated or sham operated two weeks before sacrifice and treatments were begun one week after castration. Results are from the same experiment as presented in Figs. 19 and 21. Values represent means with standard errors of the means (SEM) from 6 rats. ** indicates different from castrates and TP-treated castrates, $p < 0.01$ as per 1-level ANOVA with Newman-Keuls test 112, pp. 185-196). Castrate and TP-treated groups do not differ significantly in this experiment when it is considered alone ($q_{2,15} = 2.62$ and 2.30 for weight and protein respectively, $p \cong 0.1$ by Newman-Keuls test), but are significantly different when analyzed in combination with a replicate experiment. See Table 5B.

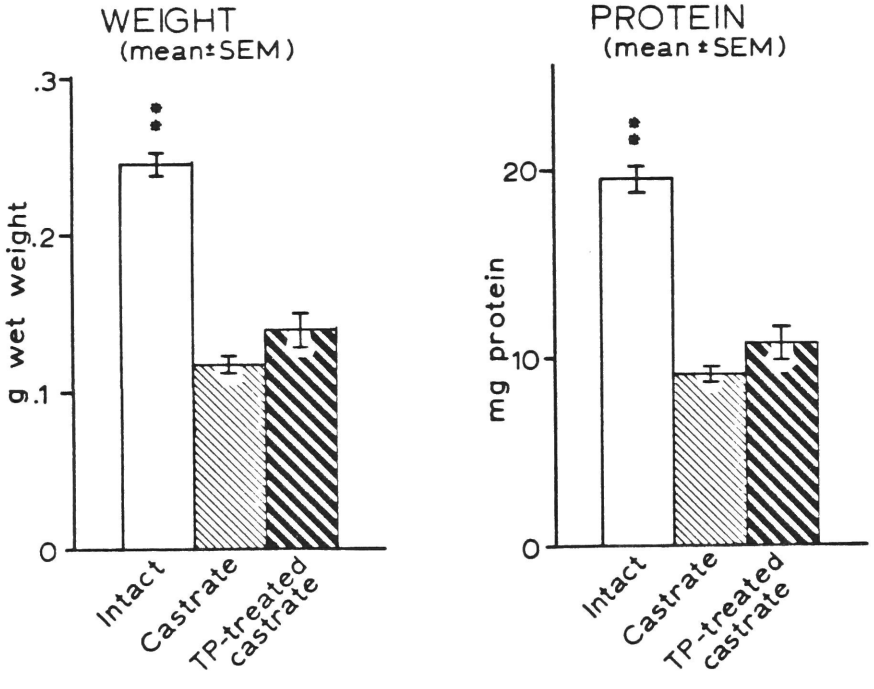
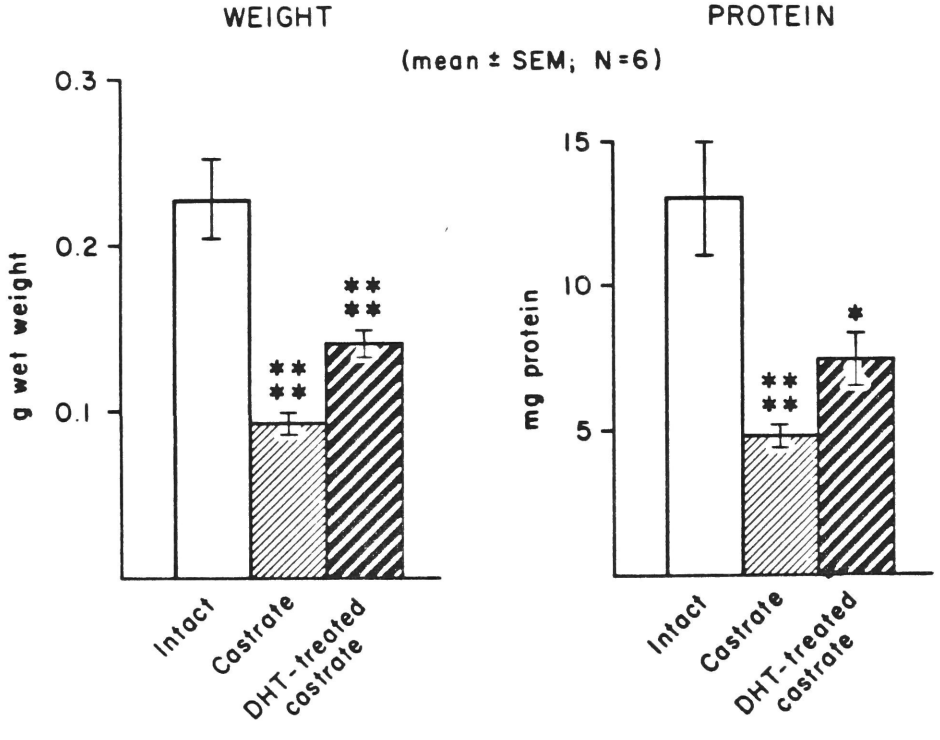


Figure 18: Results from a single experiment comparing wet weight (A) or Triton-extractable protein (B) from the levator ani of intact male rats, castrates which received silastic implants packed with cholesterol (as a control) and castrates treated with silastic implants packed with dihydrotestosterone (DHT). Males were castrated or sham-operated 19 days before sacrifice and castrated males received silastic implants 7 days after castration. Results are from the same experiment as presented in Figs. 20 and 22. Values represent means with standard errors of the means from 6 rats. * indicates different from preceding group at $p < 0.05$, as per planned comparison with Student's t-test 112, pp. 41-44). **** indicates different at $p < 0.001$ level.



together ($p < 0.05$, Table 5A). TP increased receptor number in castrates by 27.2% over untreated castrates (Table 5B) to 69.3% of intact levels (Fig. 19A). Because the changes in total AChR number paralleled the changes in muscle protein, there was no significant effect of any treatment on receptor number per protein. Castrate levels were on average 10.0% higher than intacts (ns, Table 5A), and TP-treated levels were 7.4% higher than untreated castrates (ns, Table 5B). Since TP-treatment of castrates apparently slowed the loss of AChR number more than the loss of protein, AChR number per protein was 26.0% higher in TP-treated castrates than in intacts (ns, Fig. 19B).

Nineteen days after castration there was a 64% decrease in total AChR number ($p < 0.005$, Fig. 20A). DHT treatment increased total AChR levels 68% over castrate levels to 57% of intact levels ($p < 0.01$ and $p \cong 0.2$, Fig. 20A). AChR number per protein was not significantly different in any of the groups. Castrate levels of AChR number per protein was 11% of intact, while DHT treatment increased levels by 18% to 105% of intact (ns, $p \cong 0.3$, Fig. 20B). Curiously, castration caused a 2.6 fold increase in the standard deviation of AChR per protein ($p < 0.005$, Fig. 20B). DHT caused a nonsignificant drop of 18% in the standard deviation from the value for castrates (ns, Fig. 20B).

5.3.3. Acetylcholinesterase

Two weeks after castration, total Triton-extractable AChE was unchanged from intact levels (ns, Fig. 21A). Because total protein decreased dramatically, specific AChE was 2.17 times higher in castrates than intacts ($p < 0.01$, Fig. 21B). Under these conditions, TP treatment had no significant effect on Triton-extractable AChE activity. TP-

Figure 19: Number of acetylcholine receptors in the levator ani of intact rats, castrates and castrates treated with TP injections. Results from the same experiment as presented in Figs. 17 and 21. AChR number was measured as described in the the text (Section 3.2.8) and is expressed as pmoles of [^{125}I]-α-bungarotoxin binding either in the entire levator ani (A) or per mg protein (B). Values represent means with standard errors from 6 rats. ** indicates different from castrates and TP-treated castrates, $p < 0.01$, as in Fig. 17. AChR number per muscle for castrate and TP-treated groups do not differ significantly in this experiment ($q_{2,15} = 2.74$, $p \cong 0.1$), but are significantly different when analyzed in combination with a replicate experiment. See Table 5B.

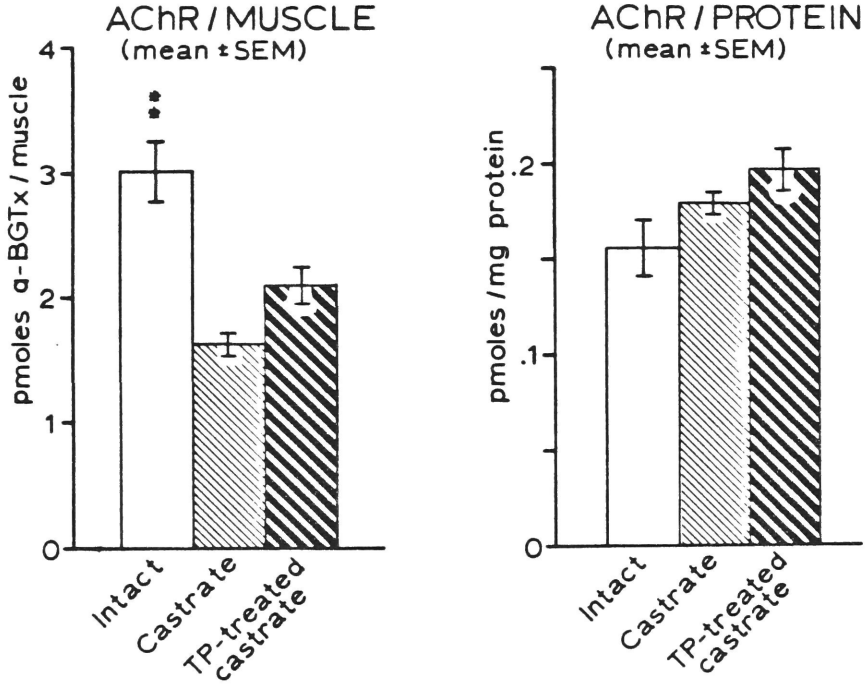


Figure 20: Number of acetylcholine receptors in the levator ani of intact rats, castrates and castrates treated 5-α-dihydrotestosterone implants. Results from the same experiment as presented in Figs. 18 and 20. AChR number was measured as described in the text (Section 3.2.8) and is expressed as pmoles of [^{125}I]-α-bungarotoxin binding either in the entire levator ani (A) or per mg protein (B). Values represent means with standard errors from 6 rats. ** indicates different from preceding group at $p < 0.01$, **** indicates $p < 0.001$, as in Fig. 18.

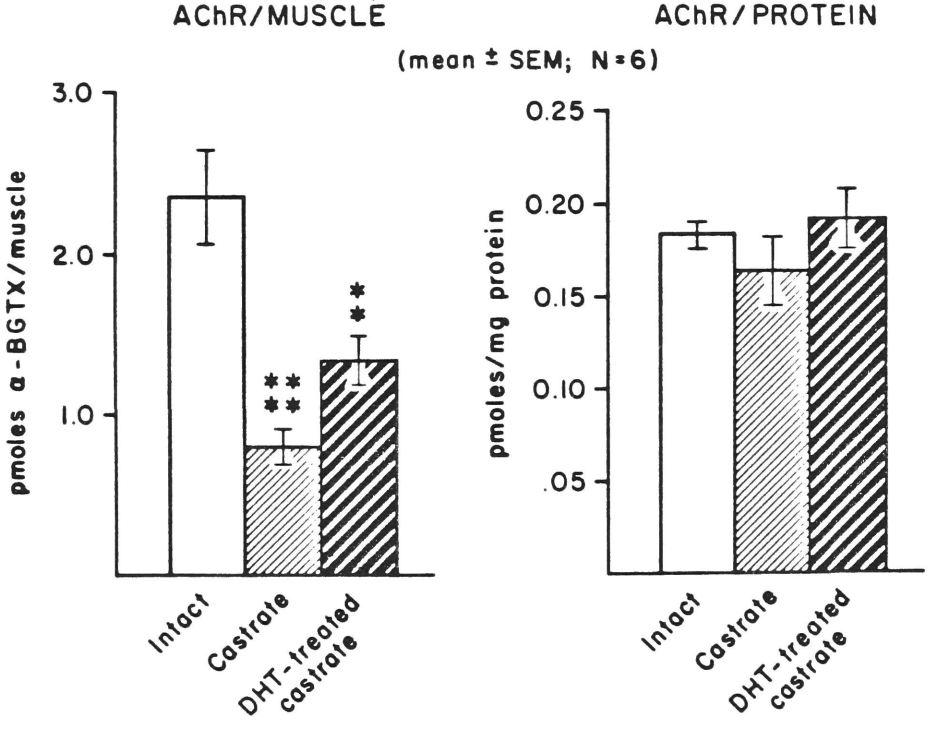
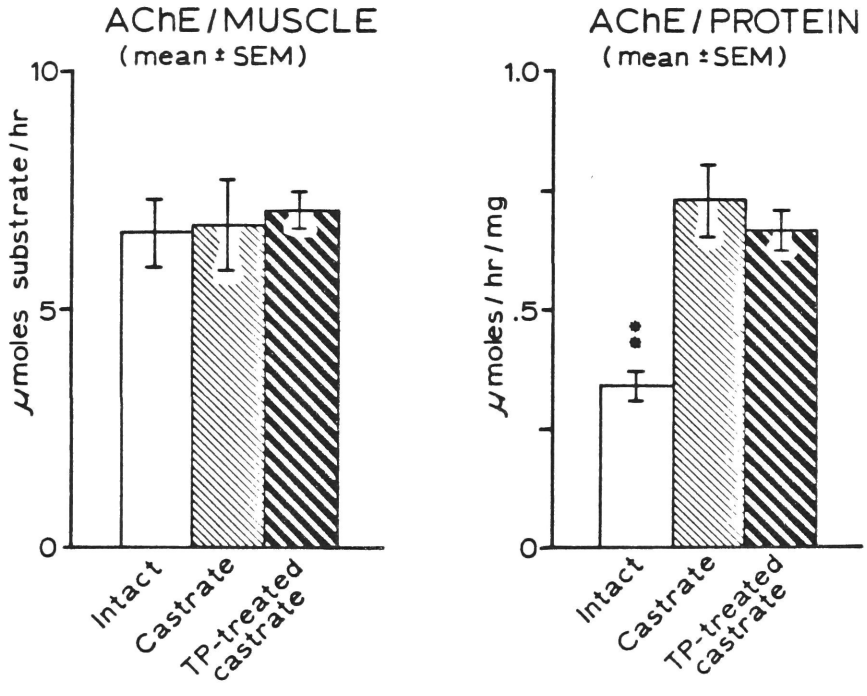


Figure 21: Activity of Triton-extractable acetylcholinesterase in the levator ani of intact rats, castrates and castrates treated with TP. Results from the same experiment as presented in Figs. 17 and 19. Enzyme activity is expressed as μmoles of [^{3}H]-acetylcholine degraded per hour in the entire levator ani (A) or per mg protein (B). Values represent means with standard errors from 6 rats. ** indicates $p < 0.01$, as in Fig. 17.



treated castrates had 4.5% more total AChE than untreated castrate levels (ns, Fig. 21A) and 8.6% less specific activity (ns, Fig. 21B).

19 days of castration also had no significant effect on total AChE activity. The castrate group had 5% less AChE activity than sham-operated intacts (ns, Fig. 22A). Again, specific activity increased dramatically after castration, to 2.6 times intact levels ($p < 0.01$ Fig. 22B). DHT treatment had no significant effect on AChE activity. DHT-treated castrates had 13% less AChE than castrates; 83% of intact levels. The DHT-treated group had 36% less AChE per protein than the castrate group ($p < 0.5$, Fig. 22B) and 1.7 times more than the intact group.

Changes in the specific activity of AChE follow the inverse of protein closely and reflect the effect of androgen alteration on protein and the absence of such an effect on total AChE activity.

5.3.4. Specificity of Effects to the Levator Ani Muscle

No effect of castration on weight, protein, AChR number or AChE activity was observed for the extensor digitorum longus muscle, a muscle which is not notably sensitive to castration or androgen treatment (121 , Table 6).

5.4. DISCUSSION

The three hour incubation of AChR extracts with [^{125}I]-α-bungarotoxin allowed sufficient time for reaction of toxin with all the AChR present. Therefore, the differences reported here represent differences in the number of toxin-binding sites (AChR), and not in the kinet-

Figure 22: Activity of Triton-extractable acetylcholinesterase in the levator ani of intact rats, castrates and castrates treated with silastic implants packed with DHT. Results from the same experiment as presented in Figs. 18 and 20. Enzyme activity is expressed as μmoles of [^{3}H]-acetylcholine degraded per hour in the entire levator ani (A) or per mg protein (B). Values represent means with standard errors from 6 rats. **** indicates $p < 0.001$, as in Fig. 18.

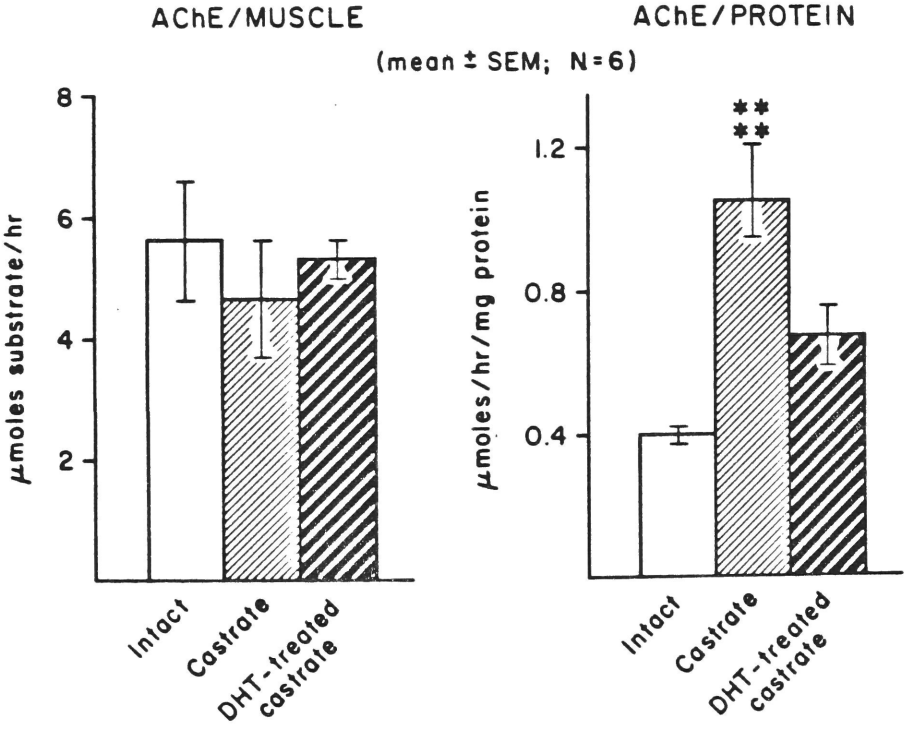


TABLE 6

EFFECT OF CASTRATION ON
THE EXTENSOR DIGITORUM LONGUS MUSCLE OF THE MALE RAT

	SHAM-OPERATED INTACTS	CASTRATES
WEIGHT (mg)	166 ± 7.3	168 ± 4.5
PROTEIN (mg)	1.11 ± 0.07	1.15 ± 0.04
TOTAL AChR (fmoles)	99.0 ± 5.2	99.7 ± 6.7
AChR / PROTEIN (fmoles/mg)	89.7 ± 1.6	86.2 ± 3.2

Table 6: Effect of castration on the extensor digitorum longus muscle of male rats.

Rats were castrated for 19 days before sacrifice. The levator ani was also removed from these animals, and this data is included in Figs. 19, 21 and 23. No significant differences between castrates and intacts were seen for any parameter measured.

ics of interaction of α-bungarotoxin and receptor. Others have reported no differences in the kinetics of interaction of α-bungarotoxin with AChR from the LA muscles of castrated and intact rats (91).

Chin and Almon (91) have also examined the effect of castration on AChR in the LA and EDL muscles. They reported that castration of adult male rats caused an increase in AChR number per gram weight in both muscles which peaked at about 2 weeks after castration and then declined. In this study, I found no significant difference in AChR number per protein in the LA of intact and castrated males, 14 and 19 days after castration, although protein may have decreased slightly more than AChR number. No effect of 19 days of castration was seen on any parameter measured for the EDL muscle. The reason for the differences in the results of these two studies are not clear, although they may be related to methodological differences. For example, rats were apparently not sacrificed simultaneously in the Chin and Almon study, and therefore may have differed in age. In addition, the scrotal castrations performed in that study may be more of a source of infection or local irritation than the abdominal castrations used here.

The short term effects of castration on the levels of AChE and AChR in the LA are surprisingly different. Whereas AChR number decreases in parallel with the decrease in total muscle protein, AChE activity remains remarkably constant, despite the pronounced atrophy of the muscle. In the syrinx, castration causes a loss of AChE activity with a half-time of disappearance of 16 days (Section 4.3.3). The constancy of total AChE activity in the LA after two weeks of castration suggests

that either this enzyme is remarkably stable, or, despite the general decrease in other proteins, new AChE is still appearing in the muscle at a high rate. Other studies of the LA have shown a significant drop in AChE after long-term castration (41; 43; and A. Harrelson, unpublished observation). Perhaps the major portion of AChE in the LA is neuronally derived and therefore is not affected as rapidly by castration. Even if castration causes a decrease in the synthesis of AChE by the motoneuron, the arrival of the enzyme at the muscle by axonal transport might continue at intact rates for some time after castration.

The AChE activity reported here represents Triton-extractable AChE, and does not include the contribution of low-salt insoluble AChE, which is lost during centrifugation (64; 70). It is entirely possible that the insoluble component, which makes up 25% of the total AChE activity in crude homogenates, constitutes the bulk of the endplate AChE activity, and that therefore the activity measured here and in previous studies is primarily nonsynaptic. Because of this, and the contamination of earlier measurements of choline-acetyltransferase in the LA by carnitine-acetyltransferase (44), the demonstration of changes in AChR number in the LA after changes in circulating testosterone levels is important evidence of effects of this hormone on a specifically synaptic product.

It remains to be determined if the effect of testosterone is specific or is the result of a general anabolic effect on this muscle. That AChR number changes in parallel with protein content suggests that either testosterone has a general, nonspecific effect on protein synthesis, or AChR number is regulated to reflect muscle fiber volume. The fact that AChE activity does not change in parallel with muscle protein

suggests that there may be some specificity to the effects of testosterone, and that AChR number may be specifically regulated to parallel muscle growth and atrophy.

The number of muscle fibers in the LA does not change after castration (30; 31). Since AChRs are restricted to the endplates in innervated fast muscles (108; 110) the changes in AChR number in the LA after castration imply that there are either changes in the number of AChRs per endplate or in the number of endplates per muscle or both. Since the fibers of the LA apparently have one terminal each (43 and Section 6.1.3), castration cannot affect the number of endplates per muscle fiber in the LA. Therefore, the number of endplates in the LA is probably constant in adulthood, and changes in total AChR number imply changes in the number of AChRs per endplate. Thus castration causes a decrease in the number of AChRs per endplate in this muscle, and treatment with androgens counteracts this effect. These changes at the level of individual endplates provide a novel system for the study of plasticity at synapses.

5.5. SUMMARY

The levator ani muscle of male rats provides a neuromuscular system in which both the muscle and its motoneurons have high levels of androgen receptors. Castration causes a loss of acetylcholine receptors in this muscle. Treatment with androgens after castration partially reverses this effect. These changes in AChR number parallel changes in muscle protein content. In contrast, castration and testosterone treatments of castrates has no effect on total, Triton-extractable acetylcholinesterase activity. Since it is known that the number of fibers in

the LA does not change after castration or testosterone treatment, these changes in AChR number are strong evidence for effects of androgens at the level of the single synapse.

6. CHAPTER VI: ANDROGEN EFFECTS ON SINGLE ENDPLATES

6.1. EFFECT OF CASTRATION ON ENDPLATES : LEVATOR ANI

6.1.1. Introduction

Alterations in the levels of synaptic components in the levator ani (LA) and the syrinx might reflect several changes at the level of individual synapses. Changes in androgen levels might cause changes in 1) the amount of each component present at a single endplate, 2) the number of endplates per muscle fiber, and/or 3) the number of muscle fibers per muscle. In the syrinx, all three hypotheses seemed possible, therefore I first examined the androgen-sensitivity of endplates in the LA of the rat. The LA provides several advantages for these studies. The muscle fibers are robust and provide excellent dissociated preparations after fixation. The endplates, as defined by cholinesterase staining, are large, have simple discrete boundaries and are relatively homogeneous in structure. Since the number of fibers per muscle and the number of endplates per fiber probably do not change with changes in testosterone levels (Section 5.4), it follows that the number of endplates per muscle is also constant. Therefore, the change in total AChR number after castration and androgen treatment can be tentatively ascribed to a change in the number of AChR per endplate. I set out to quantify this change using histological techniques.

6.1.2. Methods

CD strain intact male rats (Charles River) were castrated or sham-operated by the abdominal route, then sacrificed by decapitation ten weeks after operation, and LA muscles were removed as previously

described (Section 5.2). Muscles were pinned under moderate tension in Sylgard-filled dishes in ice-cold Millonig phosphate-buffered saline (MPBS; 0.5% glucose, 120 mM Na phosphate, pH 7.2) and fixed in 4% glutaraldehyde in MPBS for 1 hour at 4° C (122). After fixation and rinsing, the muscles were minced into squares 1 cm X 1 cm and dissociated for five sec with a Brinkman Polytron (Model PT10/35 with PT10ST probe generator) at setting 6 in 3 ml of MPBS. Clumps were removed and homogenized in another 3 ml, and the procedure was repeated. The total 9 ml of fiber preparation was combined and stored at 4° C until processed further.

Staining of fibers to demonstrate cholinesterase was performed by drying 1 ml or less of a fiber suspension onto a chromalum-gelatin coated slide and covering the dried fibers with 1 ml of Karnovsky's reagent for cholinesterase stain, then incubating for 30-50 min at room temperature (123; 124). The slides were then rinsed in water, dried on a slide-drier at 50° C, counter-stained with Fast Green, dehydrated in graded ethanol baths, cleared in xylene and mounted under Permount. 200 uM eserine, an inhibitor of AChE activity, was included in the Karnovsky's reagent in some cases as a control. Eserine at this concentration inhibits AChE and prevents the appearance of the brown reaction product of the Karnovsky reaction, but does not affect nonspecific staining.

[^{125}I]-α-bungarotoxin was obtained from New England Nuclear and used without further purification. For AChR visualization, suspensions of muscle fibers were incubated with 5 nM [^{125}I]-α-bungarotoxin in MPBS with 0.1% bovine serum albumin (BSA) for 1.5 hrs at 37° C with constant

agitation. In parallel incubations, 100 uM curare was included as a control, to block the binding of α-bungarotoxin to AChRs. Fibers were pelleted and the supernatant removed and replaced with ice-cold MPBS / 0.1% BSA. This washing was repeated until the radioactive counts remaining in the pelleted fibers no longer decreased with further washing (usually 5 to 6 washes). The fibers were then resuspended in distilled water and dried onto coated slides as above. They were then dipped in Kodak NTB2 emulsion and processed for autoradiography according to standard procedures (125). Fixation with 4% glutaraldehyde resulted in a much higher background of nonspecific labelling with [^{125}I]-α-bungarotoxin than did fixation with 2% paraformaldehyde. However, endplates were still recognizable and grain density quantifiable.

In order to estimate AChR density, the density of silver grains in endplates viewed en face was measured. This was done with the aid of an Artek Colony Counter, Model # 880 with a video camera attached to a Zeiss microscope. Endplates were located under dark-field illumination, then counted under light-field illumination at 200 X by automatically counting all grains in a rectangle 3 μm by 16 μm. This rectangular field was located in the middle of the region of dense grains and the fiber was oriented, with a rotating microscope stage, so that the long axis of the rectangle was longitudinal to the fiber (Plate 2A).

6.1.3. Results

In order to quantify one parameter associated with the size of endplates, the longitudinal length of cholinesterase stain at endplates was measured with an ocular micrometer. The diameter of each fiber was

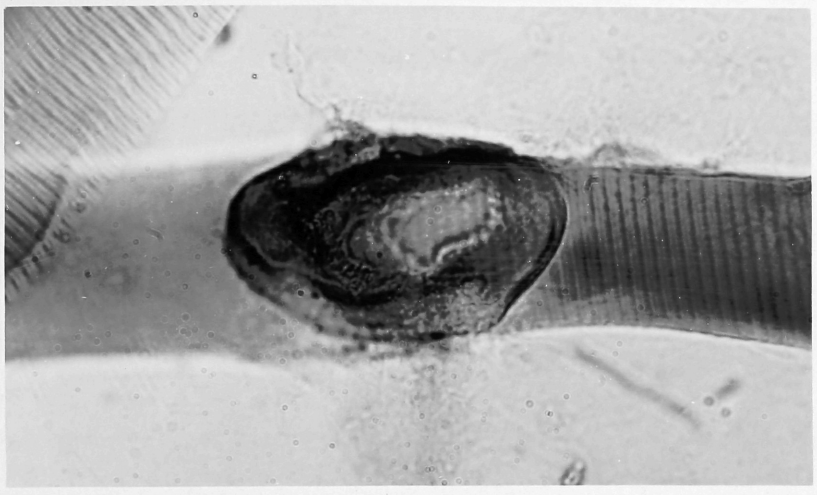
measured in a region near the endplate. Ten endplates were measured from each of three castrated rats and three sham-operated rats. Fiber diameters were significantly different. The mean diameter for intacts was 33.0 ± 1.4 (mean ± SEM, N=30), while for castrates it was 17.8 ± 1.2 (N=30). Endplate lengths were not significantly different. Mean endplate length for intacts was 51.7 ± 1.9 (N=30), while for castrates it was 50.9 ± 1.8 (N=30). There was a significant correlation between endplate length and fiber diameter for castrates ($r = 0.437$, $p < 0.01$, N=30) but not for intacts ($r = -0.262$, $p \cong 0.08$, N=30).

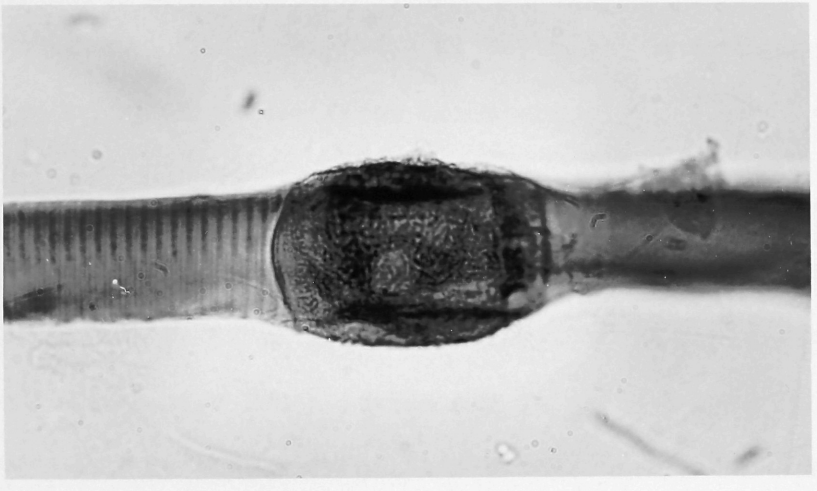
The surface area covered by the endplate is a difficult parameter to measure in the LA, since the endplate width often exceeds fiber diameter and endplates often wrap around the fibers (Plate 1). Endplate width (measured circumferentially) does not appear to be greatly altered in castrates. Many endplates on castrate fibers appear to have collapsed around the muscle fiber as it shrank in size (Plate 1C), while others occur on large swellings of the fiber.

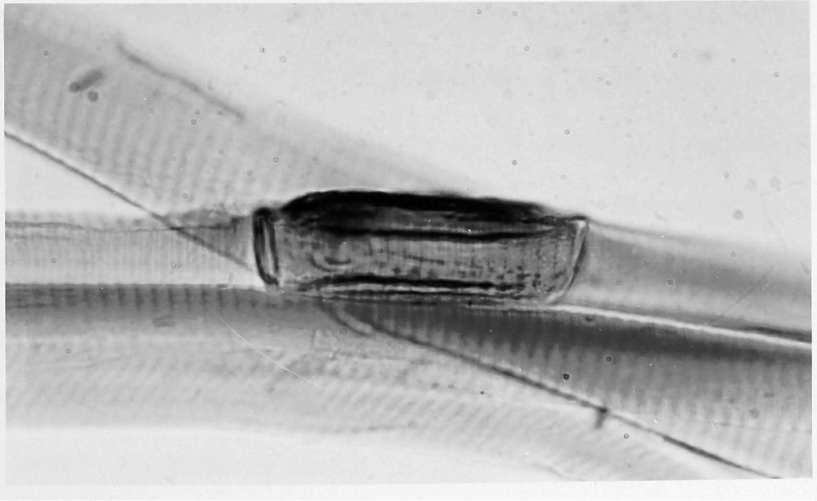
No fibers were ever found to have more than one endplate. Small spots of cholinesterase reaction product were often seen near the endplate on fibers from intacts (14 of 22 endplates), but were never observed on fibers from castrates (0 of 20 endplates).

To visualize AChRs, dissociated fibers were labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography. Grain density was measured for several endplates from each of 2 intact and 3 castrate rats. Intacts were found to have at least 13.5 ± 0.9 grains / rectangular field (mean ± SEM, N = 12), while castrates had at most 8.9 ± 1.3 grains/field (N = 15); 34% fewer than intacts ($t = 2.85$, $df = 25$, $p <$

Plate 1: Effect of castration on cholinesterase at levator ani endplates. Endplates on dissociated muscle fibers from the levator ani of (A) an intact male rat and (B and C) a male castrated 10 weeks before sacrifice. Fibers were dissociated and stained for cholinesterase as described in the text (Section 6.2.2). Magnified 875 times.







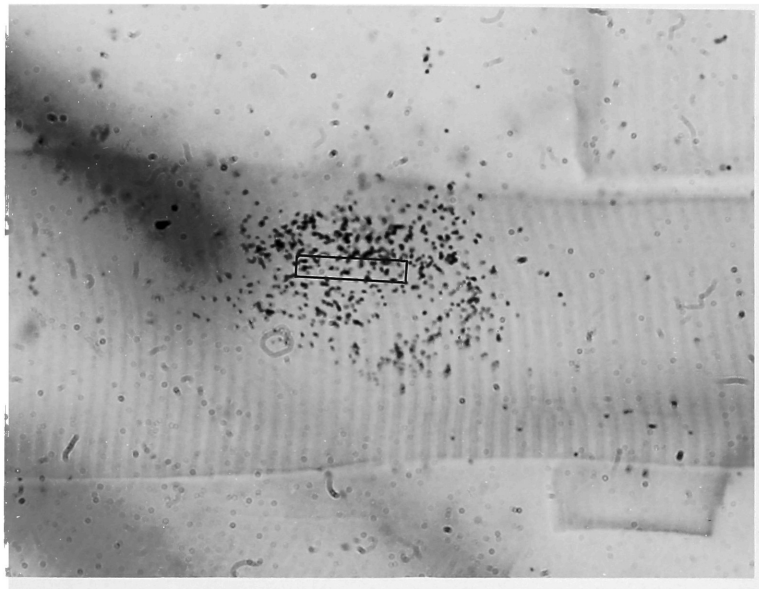
0.01; Plate 2). These values may be biased estimates, since grain counts on endplates with extremely dense AChRs may be too low, due to saturation of the automatic counting apparatus, while endplates with very low grain densities may have been missed. Overall, these two factors will tend to reduce the measured difference between castrate and intact, relative to the actual difference in AChR density.

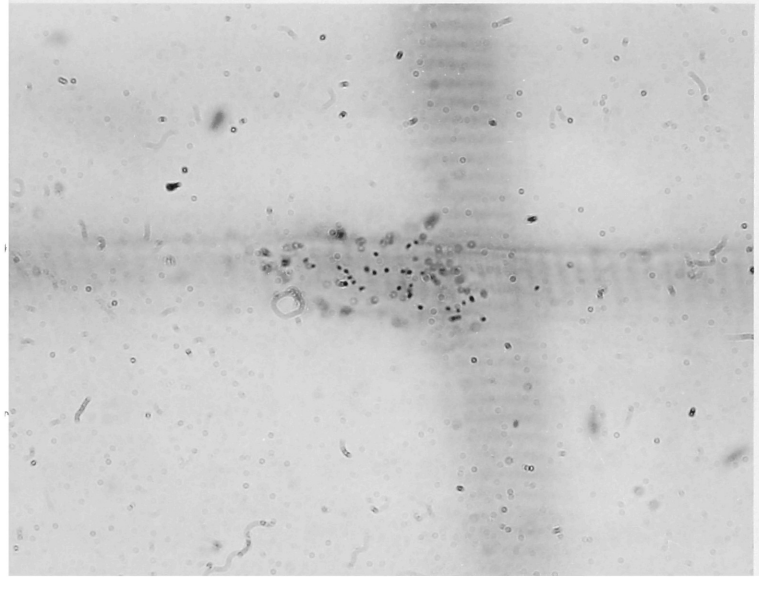
6.1.4. Discussion

The decrease in the diameter of fibers in the LA after castration which was observed in this study agrees well with the results of Venable (126), who found that the size of the LA decreased after castration as a result of a decrease in muscle fiber diameter.

Despite the large change in fiber diameter after castration, the area covered by cholinesterase at endplates in the LA does not appear to decrease substantially, if at all. Synaptic AChE is largely or entirely localized in the synaptic basal lamina and is quite stable in some respects, remaining in the extracellular matrix even after the muscle and nerve have been killed and degraded (80). Perhaps castration has no effect on the localization of AChE which has already been bound in the synaptic cleft. However, after long-term castration, total AChE activity in the LA does decreases (43 and A. Harrelson, pers. comm.). Since the size of the cholinesterase rich region of endplates does not appear to change, this decrease must either represent a decrease in non-synaptic AChE or a decrease in the density of AChE per synaptic area. This later phenomenon could be the result of decreased complexity and depth of the post-junctional folds, and this has indeed been described in the LA muscles of rats castrated 1 month after birth and

Plate 2: Effect of castration on AChRs at levator ani endplates. AChR distribution at an endplate on a dissociated muscle fiber from (A) the levator ani of an intact male rat and (B) a male castrated 10 weeks before sacrifice. The overlay in (A) illustrates the field in which grains were counted for quantification of autoradiograms. Fibers were dissociated, labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography as described in the text (Section 6.2.2). Magnified 875 times.





examined 6 months later (41) .

The correlation within castrate groups between length of endplate cholinesterase and fiber diameter agrees with correlations of endplate size and fiber size which have been published previously (127; 128; 129). This correlation does not appear to extend to the short-term effect of castration on the LA, after which the fiber diameter falls, while the area covered by synaptic cholinesterase apparently remains relatively constant.

Small spots of cholinesterase were often found near the endplate on fibers from intact rats, but were never observed on fibers from castrates. These spots resemble those seen in other muscles at the ends of small sprouts growing out from motor nerve terminals (130). In this light, it is interesting that testosterone may induce sprouting from nerve terminals in the LA muscle of castrated rats (119). Perhaps testosterone stimulates terminal sprouting in the LA.

In mammalian muscles, AChRs at the neuromuscular junction are largely restricted to the ridges of the postjunctional folds, those regions closest to the presynaptic terminal. The decreased density of autoradiographic grains over endplates in the LA after castration suggests that either the density of AChRs on the receptor-rich regions of the postjunctional folds has decreased, or that the proportion of the postsynaptic region made up by receptor-rich membrane has decreased. It is impossible to distinguish between these two possibilities with the techniques used in these studies. Another alternative must also be considered. The synaptic cleft of the neuromuscular junction is filled with a dense extracellular matrix or basal lamina. Some of this

material remains at the endplate after fiber dissociation and this material may restrict the access of molten photographic emulsion to the postsynaptic membrane. It is possible that this effect is more pronounced in preparations from castrates than in those from intacts, although there is no a priori reason to assume this is so.

In the LA, a decrease in the density of AChRs in the synaptic region may be partly responsible for the drop in AChRs after castration. However, the area of the endplate which is densely covered with AChRs may also decrease. Although the area covered by cholinesterase stain was not observed to change after castration, synaptic AChE and AChRs are not necessarily coextensive. It may be that in the area of the synapse which is packed with AChRs can decrease without a change in the localization of junctional AChE. Such a decrease in AChR-rich area may be another source of the decrease in total AChRs after castration, in addition to the decrease in density of AChR within the receptor-rich region.

6.1.5. Summary

In the LA, the decrease in AChR number following castration which was detectable biochemically reflects a change in the number of AChRs at individual synapses, and this change can be demonstrated histologically. In addition, this histological study revealed new information about the nature of this change, indicating that the decrease in AChRs occurs in part by a reduction in the density of receptors in the synaptic region.

6.2. ENDPLATE MORPHOLOGY IN SYRINGEAL MUSCLES

6.2.1. Introduction

Biochemical studies have shown that androgens act to increase the activity of AChE (58) and the total number of AChRs in syringeal muscle (Section 4.3.2). Several hypotheses can be proposed to explain these hormone-related alterations. As a first step to test these various possibilities, I decided to look for synaptic and muscle fiber differences in the syrinxes of adult male and female zebra finches. My rationale was that the two sexes offer natural, physiological extremes in adult blood androgen levels (58). Syringeal parameters that did not differ between the sexes would be unlikely candidates for hormonal regulation in adulthood. Conversely, parameters that did differ between the two sexes might differ by virtue of hormonal regulation in adulthood, or by virtue of organizational effects of steroids acting during development.

6.2.2. Methods

6.2.2.1. Materials

Zebra finches were the generous gift of Dr. Cheryl Harding (American Museum of Natural History) and were bred from outbred stock. All birds were in good health and in full adult plumage when sacrificed. [^{3}H]-α-bungarotoxin was obtained from Amersham. [^{125}I]-α-bungarotoxin was obtained from New England Nuclear and used without further purification.

6.2.2.2. Tissue Preparation for Muscle Fiber Counts

Syrinxes were fixed for two days in Bouin's-Hollande sublime (5%

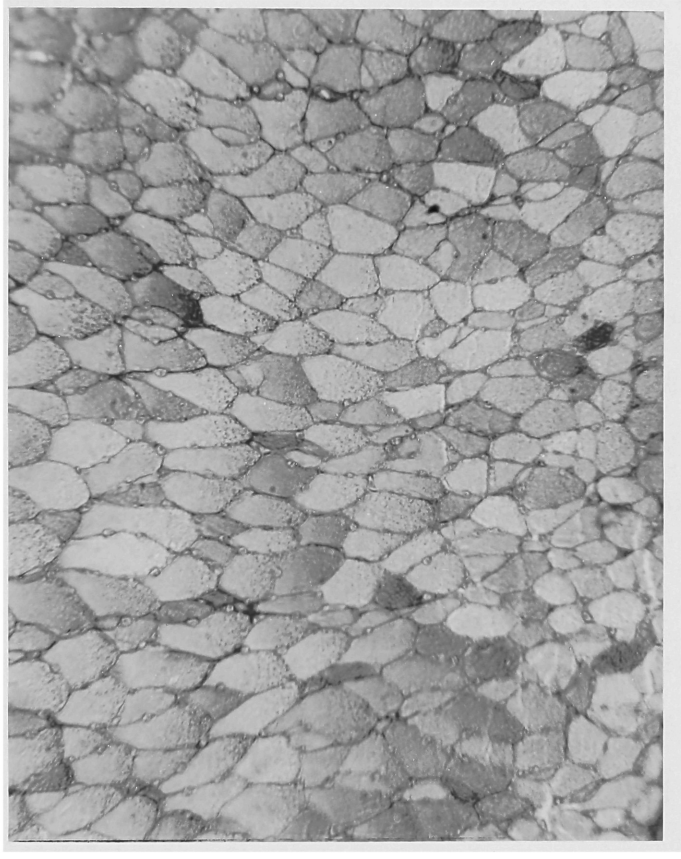
picric acid, 3% paraformaldehyde, 2.1% copper acetate, 1.7% trichloroacetic acid and 0.75% mercuric chloride) and decalcified in Jenkin's reagent (131) for 7-9 days, then embedded in Paraplast according to standard procedures and cut at 12 µm with a rotary microtome and steel knife. To visualize fiber boundaries, sections were stained using the periodic acid-Schiff's (PAS) procedure (131). After periodate oxidation, Schiff's reagent reacts with glycosylated components, which are particularly concentrated in the extracellular matrix. This outlines each muscle fiber with dense pink stain, making counting possible (Plate 3). Haematoxylin was used as a counterstain. With this counterstain, nonmuscle cells can be differentiated from muscle fibers by their large, densely stained central nuclei. Fibers were counted at 40X with a camera lucida and drawings were made to keep track of fibers counted.

Fiber counts were performed on the ventral muscle bundles (m. syringeus ventralis and m. tracheolateralis ventralis; 45) in syrinxes cut in cross-section. These counts were made at a point approximately 200 um rostral to the union of the bronchial lumina. The influence of the exact rostro-caudal level on fiber counts was examined. A section of a male syrinx at the level previously described had 2320 fibers in its right ventral bundle while a section 192 µm rostral to this had 2354, a difference of 1.5%.

6.2.2.3. Visualization of Single Neuromuscular Junctions

Inspired by the technique of *in vitro* labelling of neurotransmitter receptors (132), I used a similar approach for *in vitro* labelling of AChRs on frozen sections of muscle. 20 µm frozen sections of muscle were mounted on chromalum coated slides and incubated in [^{125}I]- or

Plate 3: Muscle fibers in the syrinx. A photomicrograph of a cross-section of the ventral syringeal muscles from a male zebra finch, illustrating the delineation of fiber boundaries. 12 µm paraffin section stained by the Periodic Acid-Schiff procedure and counter-stained with Ehrlich's Haemotoxylin. Magnified 350 times.



[^{3}H]-α-bungarotoxin (Amersham) in the presence or absence of 25 uM curare as a competitor. Incubation and wash was in 100 mM sodium phosphate, pH 7.2 with 0.1% BSA. In preliminary experiments, after incubation and washing, 4 sections were wiped from each slide and counted in 5 ml of Liquiscint (National Diagnostics) in a Packard Scintilation counter. In this way, specific and non-specific binding were calculated. The concentration of toxin, temperature and time of incubation, and wash conditions were all systematically varied. The protocol used in subsequent labelling was chosen to give saturation of specific binding with a minimum of nonspecific. On this basis, a 1.0 hour incubation in 1 nM radiolabelled α-bungarotoxin followed by washing with a quick dip and then a 15 min soak was chosen as a standard procedure. For processing for autoradiography, after incubation and washing, slides were dipped in distilled water, dried on a 50° C hot plate and dipped in NTB3 (Kodak) emulsion. After appropriate exposure times, slides were developed according to standard procedures (125).

To further analyze the distribution and structure of synapses in the syrinx, a technique for visualizing synapses on single fibers was developed. This was based on the procedure of Robbins et al. (122) for dissociating muscle fibers. Muscles and whole syrinxes were removed, pinned under moderate stretch in Sylgard-filled dishes and fixed for 30 min in 2% paraformaldehyde over ice, for 20 min in 2% paraformaldehyde at room temperature or for 30 min in 1% paraformaldehyde / 1% glutaraldehyde at room temperature. Specific methods are detailed in figure legends. All fixatives were made up in Karnovsky's Phosphate-buffered saline (KPBS, 128 mM dextrose, 90mM sodium phosphate, pH 7.2). Preliminary experiments indicated that there was no qualitative difference in

the binding of bungarotoxin to fibers prepared after fixation with 2% paraformaldehyde and fibers prepared after fixation with 2% paraformaldehyde, 100 mM lysine and 10 mM Na periodate (133). Tissue was rinsed with KPBS and syringeal muscle was carefully dissected away from cartilage. Muscle fibers were then dissociated with a Polytron at setting 5.5 as described above (Section 6.2.1). Muscle was dissociated for 15 or 30 seconds in 2 ml of KPBS, the Polytron bit was rinsed twice for 15 sec with 2 ml of KPBS, and the 6 mls of fiber suspension were combined and stored at 4° C until processed further.

For visualization of cholinesterase and AChR distributions on muscle fibers, preparations of dissociated fibers were stained histochemically or processed for autoradiography. For cholinesterase stain, 1 ml or less of this fiber suspension was dried onto cleaned, chromalum-gelatin-coated slides on a slide-drier at 50° C. The dried fibers were then covered with 1 ml of Karnovsky's reagent for AChE stain (123; 124) and incubated for 20 min at room temperature and processed as described above (Section 6.1.2).

For AChR visualization, suspensions of muscle fibers were labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography as described above (Section 6.1.2), except that KPBS was substituted for MPBS.

Lengths of cholinesterase stain and fiber diameters were measured with a camera lucida and computer aided digitizing tablet at 320X. Grain counts of autoradiograms were performed by eye under oil immersion at 1250X. All grains were counted within a rectangular area 1.9 X 38.1 um, oriented with long axis parallel to the longitudinal axis of the

fiber (Plate 9A). The longitudinal extent of the AChR-rich membrane was measured at 1000X by taking the boundaries of this region as the points on the fiber where the longitudinal distance between grains fell to 2 μm; half the average intergrain distance in nonsynaptic regions of the fibers.

6.2.3. Results

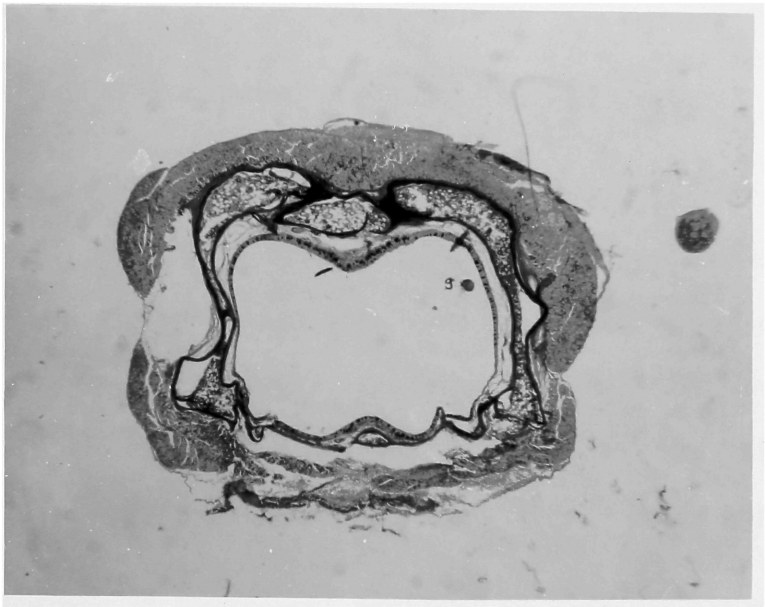
6.2.3.1. Fiber Numbers

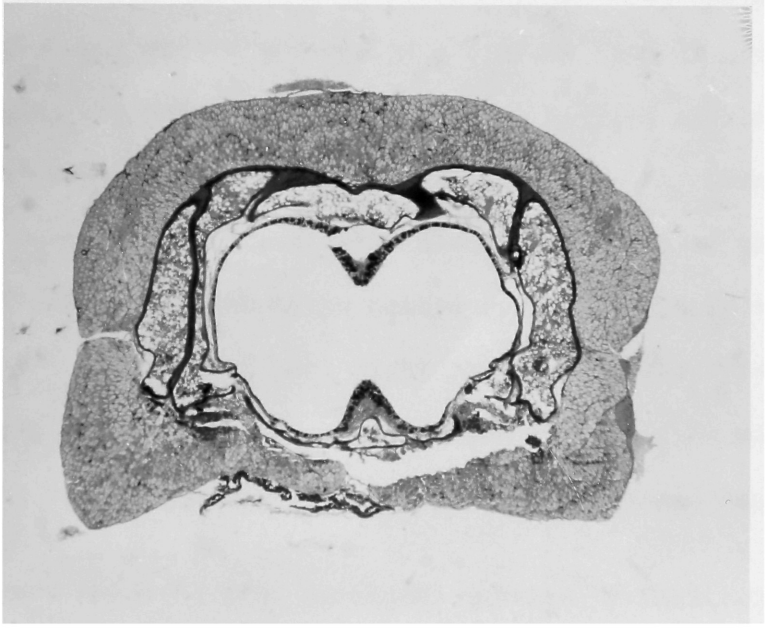
Cross sections of male and female zebra finch syrinx were examined for evidence of a sex difference in fiber number. Two females had 2377 and 2272 fibers in their right ventral muscle bundles, while two males had 2320 and 2515 fibers, for a difference of 3.9 % (Plate 4).

In the canary, there was a small difference in the numbers counted in the right and left muscle bundles in the syrinxes. The male's right ventral bundle had 2049 fibers, while his left had 2304, a difference of 11.7%. Similarly, the female's ventral bundles had 1840 (right side) and 2082 (left side) fibers, for a difference of 12.3%. This suggestion of a right-left difference is interesting in light of the lateralization of song production in the canary (134).

Since the muscle masses of male syrinxes are much greater than those of females and sex differences in fiber number apparently contribute little to differences in muscle mass, it follows that the size of muscle fibers should be larger in males. This was confirmed by measurement of fiber diameters in preparations of dissociated fibers. Average fiber diameters for two female zebra finches was 11.6 ± 0.5 μm and 9.4 ± 0.5 μm (mean ± SEM, N = 42 and 55). For a male, fiber diameter was

Plate 4: Sex differences in syrinx size. Transverse sections of the syrinx, of a female (above) and male (below) zebra finch, illustrating the sexual dimorphism in muscle mass. Sections prepared as in Plate 3. Magnified 29.8 times.





significantly greater, with a mean of 19.9 ± 1.0 μm (N = 41). A male zebra finch castrated 12 months before sacrifice had significantly lower fiber diameters than the intact male, with a mean of 12.2 ± 0.7 μm (N = 58; Fig. 23).

6.2.3.2. Pattern of Terminals on Syringeal Muscle Fibers

If differences in fiber number are not responsible for differences in the amount of synaptic components between males and females, this would imply that synapse number per fiber or the amount of synaptic material per synapse is different in males and females. In order to visualize synapses, horizontal sections of syrinxes were labelled with radiolabelled α-bungarotoxin and processed for autoradiography.

Autoradiograms of horizontal sections of syrinxes labelled with radiolabelled-α-bungarotoxin revealed a diffuse band of dense clusters of silver grains extending laterally from the midline across the mid-region of the ventral syringeal muscle mass (Plate 5). Dense clusters of receptors were also seen scattered along the length of the tracheolateralis muscles and throughout the entire syringeal muscle mass. This suggested that some muscle fibers might have multiple endplates. To further explore this issue, the distribution of endplates was examined on dissociated fibers processed to demonstrate cholinesterase and AChRs.

Fibers were prepared from syringeal muscles by fixation and dissociation with a Polytron as described above. These dissociated fibers were then stained to demonstrate cholinesterase or labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography. The distribution of cholinesterase stain on these fibers suggests that there is a

Figure 23: Histograms of fiber diameters for the syringeal muscles from four zebra finches. Data from an intact male, 2 intact females and a male castrated 12 months before sacrifice are shown. Diameters were measured in preparations of dissociated muscle fibers prepared as described in the text. Axes are labelled in micrometers. Intact fibers are significantly larger than all other groups (female 1, $t = 2.31$, $df = 81$, $p < 0.001$; female 2, $t = 3.49$, $df = 96$, $p < 0.001$ and castrated male, $t = 6.19$, $df = 97$, $p < 0.001$)

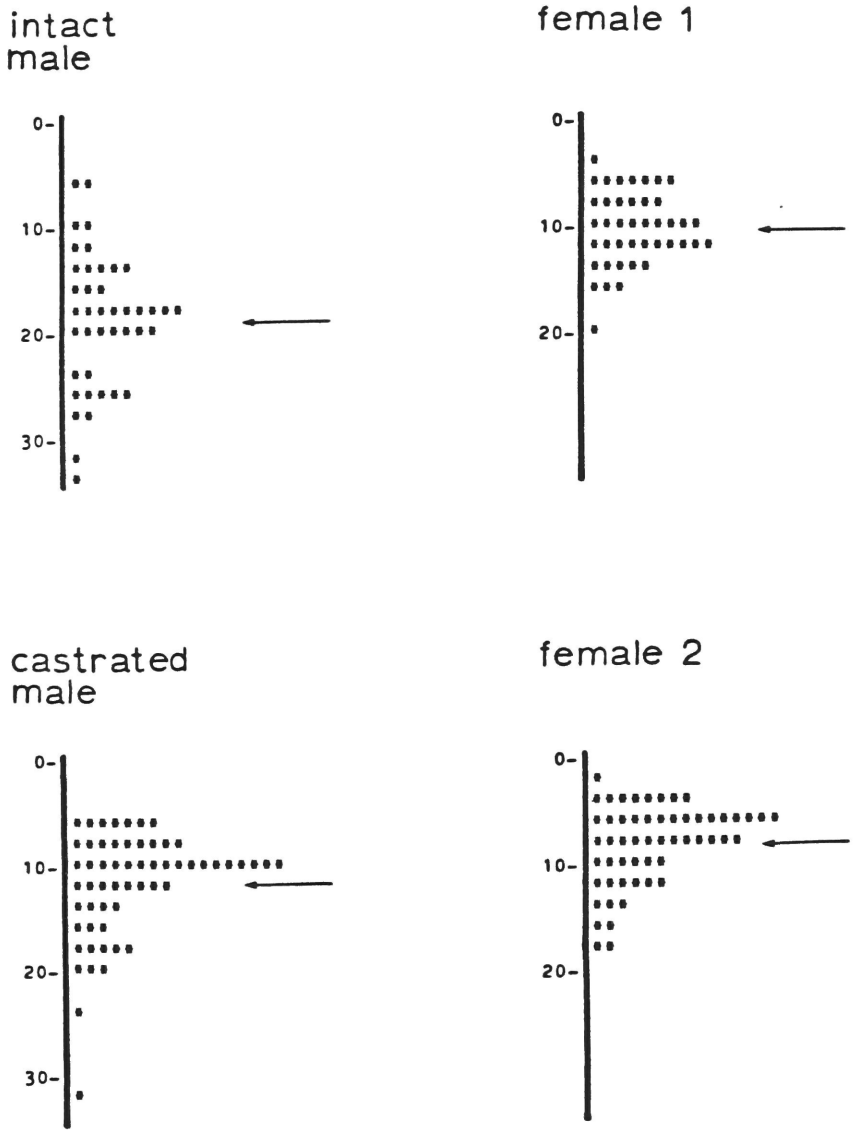


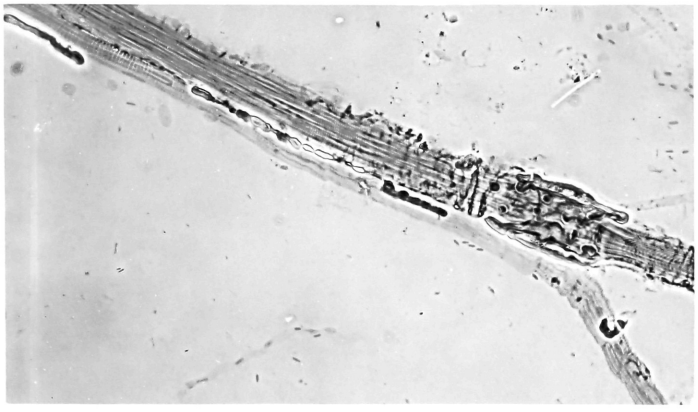
Plate 5: AChR distribution in the syrinx. A horizontal section of the syrinx of a male zebra finch, illustrating the distribution of AChRs. A 32 μm frozen section was incubated in [^{3}H]-α-bungarotoxin and processed for autoradiography as described in Section 6.2.2.3. In this photomicrograph, prepared with dark-field illumination, silver grains indicating AChRs appear as bright specks. Endplates appear as dense accummulations of these specks. Magnified 85.2 times.

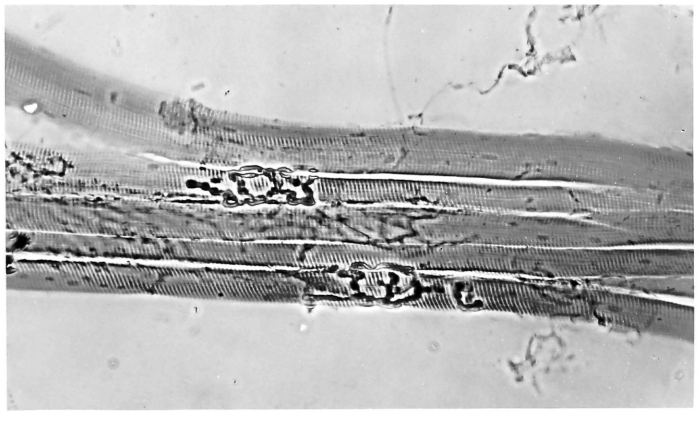
large amount of heterogeneity in the pattern of innervation of different muscle fibers in the syrinx. The majority of stain appears as large, dense ramifications of finger-like projections, which presumably represent single synapses (Plates 6A). Fibers with one of these large endings were never also observed to have other dense patches of stain, suggesting that these fibers are single-terminal fibers. Other fibers have cholinesterase scattered along their lengths, in a pattern that suggests that these are multi-terminal fibers (Plates 6A). What may represent a third class of endplate appears to be a single long track of cholinesterase resembling the Endbuschel ending of frog muscles (135).

The distributions of reaction product on fibers stained for AChE is similar to the distribution of silver grains seen in autoradiograms of fibers labelled with [^{125}I]-α-bungarotoxin. The majority of fibers had large dense clusters of AChR, which in some cases appeared to form lattice-like patterns resembling the pattern of cholinesterase stain described above. Other fibers had smaller clusters scattered along their lengths (Plate 7A). This class of fibers also had a higher background of silver grains over their entire surfaces. This diffuse labelling did not appear in controls in which competitors blocked specific binding, indicating that it represents specific binding to AChRs.

For comparison, I examined the pattern of cholinesterase and AChRs in the anterior and posterior latisimus dorsi muscles (ALD and PLD) and the hyomandibularis muscle, a muscle of the hyoid complex (136). The ALD muscle in birds is a postural muscle of the wing and is known to be a slow tonic muscle with multiple terminals on each fiber (137; 138). The PLD is a fast twitch muscle of the wing which has single terminals on

Plate 6: Cholinesterase distribution on muscle fibers from a zebra finch. Muscles from a male zebra finch were fixed for 30 min in 2% paraformaldehyde in Krebs-phosphate buffered saline at room temperature, then fibers were dissociated and stained as described in Section 6.2.2.3. (A) A densely staining endplate on one muscle fiber from the syrinx and scattered endplate staining on an adjacent fiber. (B) Two endplates characteristic of those seen in the posterior latisimus dorsi muscle of the same bird. (C) Scattered endplate staining on fibers from the anterior latisimus dorsi muscle of the same bird. Magnified 213 times.





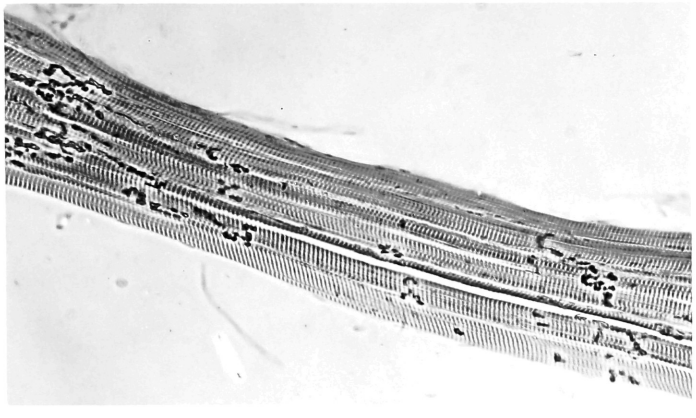
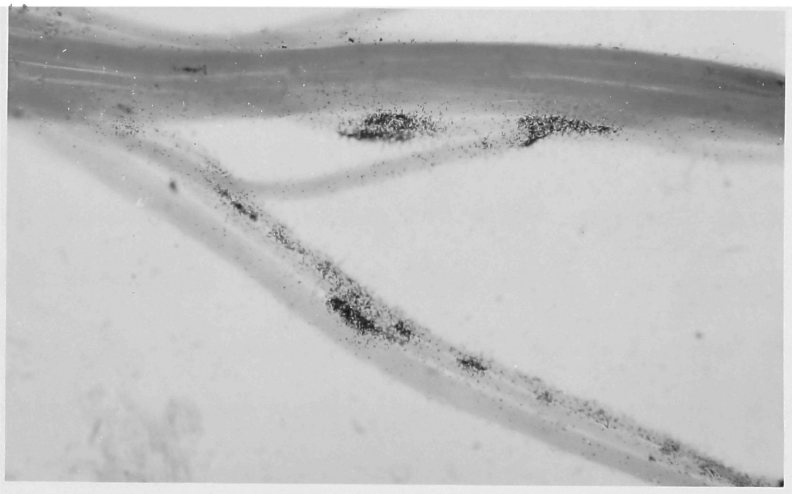
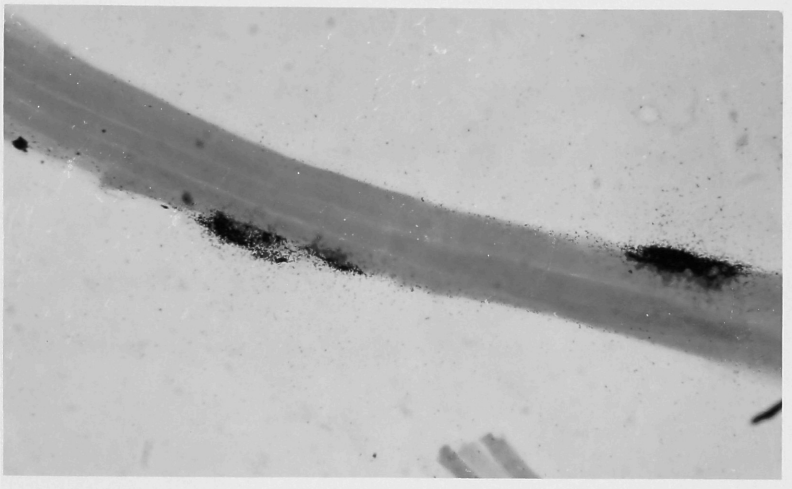
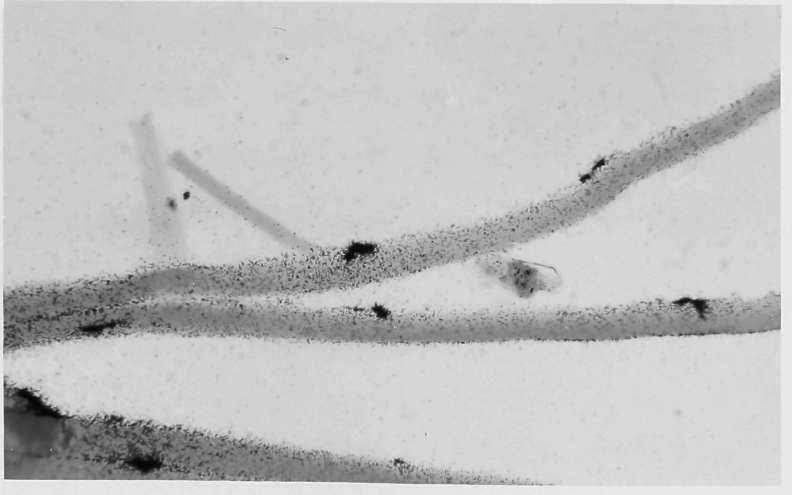


Plate 7: AChR distribution on muscle fibers from a zebra finch. Muscles from a female zebra finch were fixed for 30 min. in 2% paraformaldehyde in Krebs-phosphate buffer over ice. Fibers were then dissociated, labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography as described in Section 6.2.2.3. (A) Muscle fibers from the syrinx, showing several fibers with densely labelled endplates and a single fiber with scattered labelling. (B) Fibers from the hyomandibularis muscle of the same bird, illustrating the densely labelled endplates characteristic of this muscle. (C) Fibers from the anterior latisimus dorsi muscle showing the scattered terminals and high extra-junctional labelling characteristic of this muscle. Magnified 213 times.







each fiber. Muscles of the hyoid are innervated by the ascending branch of the XIIth nerve and are thought to be embryologically related to the syringeal muscles (57).

Fibers of the PLD and most fibers of the hyoid muscles had large, isolated patches of cholinesterase and AChRs, suggesting single discrete endplates and resembling endplates observed on some syringeal fibers (Plates 6B and 7B). In contrast, most fibers in the ALD preparation had diffuse scattered cholinesterase staining and clusters of AChRs (Plates 6C and 7C). These fibers had a high density of specific extrajunctional labelling with [^{125}I]-α-bungarotoxin. The high density of extrajunctional AChR on fibers from the ALD and on presumptive multi-terminal fibers from the syrinx is consistent with the diffuse sensitivity of tonic, multi-terminal muscle fibers to acetylcholine (139; 138). This distribution of clustered and diffuse AChRs closely resembles the pattern of AChRs observed on denervated fibers (140).

6.2.3.3. Sex Differences

To examine the morphological basis of the androgen-sensitivity of AChR number in the syrinx, dissociated fibers were prepared from the syrinxes of two intact females, an intact male and a male castrated 12 months before sacrifice. Syrinxes were fixed and dissociated with a Polytron as described above (Section 6.2.3.3). Dissociated fibers were then processed to demonstrate cholinesterase and AChRs (Section 6.1.2).

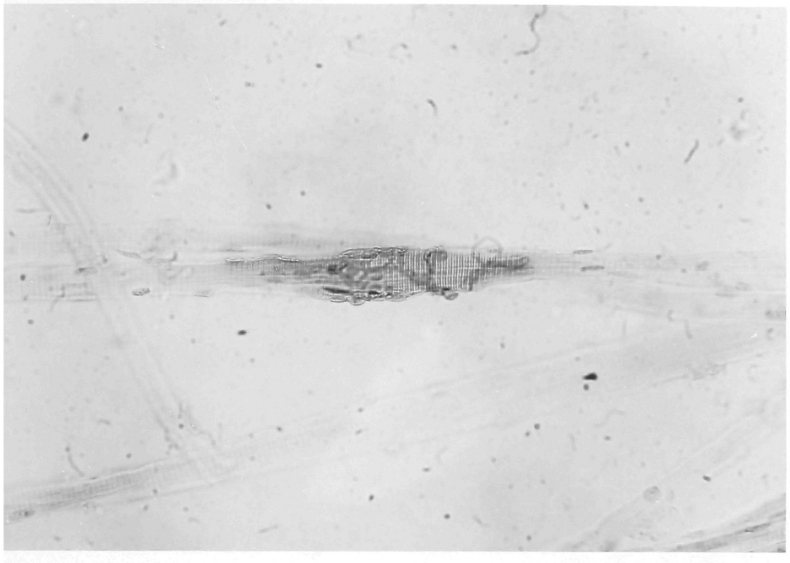
Unlike the discrete endplates in the LA, the larger endings on presumptive single-terminal fibers in the syrinx have complex structures, with numerous fingerlike ramifications of cholinesterase encasing

the muscle fiber. An examination of these large endings showed only a small difference between males and females in the length of muscle fiber over which cholinesterase extends (Plate 8 and Fig. 24). These large endplates on fibers from the intact male had a mean length of 91.5 ± 4.4 μm (mean ± SEM, N = 20), while a female had endplates which were 81.7 ± 4.7 μm long (N = 18).

I also measured the diameter of the fibers at the midpoint of the region stained by cholinesterase. These diameters were greater in the male than the female, corresponding to the differences in fiber diameter described above. Mean fiber diameters for male endplates was 27.4 ± 2.05 μm (N = 20), while for female endplates, the mean was 16.7 ± 0.8 μm (N = 18). These values are greater than the mean fiber diameters for these fibers, since fibers tend to be somewhat enlarged at the endplate region and presumptive single-terminal fibers have larger diameters than do multi-terminal fibers. There was no correlation between fiber diameter and endplate length ($r = -0.102$, N = 20 for a male and $r = -0.245$, N = 18 for a female).

In both males and females, terminals of this type appear to completely encase the muscle fiber, wrapping around it circumferentially. In an effort to quantify the size of the endplates, the length of the cholinesterase stain and the diameter of the fiber at the midpoint of cholinesterase stain were used to calculate the surface area of a cylinder which would completely enclose all of the cholinesterase stain at a terminal (area = pi X diameter X length). The mean surface area of such cylinders for the male was 7814 ± 667 μm^2 (N = 20), while for the female it was 4228 ± 259 μm^2 (N = 18).

Plate 8: Sex differences in cholinesterase distribution at endplates in the syrinx. Endplates demonstrated by cholinesterase stain on dissociated fibers from the syringeal muscles of (A) a male and (B) female zebra finch. Fibers were dissociated and stained as described in Section 6.2.2.3. Magnified 350 times.



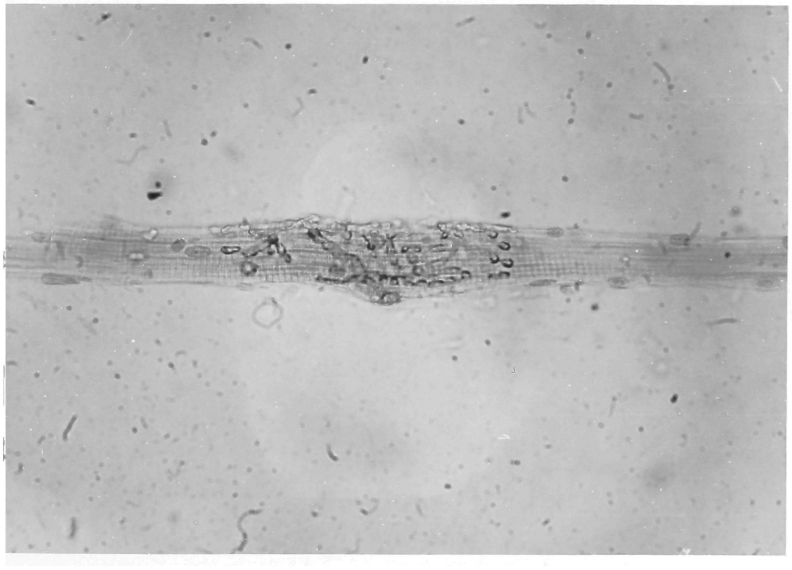


Figure 24: Drawings of endplates visualized by cholinesterase stain on dissociated fibers from the syringeal muscles of a male (A) and female (B) zebra finch. Drawings were made with a camera lucida. Magnified 680 times.

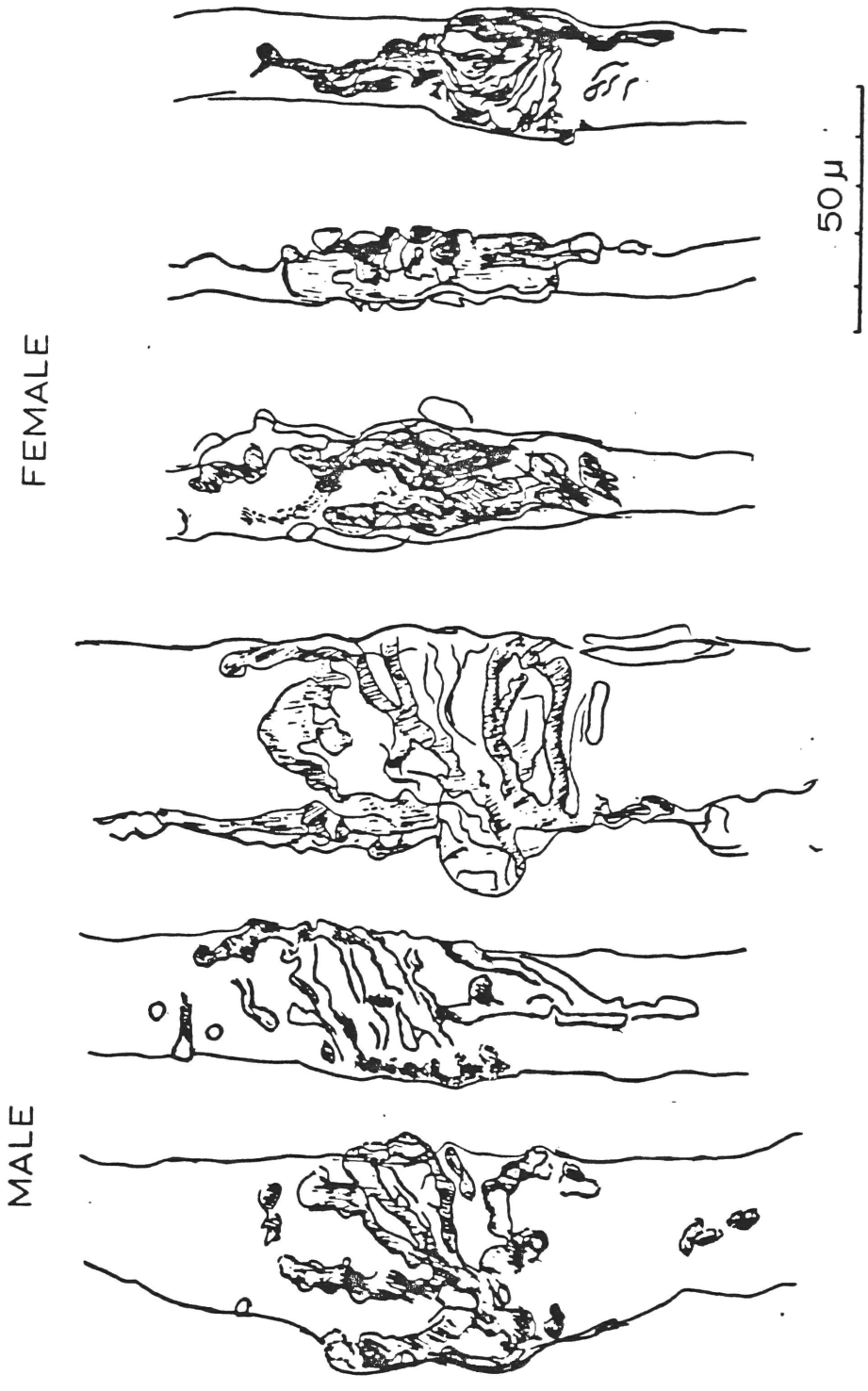
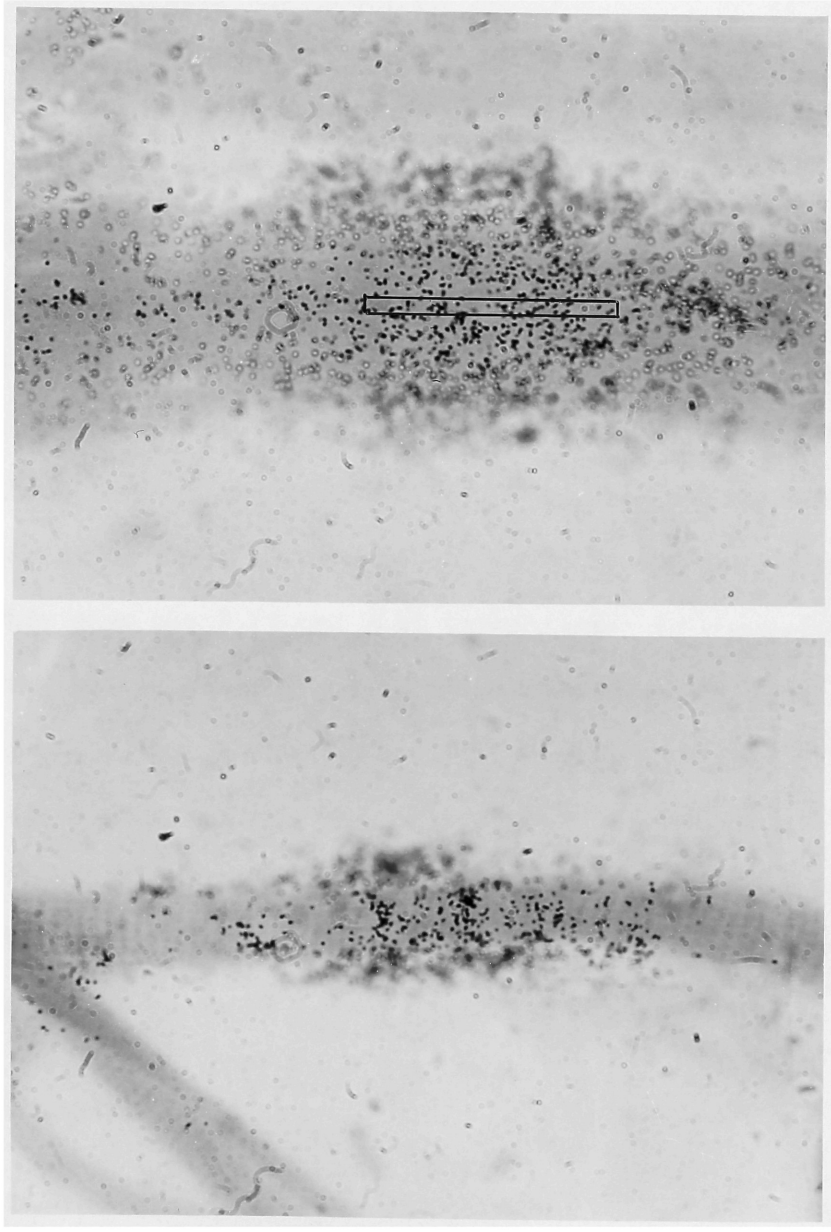


Plate 9: Sex differences in AChR distribution at endplates in the syrinx. Distribution of AChRs at single endplates from syringeal muscles of (A) a male and (B) female zebra finch. The overlay in (A) illustrates the rectangular field in which grains were counted for quantification of AChR density. Fibers were dissociated, labelled with [^{125}I]- α-bungarotoxin and processed for autoradiography as described in Section 6.2.2.3. Magnified 875 times.



Examining autoradiograms of dissociated fibers labelled with [^{125}I]-α-bungarotoxin I measured the density and extent of silver grains in the AChR-rich region of endplates on presumptive single-terminal fibers, in males and females (Plate 9). The length of the AChR-rich region differed little between male and female (Table 7). Mean length was 19% and 54% greater in a male than in two females. A male castrated one year before sacrifice had a mean similar to those of the females and 80% of the intact male's value. The diameters of the fibers at the endplates differed greatly, as described above. The male fibers were 1.78 and 2.04 times larger than fibers of two females. The castrated male's fibers had diameters intermediate between male and female values; 67% of the intact value. The area of the cylindrical surface enclosing the AChR-rich region was calculated as described above. This area was 2.71 and 2.45 times larger in the male than in the two females. Again, the castrate's value fell between intact male and female and was 53% of intact (Table 7). Finally, the density of AChR in the synaptic region, as represented by silver grains per unit area also differed in males and females. The male endplates had 48% and 73% more grains than did the two females. The castrated male had densities similar to the intact male; 5% less than intact (Table 7).

6.2.4. Discussion

Previous studies indicated that in the syrinx, the activity of CAT (58) and of the innervation-sensitive "endplate" form of AChE (Section 2.3.4) and the number of AChRs (Section 4.3) decrease following castration and increase with testosterone treatment. This indicates that the amount of synaptic material in the syringeal muscles is under the con-

TABLE 7

TESTOSTERONE EFFECTS AT SINGLE ENDPLATES
IN THE ZEBRA FINCH SYRINX:
A MODEL

	FEMALES	CASTRATE	MALE
ENDPLATE "LENGTH"	68.3 ± 4.4 88.0 ± 5.8	84.3 ± 4.3	105.0 ± 5.3 μm
FIBER DIAMETER AT ENDPLATE	12.7 ± 0.8 11.1 ± 0.6	15.2 ± 0.9	22.6 ± 1.6 μm
SURFACE AREA (π XlengthXdiam.)	2781 ± 288 3072 ± 243	3985 ± 268	7528 ± 703 μm^2
AChR DENSITY (Grain density)	0.527 ± 0.063 0.453 ± 0.052	0.745 ± 0.082	0.782 ± 0.102 grn/μm^2
AChR/ENDPLATE	1446 ± 211 1397 ± 202	3054 ± 438	5544 ± 722 grains

Table 7: A model for the calculation of the sex difference of AChRs per endplate in synapses in the syringeal muscles of the zebra finch. Muscle fibers were dissociated, labelled with [^{125}I]-α-bungarotoxin and processed for autoradiography as described in the text (Section 6.2.1). The dimensions of a cylinder which would completely encompass the region of dense silver grains at an endplate were determined for endplates in the syrinxes of two females, an intact male and a male castrated one year prior to sacrifice. The density of grains at these same endplates were measured as described in the text (Section 6.3.1). Autoradiograms of labelled fibers were exposed for 14 hours. The means of grain density and of the surface area of the cylinder encompassing dense accumulations of AChR were used to estimate the total number of AChR per endplate, (measured as grains in autoradiograms), under the assumption that grain density is uniform over the entire area of the cylinder. Entries are means ± standard error of the mean for 15 endplates from each syrinx.

trol of androgens. Several hypotheses can be proposed to explain alterations in the levels of synaptic components which follow changes in androgen levels in the syrinx. These alterations might reflect changes in 1) the number of muscle fibers per muscle 2) the number of synapses per muscle fiber, and 3) the amount of synaptic material present at a single synapse. There is some precedent for each of these possibilities. Increases in the number of muscle fibers in adult muscles have been reported in pathological situations and after chronic stretch (141; 142; 143). However, in the LA muscle of the mouse and rat, castration and testosterone-treatment after castration cause marked changes in muscle size without any change in muscle fiber number (30; 31). The possibility must also be considered that castration and androgen treatment cause changes in the number of endplates per muscle fiber in the syrinx. Many muscles are innervated with multiple terminals on each muscle fiber, especially in nonmammalian vertebrates (135). Finally, alterations in androgen levels may cause changes at the level of individual endplates in the syrinx. In other muscles, there is a positive correlation between endplate size and muscle fiber diameter (129; 128). Thus, endplate size may be regulated to match muscle fiber diameter. Changes in the levels of circulating androgens cause changes in muscle fiber diameter in the syrinx. In the LA of the rat, castration causes a decrease in total AChR number which is entirely the result of a decrease in the number of AChRs per endplate (Section 5.3.2 and Section 6.2.3). Thus, any or all of the mechanisms outlined above may be responsible for the effects of androgens on the amount of synaptic material in the syrinx.

Measurements of fiber number in the syringeal muscles of zebra

finches suggest that there is no marked difference in the number of muscle fibers of the ventral syringeal muscles of males and females. Rather, measurements of fiber diameters suggest that males have more syringeal muscle mass by virtue of having larger muscle fibers. From this we may infer that muscle fiber number is not likely to be regulated by adult blood androgen levels. Therefore, hormonal regulation of AChR number and CAT and AChE activity probably occurs by virtue of changes in the amount of synaptic material per fiber and not by virtue of changes in fiber number.

The similarities in the pattern of silver grains in AChR autoradiograms and reaction product on fibers stained for cholinesterase suggests that these patterns represent the true distribution of endplates on the muscle fibers. This distribution indicates that syringeal muscles contain both single and multi-terminal fibers. This is an intriguing observation in light of the fact that the hypoglossus motoneurons provide the only known source of innervation for syringeal muscles (52). Perhaps the two patterns of innervation reflect the dual role of the syrinx, which has both respiratory and vocal functions (47).

Mixed fiber types within single vertebrate muscles occur in the extra-ocular muscles of mammals (135; 144) and the "complexus muscle" of chickens (145). Multi-terminal muscle fibers are typically slow-tonic fibers and exhibit graded contractures instead of rapid, all-or-none twitch. In the adult, the multi- or single-terminal character of a muscle fiber may be relatively immutable. In chickens, cross-innervation between the multi-terminal anterior latisimus dorsi (ALD) and the singly innervated posterior latisimus dorsi (PLD) does not alter

the pattern of terminals on these muscles (146). However, it is conceivable that the changes in AChR number and AChE activity which follow alterations in levels of circulating androgens result in part from changes in the proportion of single and multi-terminal fibers or from changes in the number of AChRs on multi-terminal fibers. The present study does not test this possibility.

The sexual dimorphism in synaptic structure revealed by this study occurs in the class of large, single-terminals. Although these endplates, which wrap entirely around the muscle fiber, show comparable longitudinal extent in males and females, there are sex differences in the area encompassed by the ramifications of the terminal. Fiber diameters at these endplates are 1.9 times larger in male than in female syrinx, and the surface area of the fiber over which the ramifications of the endplate extend may therefore be up to 2.6 times larger in males. There also is an apparent difference in the density of AChR at these endplates, measured as density of silver grains averaged throughout the region of the fiber over which the endplate extends. Grain counts indicate that these large terminals have 1.6 times higher densities of AChR in male relative to female syrinx. Combining the AChR density estimated by grain counting and the area of the cylinders encompassing the AChR-rich region, the overall difference in AChR number per synapse would be predicted to be about four fold (Table 7). This may represent a large portion of the 3.8 fold difference in the total number of AChR in male and female syrinxes measured biochemically (Section 4.3.1). Perhaps another contribution arises from differences in the proportion of multi-terminal to single terminal fibers or differences in the number of AChRs on multi-terminal fibers.

It is interesting that the density of AChRs at syringeal endplates appears to remain near intact values after castration. This preliminary finding suggests that the difference in grain density observed between intact male and female endplates is not a technical artifact related to the difference in fiber diameter in these samples, since fibers from the castrated male have diameters similar to those of females, but grain densities similar to the intact male. However, a definite determination of whether AChR density at endplates in the syrinx is androgen-sensitive in the adult or only during development must await a larger sample.

6.3. SUMMARY

The effects of alteration in circulating levels of androgens on single synapses in the levator ani and the songbird syrinx have been explored using histological techniques. In the LA, where changes in muscle fiber number or number of endplates per fiber are not known to occur, castration causes a decrease in the number of AChRs at single endplates in part by causing a decrease in the density of AChRs per postsynaptic area. In contrast, castration has little or no effect over the short term on the localization of synaptic AChE at the endplate. In the syrinx, there is little or no difference in the number of muscle fibers of male and female zebra finches, but male muscle fibers have larger diameters. This suggests that the changes in synaptic components observed after alterations in androgen levels represent changes in the amount of synaptic material per muscle fiber. The distribution of cholinesterase and AChRs suggests there are at least two types of muscle fiber in syringeal muscle: single-terminal and multi-terminal. A preliminary study of endplates on presumptive single-terminal fibers indi-

cates that these endplates are larger in males than in females. These endplates also have a higher density of AChRs in males than in females. A castrated male resembled intact females in the dimensions of these endplates and differed from an intact male, suggesting that these dimensions are plastic and reflect levels of circulating androgens in the adult. In contrast, the density of AChRs at the castrate's endplates resembled that of an intact male.

7. CHAPTER VII: DISCUSSION AND SIGNIFICANCE

7.1. MECHANISM OF TESTOSTERONE ACTION

I have explored the effects of changes in the levels of circulating hormones on the neuromuscular junctions in two androgen-sensitive muscles, the syrinx of the songbird and the levator ani of the rat. These studies demonstrated that castration and testosterone-treatment cause changes in the number of AChRs and the activity of AChE in these two muscles. Previous studies demonstrated that the activity of choline-acetyltransferase (CAT) in the syrinx and in its motor nerve is also testosterone-sensitive. The results of these investigations provide strong evidence for structural plasticity at individual synapses in response to changing levels of blood testosterone. These synaptic modifications must involve a variety of pre- and post-synaptic modifications. Acetylcholine receptors (AChRs) at the neuromuscular junction are produced only by muscle cells (110, however, see 147). In contrast, CAT at the junction is normally only synthesized by the motoneuron (44). AChE at the neuromuscular junction is probably derived both from the presynaptic motor nerve and from the postsynaptic muscle cell (75; 148; 74). Because of the different cellular sources of these three synaptic components, testosterone must effect their levels by different mechanisms.

Gutmann and colleagues first discussed the possible mechanisms of hormone action on androgen-sensitive muscles in the context of their study of testosterone action on CAT in the LA (43). They considered the possibilities that 1) testosterone directly stimulates synthesis of CAT by the motoneurons innervating the perineal muscles, 2) testosterone

acts primarily on the muscles, causing the muscle to exert a retrograde trophic influence on the motoneurons or 3) that testosterone increases impulse activity in the appropriate motoneurons, presumably by acting on higher centers in the nervous system. To restate these alternatives more generally, hormones may act on synapses by 1) primary actions on postsynaptic cells, 2) primary actions on presynaptic cells and 3) secondary actions on these cells mediated by effects on electrical activity of neurons "up-stream" from the motor unit. In the syrinx and levator ani, the muscles and the motoneurons which innervate them have putative androgen receptors (see Section 1.2). In addition, testosterone rapidly exerts a variety of effects on the central nervous system which alter behavior and may cause changes in the activity of these muscles. In short, the three hypotheses outlined by Tucek et al. are all still relevant and deserve to be considered in the light of recent evidence.

7.1.1. Effects Mediated by Muscle Activity

Androgen effects on androgen-sensitive muscles may be secondary to changes in the amount of activity of these muscles, and these changes in turn result from changes in the behavior of the animal elicited by alterations in testosterone levels. In the syrinx, increased testosterone levels induce singing and thereby increase the amount of activity of the syringeal muscles. When adult male zebra finches are castrated, the rate of song production gradually decreases to much lower levels (149). Testosterone replacement then reinstates singing (51). These changes in the pattern of use of the syrinx may be responsible for the changes in the levels of cholinergic enzymes and receptors. In rat mus-

cle, increases in total AChE follow increases in muscle use (150; 151; 152). In nerve-muscle co-cultures, neuromuscular activity increases the concentration of AChE at the endplate (83).

Thus, testosterone may act on synaptic components indirectly via its effects on muscle activity. However, several facts argue against the view that this indirect mechanism is the only factor involved in testosterone action on syringeal AChE. Changes in muscle use cause changes in total activity of muscle AChE, but do not change specific activity. However, in the syrinx, changes in testosterone levels cause changes in specific activity as well as total (58). In addition, female zebra finches do not sing when implanted with testosterone as adults, implying that there is little increase in syringeal muscle activity, but specific syringeal AChE does increase after implantation (58). Decreases in specific AChE after castration and increases after testosterone therapy are observed in the right half of the canary syrinx, which is little used for song (58). In addition, when castrated males are treated with the non-aromatizable androgen, α-dihydrotestosterone, the weight of the syrinx increases, but the rate of singing does not (C. Harding and V. Luine, in press). Finally, AChE activity increases after testosterone treatment even in denervated syringeal muscle (Section 4.3.4), indicating that some effects of testosterone are possible without the influence of nervous activity.

We might also consider the possible contribution of changes in use to the androgen-sensitivity of AChR number. The pattern of neuromuscular activity has been shown to be critical in determining the metabolism of the AChR molecule (153; 154; 117). However, increased use generally

causes a decrease in AChR number, rather than an increase (111).

Considering all of these observations together, it seems unlikely that changes in the amount or pattern of activity are responsible for much of the androgen-sensitivity of the cholinergic systems in these muscles. Rather, more direct effects on the muscle and motoneurons may be the main mechanisms of testosterone's action.

7.1.2. Effects Mediated by Motoneurons

Testosterone may exert primary effects on motoneurons by mechanisms which do not involve electrical activity. As discussed above (Sections 1.2 and 1.3), the motoneurons which innervate the syrinx and LA both accumulate androgens specifically (54; 56; 39). Indeed, the presence of androgen receptors in their motoneurons may be a general property of androgen-sensitive muscles. In the frog, Xenopus, the cranial motoneurons which innervate the androgen-sensitive muscles of the larynx also accumulate androgens specifically (155).

We might hypothesize that the levels of AChE and CAT in androgen-sensitive muscles might be affected by actions of androgens on motoneurons. In the syrinx, castration decreases specific activities of CAT and AChE in the tracheosyringealis portion of the hypoglossal nerve and testosterone treatment restores these activities to intact levels (58). It seems likely that the effect of testosterone on CAT activity in the syrinx is due to an effect on the amount of CAT produced by the syringeal motoneurons and transported down the motor nerve. In the preoptic area of the hypothalamus, immunochemical studies have shown that increased concentration of CAT is responsible for increased CAT

activity after estrogen treatment (156). This increase in CAT concentration is suspected to be the result of increased CAT synthesis caused by genomic activation by the hormone-receptor complex. A similar mechanism may be involved in the effect of testosterone on CAT in the syringeal motor nerve.

The increase in syringeal AChE activity may also be the result of primary actions of testosterone on the motoneurons, at least in part. Testosterone increases the activity of all molecular forms of AChE, but since all forms are transported along motor axons (75; 76; 157), all forms of AChE may be derived from motoneuronal synthesis and axonal transport. However, the fact that testosterone increases syringeal AChE activity even after denervation argues that not all of the effects of the hormone are mediated by the motoneurons (Section 4.3.4).

The levels of AChR in the syrinx and LA may also be affected by actions of testosterone on the motoneurons. It is believed that trophic factors which affect the number of AChR on the muscle fiber are delivered to muscle by motor nerves, and candidate molecules have been identified (158; 159; 160). It may be that androgens affect the rate of delivery of these factors from the motoneurons to the LA and syringeal muscles. The fact that no effect of testosterone treatment was seen on denervated syringeal muscle supports this hypothesis, although other interpretations of this result ought to be considered (Section 4.4).

Considering testosterone's effects on the levels of CAT, AChE and AChRs, it seems likely that primary actions on motoneurons are responsible for some, but not all of the effects of testosterone on the neuromuscular junctions in these androgen-sensitive muscles.

7.1.3. Effects Mediated by Muscle Androgen Receptors

In addition to actions of testosterone on muscle activity and on motoneurons, direct actions of the hormone on the muscles themselves may be responsible for many of the effects of testosterone on androgen-sensitive muscles. As discussed above, (Sections 1.2 and 1.3), the LA and syringeal muscles both contain high affinity binding sites for androgens, with affinity and specificity similar to androgen receptors from other androgen-sensitive tissues (32; 33; 34; 57).

Gutmann and colleagues demonstrated that the LA, isolated from neural and endocrine systems in long-term organ culture, retains its androgen-sensitivity, as demonstrated by increased incorporation of labelled precursor into protein and RNA in the presence of testosterone in the culture medium (161; 162). In other studies (163), no testosterone sensitivity of weight or contraction time was detected in soleus muscles implanted as free grafts into the site of the LA in adult rats. In contrast, when the LA muscle was grafted in the bed of the extensor digitorum longus in adult rats, the muscle retained testosterone-sensitivity of weight and contractility (164). These results, when considered together, provide strong evidence that most of the effects of testosterone on the growth and contractile physiology of the LA are not mediated by neuronal influences, muscle use or effects on other perineal structures, but rather are primary effects on the muscle itself. It seems likely that these primary effects are mediated by muscle androgen receptors. However, these studies do not address the question of what mechanisms are involved in the effects of testosterone on cholinergic enzymes and AChRs. The small but significant increase in AChE activity

which occurs after testosterone treatment of denervated females indicates that some of the effects of testosterone on this enzyme are mediated by primary actions on the muscle, and do not involve the nerve (Section 4.3.4). Since AChRs at the neuromuscular junction are synthesized by the muscle, it seems likely that testosterone causes AChR number to increase in the syrinx and levator ani by primary actions on the muscle cells, perhaps by direct genomic activitation of synthesis of mRNAs for the AChR subunits.

The task of teasing apart the mechanisms involved in testosterone's effects on the neuromuscular junctions in these muscles is an important one, as it may provide insight into the cellular basis of synaptic regulation and plasticity in general. Results of this and previous studies indicate that the primary effects of testosterone on these muscles are partially responsible for the changes in muscle protein and AChE activity. It also seems likely that changes in muscle activity is not the primary factor mediating most of the effects of testosterone on AChR number and AChE and CAT activity. However, it seems likely that all three mechanisms of testosterone action, presynaptic, postsynaptic and mechanisms mediated by changes in electrical activity, are responsible for the total range of changes which occur at the neuromuscular junction.

7.2. CELLULAR MECHANISMS

If testosterone has primary actions on the muscle and motoneurons in androgen-sensitive systems, it is likely that testosterone or its metabolites act by a classical genomic mechanism; that is, by combining with steroid receptors, and in complex with the receptors, binding to

chromatin and inducing mRNA for AChE and AChR. Such a mechanism has been shown or is implicated as the route of steroid effects on many products in other tissues (11; 12; 13). In particular, in the prostate, androgens have been shown to increase the diversity of mRNA and to increase the amount of translation of specific mRNAs in cell free translation systems, presumably as a result of induction of specific mRNAs (165; 166).

Other less direct actions of testosterone might also be imagined. Testosterone might induce mRNAs for other products, and these products might secondarily induce an increase in synaptic components. For example, T has been shown to induce inhibitors of phosphodiesterase in the prostate (167), and this could cause an increase in the levels of the cyclic nucleotide intracellular messengers: cAMP and cGMP. In the uterus, estrogen induces the enzyme ornithine decarboxylase, the rate-limiting enzyme in the synthesis of polyamines (168). In turn, polyamines have been shown to inhibit phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) (169). Phosphorylation of this factor inhibits initiation of translation of mRNAs, so the end result of this chain of events might be to increase the translation of all mRNAs. Such a mechanism has been proposed to explain induction by testosterone of certain products in the kidney, where increases in polyamines are necessary and perhaps sufficient for expression of some effects of testosterone (170). Other mechanisms for steroid hormone action have been identified (14; 15). Such involved mechanisms for steroid action cannot be ruled out in this, or indeed in most cases of steroid sensitivity. To date, no conclusive evidence exists as to which mechanisms are involved in any of the effects of steroids on the nervous system (15).

7.2.1. Anabolic Effects of Androgens

The effects of testosterone on the LA and syringeal muscles are similar in some ways to the so-called myotrophic anabolic actions of androgens on other muscles. There are, none the less, several differences. The time course of the response of the syrinx and LA to testosterone treatment are much more rapid than anabolic effects on other muscles, which generally take months to express themselves (24). In addition, the magnitude of effects on the weight of other skeletal muscles is much smaller than the effects seen here, amounting to changes of at most 5 to 15% (171; 121). These differences may reflect the fact that androgen receptors are several times more abundant in the LA and syringeal muscles than in other rat skeletal muscles.

It is worth noting that the anabolic response to androgens is not a universal property of all muscles in most species. For example, in the guinea pig, the muscles of the head, neck and shoulder respond to androgen treatment after castration much more than do other body muscles (171). In the zebra finch, males are not larger than females and most muscles may be unresponsive to androgens. The muscles of the hyoid and larynx are not affected by castration (58, and above, Section 4.3.2). These differences in the degree of responsiveness of different muscles suggests that the myotrophic effects of androgens are not general effects on all muscles, but rather, represent adaptive responses of particular muscles which are selected to be larger in males.

It seems likely that the anabolic effects of androgens on muscles are mediated by androgen receptors. The androgen receptors in rat limb muscle are apparently biochemically indistinguishable from androgen

receptors in other steroid-sensitive organs (35, however, see also 11 and 12). This suggests that the mechanism of myotrophic action of androgens may be identical to the mechanism of their actions on other tissues which androgens induce to grow. Although there are pharmacologically characterizable differences between muscle and other tissues in their relative responsiveness to various androgen derivatives, these pharmacological differences may result from differences in the concentrations of various androgen metabolizing enzymes in these tissues, rather than from differences in androgen receptors or in the mechanism of androgen action (173).

Thus, the mechanism of production of myotrophic effects may not be distinguishable from those of androgenic effects. Furthermore, the actions of androgens on the LA and syrinx may be generalizable to other muscles which are less sensitive to androgens.

7.3. REGULATION AND COORDINATION OF HORMONAL EFFECTS

7.3.1. Coordination of Pre- and Postsynaptic Effects

In order to maintain the function of a synapse, the concentration of the enzymes, receptors, channels and structural components which make up the synaptic machinery must be coordinatedly regulated. Perhaps the most interesting aspect of the mechanism of hormone action on synaptic components is the mechanisms which insure coordination of the various effects during synapse modification. In other cases of long term synaptic plasticity, this coordination may be achieved by the passage of special messengers, trophic factors (174), from pre- to post- and post- to pre-synaptic cells. Presumably, these trophic factors regulate the

nature and extent of changes on the pre- and postsynaptic sides of the synapse. In the case of hormonally induced alterations in neural systems, coordination may be achieved by having the same hormone cause different but appropriate effects on the pre- and post-synaptic neurons. In androgen-sensitive muscles, the fact that both muscle and motoneurons have androgen receptors and are apparently involved in synaptic modification, suggests that these androgen-sensitive neuromuscular systems have evolved so that the hormone itself can insure that the alterations of the synapse occurs with coordinated pre- and post-synaptic changes. Perhaps other exogenous signals, such as neuromodulators, also cause synaptic modifications by simultaneously causing appropriate adjustments by pre- and post-synaptic neurons. Androgen-sensitive muscles such as the syringeal muscles and the levator ani may provide powerful systems in which to examine the mechanisms of coordination of pre- and post-synaptic change during synaptic modification.

7.3.2. Fiber Size and Endplate Physiology

For synaptic function to be maintained during modification, not only must pre- and postsynaptic effects be coordinated, but so must the effects on a single cell. In fact, the effects of testosterone on the synapses in androgen-sensitive muscles may be a reflection of this requirement.

In both the syrinx and LA, the number of AChR per muscle protein remains relatively constant, throughout hormone-induced alterations. This may simply reflect a lack of specificity of the effect of testosterone on protein synthesis. Alternately, this fact may be the key to understanding the significance of the effects of testosterone in terms

of the physiology of synaptic transmission. In these two muscles, muscle protein is correlated with the volume of the individual muscle fibers, since fiber number is constant. But as fiber volume increases, the fiber surface area increases and with more surface area, leakage of synaptic current from the fiber will also increase; that is, the input resistance of the fiber will fall. If fiber length does not change, then surface area will change proportionately to the square root of volume. In a simple cylinder of leaky membrane, input conductance will increase proportionately with diameter to the 3/2 power (175), or volume to the 3/4. Since the magnitude of a voltage change caused by a membrane current is the product of the current and the input resistance, it will take proportionately more current to change the membrane potential the same amount in a fiber with a larger diameter. In the muscle fiber, this effect is complicated by capacitative effects and the internal t-tubule membrane system, which provides another source of leakage current and capacitance. For low frequencies, this extra leakage current makes membrane conductance a linear function of muscle fiber diameter (176). From this relationship, we can predict that the amount of current needed to depolarize the muscle to threshhold will change proportionately to a function between the 3/4 and unit power of muscle fiber volume. From this simple model, we might predict that as fiber diameter increased, more synaptic current would be required for depolarization to threshhold, and therefore more open AChR channels would be required. Fulfilling this requirement may involve an increase in the size of the endplate, or an increase in the density of synaptic material at the endplate. This may be reflected in increases in the activity of cholinergic enzymes, as well as in the number of AChRs.

It may be argued that the effect of changing muscle fiber diameter on input conductance is not usually important, since there is a large safety factor at most neuromuscular junctions, such that far more AChR channels are opened following transmitter release than are required to depolarize the muscle to threshhold. However, this margin of safety may be required to prevent failure of synaptic transmission in the face of repetitive stimulation, which can cause desensitization of receptors and decreases in quantal number. Perhaps the safety margin is closely regulated and actively maintained such that the depolarization caused by synaptic stimulation remains constant during changes in muscle size. In fact, endplate size has been found to be significantly correlated with fiber diameter by several investigators in many muscles (127; 128), as would be expected if this hypothesis were true.

Adjustments in endplate efficacy may be critical in muscles which periodically undergo changes in use and size. Perhaps the hormonal sensitivity of neuromuscular junctions in androgen-sensitive muscles is an adaptive response to just such a requirement.

7.4. CONCLUSION

I have explored the effects of changes in the levels of circulating hormones on the neuromuscular junctions in two androgen-sensitive muscles, the syrinx of the songbird and the levator ani of the rat. The results of these investigations provide strong evidence for structural plasticity at individual synapses in response to changing levels of hormone. Testosterone probably has effects on the neuromuscular junctions in these muscles by a combination of primary effects on the presynaptic motoneurons and the postsynaptic muscle fibers, as well acting

indirectly by increasing the electrical activity in the entire motor unit. These effects may reflect an overall adaptive response to changing requirements for synaptic function. The study of synapses in androgen-sensitive muscles may have broader applications for the study of the metabolism of synaptic components, the coordination of pre- and post-synaptic functions and the cellular mechanisms underlying modifications of synaptic structure. Androgen-sensitive neuromuscular junctions, such as those in the syrinx and the levator ani, may provide powerful systems in which to study the mechanism and adaptive significance of synaptic plasticity.

1. REFERENCES

References

1. Kandel, E. R., Cellular Basis of Behavior: An Introduction to Behavioral Neurobiology, Freeman, San Francisco (1976).

2. Castellucci, V. F., T. J. Carew, and E. R. Kandel, "Cellular analysis of long-term habituation of the gill-withdrawal reflex of Aplysia californica," Science Vol. 202 pp. 1306-1308 (1978).

3. Carew, T., V. F. Castellucci, and E. R. Kandel, "Sensitization in Aplysia: Restoration of transmission in synapses inactivated by long-term habituation," Science Vol. 205 pp. 417-421 (1979).

4. Lynch, G., M. Browning, and W. F. Bennett, "Biochemical and physiological studies of long-term synaptic plasticity," Federation Proceedings Vol. 38 pp. 2117-2122 (1979).

5. Lee, K. S., F. Schottler, M. Oliver, and G. Lynch, "Brief bursts of high-frequency stimulation produce two types of structural change in rat hippocampus," J. Neurophysiol. Vol. 44 pp. 247-258 (1980).

6. Raisman, G., "Neuronal plasticity in the septal nuclei of the adult rat," Brain Res. Vol. 14 pp. 25-48 (1969).

7. Lynch, G., S. A. Deadwyler, and C. Cotman, "Postlesion axonal growth produces permanent functional connections," Science Vol. 180 pp. 1364-1366 (1973).

8. Cotman, C. W. Ed., Neuronal Plasticity, Raven Press, New York (1978).

9. Holland, R. L. and M. C. Brown, "Postsynaptic transmission block can cause terminal sprouting of a motor nerve," Science Vol. 207 pp. 649-651 (1980).

10. Reis, D. J. and T. H. Joh, "Genetic, structural, and molecular mechanisms governing the long-term regulation of tyrosine hydroxylase, a neurotransmitter synthesizing enzyme in the brain," pp. 13-34 in Neuronal Information Transfer, (1979).

11. O'Malley, B. W. and A. R. Means, "Female steroid hormones and target cell nuclei," Science Vol. 183 pp. 610-620 (1974).

12. McKnight, G. S., P. Pennequin, and R. T. Schimke, "Induction of ovalbumin mRNA sequences by estrogen and progesterone in chick oviduct as measured by hybridization to complementary DNA," J. Biol. Chem. Vol. 250 pp. 8105-8110 (1975).

13. Rae, P. M. M., "Aspects of cytoplasmic and environmental influences on gene expression," pp. 301-346 in Cell Biology: A Comprehensive Treatise vol. 3, Gene Expression: The Production of RNA's, ed. L. Goldstein and D. M. Prescott,Academic Press, New York (1980).

14. Szego, C. M. and R. J. Pietras, "Membrane recognition and effector sites in steroid hormone action," pp. 307-463 in Biochemical Action of Hormones, Volume VII, ed. G. Litwack,Academic Press, New York (1981).

15. McEwen, B. S., A. Biegon, T. C. Rainbow, C. Paden, L. Snyder, and V. DeGroff, "The interaction of estrogens with intracellular receptors and with putative neurotransmitter receptors: Implications for the mechanism of activation of sexual behavior and ovulation," in Steroid Hormone Regulation of the Brain, ed. K. Fuxe, Pergamon Press, Oxford, New York (1981).

16. Pfaff, D. W., Estrogens and Brain Function: Neural Analysis of a Hormone-Controlled Mammalian Reproductive Behavior, Springer-Verlag, New York (1980).

17. McEwen, B. S. and V. N. Luine, "Specificity, mechanisms and functional significance of steroid-receptor interactions in the brain and pituitary," pp. 239-267 in Biologie Cellulaire Des Processus Neurosecretoires Hypothalamiques, Colloq. Int. C.N.R.S. No. 280, ed. J. -D. Vincent and C. Kordon, Collogues Internationaux da C. N. R. S. (1978).

18. McEwen, B. S. and B. Parsons, "Gonadal steroid action on the brain: Neurochemistry and neuropharmacology," Ann. Rev. Pharmacol. Toxicol. Vol. 22 pp. 555-598 (1982).

19. Nottebohm, F., "Testosterone triggers growth of brain vocal control nuclei in adult female canaries," Brain Res. Vol. 189 pp. 429-436 (1980).

20. Nottebohm, F., S. Kasparian, and C. Pandazis, "Brain space for a learned task," Brain Res. Vol. 213 pp. 99-109 (1981).

21. DeVoogd, T. and F. Nottebohm, "Gonadal hormones induce dendritic growth in the adult avian brain," Science Vol. 214 pp. 202-204 (1981).

22. Lindstrom, J., R. Anholt, B. Einarson, A. Engel, M. Osame, and M. Montal, "Purification of acetylcholine receptors, reconstitution into lipid vesicles, and the study of agonist-induced cation channel regulation," J. Biol. Chem. Vol. 255 pp. 8340-8350 (1980).

23. Katz, B., Nerve, Muscle and Synapse, McGraw-Hill, New York (1966).

24. Kochakian, C. D., "Body and organ weights and composition," pp. 73-154 in Anabolic-Androgenic Steroids, ed. C. D. Kochakian,Springer-Verlag, Berlin (1976).

25. Hayes, K. J., "The so-called 'levator ani' of the rat," Acta Endocrinol. Vol. 48 pp. 337-347 (1965).

26. Cihák, R., E. Gutmann, and V. Hanzlíková, "Involution and hormone-induced persistence of the M. sphincter (levator) ani in female rats," J. Anat. Vol. 106 pp. 93-110 (1970).

27. Wainman, P. and G. C. Shipounoff, "The effects of castration and testosterone propionate on the striated perineal musculature in the rat," Endocrin. Vol. 29 pp. 975-978 (1941).

28. Eisenberg, E. and G. S. Gordan, "The levator ani muscle of the rat as an index of myotrophic activity of steroidal hormones," J. Pharmacol. Exp. Ther. Vol. 99 pp. 38-44 (1950).

29. Nimni, M. E. and E. Geiger, "Non-suitability of levator ani method as an index of anabolic effect of steroids," Proc. Exp. Soc. Exp. Biol. Vol. 94 pp. 606-610 (1957).

30. Venable, J. H., "Constant cell populations in normal, testosterone-deprived and testosterone-stimulated levator ani muscles," Am. J. Anat. Vol. 119 pp. 263-270 (1966).

31. Galavazi, G. and J. A. Szirmai, "Cytomorphometry of skeletal muscle: The influence of age and testosterone on the rat M. levator ani," Z. Zellforsch. Vol. 121 pp. 507-530 (1971).

32. Jung, I. and E.-E. Baulieu, "Testosterone cytosol 'receptor' in the rat levator ani muscle," Nature New Biol Vol. 237 pp. 24-26 (1972).

33. Krieg, M., R. Szalay, and K. D. Voigt, "Binding and metabolism of testosterone and of 5 α-dihydrotestosterone in bulbocavernosus / levator ani (BCLA) of male rats: in vivo and in vitro studies," J. Steroid Biochem. Vol. 5 pp. 453-459 (1974).

34. Dube, E. J. Y., R. Lesage, and R. R. Tremblay, "Androgen and estrogen binding in rat skeletal and perineal muscles," Can. J. Biochem. Vol. 54 pp. 50-55 (1976).

35. Michel, G. and E. -E. Baulieu, "Androgen receptor in rat skeletal muscle: Characterization and physiological variations," Endocrinology Vol. 107 pp. 2088-2098 (1980).

36. Dahlberg, E., M. Snochowski, and J.-Å. Gustafsson, "Regulation of the androgen and glucocorticoid receptors in rat and mouse skeletal muscle cytosol," Endocrin. Vol. 108 pp. 1431-1440 (1981).

37. Max, S. R. and J. F. Knudsen, "Effect of sex hormone on glucose-6-phosphate dehydrogenase in rat levator ani muscle," Molec. and Cellular Endocrin. Vol. 17 pp. 111-118 (1980).

38. Knudsen, J. F. and S. R. Max, "Aromatization of androgens to estrogens mediates increased activity of glucose 6-phosphate dehydrogenase in rat levator ani muscle," Endocrin Vol. 106 pp. 440-443 (1980).

39. Breedlove, S. M. and A. P. Arnold, "Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord," Science Vol. 210 pp. 564-566 (1980).

40. Gutmann, E., "Development and maintenance of neurotrophic relations between nerve and muscle," pp. 233-243 in Ciba Foundation Symposium on Growth of the Nervous System, ed. G. E. W. Wolstenholm and M. O'Connor,Churchill, London (1968).

41. Gutmann, E., S. Tucek, and V. Hanzlikovâ, "Changes in the choline acetyltransferase and cholinesterase activities in the levator ani muscle of rats following castration," Physiologia bohemoslov. Vol. 18 pp. 195-203 (1969).

42. Bass, A., E. Gutmann, V. Hanzlikovâ, I. Hajek, and I. Syrovy, "The effect of castration and denervation upon the contraction properties and metabolism of the levator ani muscle of the rat," Physiologia bohemoslov. Vol. 18 pp. 177-194 (1969).

43. Tuček, S., D. Kostirovâ, and E. Gutmann, "Testosterone-induced changes of choline acetyltransferase and cholinesterase activities

in rat levator ani muscles," J. Neurol. Sci. Vol. 27 pp. 353-362 (1976).

44. Tuček, S., "The synthesis of acetylcholine in skeletal muscles of the rat," J. Physiol. Vol. 322 pp. 53-69 (1982).

45. Warner, R. W., "The anatomy of the syrinx in passerine birds," J. Zool. Lond. Vol. 168 pp. 381-393 (1972).

46. Greenewalt, C. H., Bird Song: Acoustics and Physiology, Smithsonian Inst., Washington, D.C. (1968).

47. Nottebohm, F.N., The role of sensory feedback in the development of avian vocalizations, Doctoral dissertation; University of California at Berkeley, Berkeley, California (1966).

48. Häcker, V., Der Gesang der Voegel, Seine Anatomischen und Biologischen Grundlagen, Verlag von Gustav Fischer, Jena (1900).

49. Arnold, A. P., Behavioral effects of androgens in zebra finches (Poephila guttata) and a search for its site of action, Doctoral dissertation; Rockefeller University, New York, New York (1974).

50. Gurney, M. E. and M. Konishi, "Hormone-induced sexual differentiation of brain and behavior in zebra finches," Science Vol. 208 pp. 1380-1383 (1980).

51. Arnold, A. P., "The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (Poephila guttata)," J. Exp. Zool. Vol. 191 pp. 309-326 (1975).

52. Nottebohm, F., T. M. Stokes, and C. M. Leonard, "Central control of song in the canary, Serinus canarius ," J. Comp. Neurol. Vol. 165 pp. 457-486 (1976).

53. Nottebohm, F., "Brain pathways for vocal learning in birds: A review of the first 10 years," Progress in Psychobiology and Physiological Psychology Vol. 9 pp. 85-125 (1980).

54. Arnold, A. P., F. Nottebohm, and D. W. Pfaff, "Hormone concentrating cells in vocal control and other areas of the brain of the zebra finch (Poephila guttata)," J. Comp. Neurol. Vol. 165 pp. 487-512 (1976).

55. Nottebohm, F. and A. P. Arnold, "Sexual dimorphism in vocal control areas of the songbird brain," Science Vol. 194 pp. 211-213 (1976).

56. Arnold, A. P. and A. Saltiel, "Sexual difference in pattern of hormone accumulation in the brain of a songbird," Science Vol. 205 pp. 702-705 (1979).

57. Lieberburg, I. and F. Nottebohm, "High-affinity androgen binding proteins in syringeal tissues of songbirds," Gen. Comp. Endocrin. Vol. 37 pp. 286-293 (1979).

58. Luine, V., F. Nottebohm, C. Harding, and B. S. McEwen, "Androgen affects cholinergic enzymes in syringeal motor neurons and muscle," Brain Res. Vol. 192 pp. 89-107 (1980).

59. Massoulié, J. and F. Rieger, "L'acétylcholinésterase des organes électriques de Poissons (torpille et gymnote); complexes membranaires," Eur. J. Biochem. Vol. 11 pp. 441-455 (1969).

60. Hall, Z. W., "Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle," J. Neurobiol. Vol. 4 pp. 343-361 (1973).

61. Vigny, M., J. Koenig, and F. Rieger, "The motor end-plate specific form of acetylcholinesterase: Appearance during embryogenesis and reinnervation of rat muscle," J. Neurochem. Vol. 27 pp. 1347-1353 (1976).

62. Vigny, M., L. Di Giamberardino, J. Y. Couraud, F. Rieger, and J. Koenig, "Molecular forms of chicken acetylcholinesterase: Effect of denervation," FEBS Lett. Vol. 69 pp. 277-280 (1976).

63. Carson, S., S. Bon, M. Vigny, J. Massoulié, and M. Fadeau, "Distribution of acetylcholinesterase molecular forms in neural and non-neural sections of human muscle," FEBS Lett. Vol. 97 pp. 348-352 (1979).

64. Bon, S., M. Vigny, and J. Massoulié, "Asymmetric and globular forms of acetylcholinesterase in mammals and birds," Proc. Natl. Acad. Sci. USA Vol. 76 pp. 2546-2550 (1979).

65. Rosenberry, T. L. and J. M. Richardson, "Structure of 18S and 14S acetylcholinesterase. Identification of collagen-like subunits that are linked by disulfide bonds to catalytic subunits," Biochem. Vol. 16 pp. 3550-3558 (1977).

66. Vigny, M., S. Bon, J. Massoulié, and V. Gisiger, "Subunit structure of mammalian acetylcholinesterase: catalytic subunits, dissociating effect of proteolysis and disulfide reduction on the polymeric

forms," J. Neurochem. Vol. 33 pp. 559-565 (1979).

67. Koenig, J. and M. Vigny, "Formes moléculaires d'acétylcholinésterase dans le muscle lent et le muscle rapide du Poulet," C. R. Soc. Biol. Vol. 172 pp. 1069-1074 (1978).

68. Silman, I., J. M. Lyles, and E. A. Barnard, "Intrinsic forms of acetylcholinesterase in skeletal muscle," FEBS Lett. Vol. 94 pp. 166-170 (1978).

69. Bacou, F., P. Vigneron, and J. Massoulié, "Acetylcholinesterase forms in fast and slow rabbit muscle," Nature Vol. 296 pp. 661-663 (1982).

70. Rotundo, R. L. and D. M. Fambrough, "Molecular forms of chicken embryo acetylcholinesterase in Vitro and in Vivo: Isolation and characterization," J. Biol. Chem. Vol. 254 pp. 4790-4799 (1979).

71. Johnson, C. D. and R. L. Russell, "A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations," Anal. Biochem. Vol. 64 pp. 229-238 (1975).

72. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," J. Biol. Chem Vol. 193 pp. 265-275 (1951).

73. Emmerling, M. R., C. D. Johnson, and B. H. Lipton, "Multiple forms of acetylcholinesterase in quail muscle cell culture," Abstracts: Soc. Neurosci. Vol. 5 p. 479 (1979).

74. Weinberg, C. B. and Z. W. Hall, "Junctional forms of AChE restored at nerve-free endplates," Dev. Biol. Vol. 68 pp. 631-635 (1979).

75. Di Giamberardino, L. and J. Y. Couraud, "Rapid accumulation of high molecular weight acetylcholinesterase in transected sciatic nerve," Nature Vol. 271 pp. 170-172 (1978).

76. Brimijoin, S., "Axonal transport and subcellular distribution of molecular forms of acetylcholinesterase in rabbit sciatic nerve," Mol. Pharm. Vol. 15 pp. 641-648 (1979).

77. Lwebuga-Mukasa, J. S., S. Lappi, and P. Taylor, "Molecular forms of acetylcholinesterase from Torpedo californica: Their relationship to synaptic membranes," Biochem. Vol. 15 pp. 1425-1434 (1976).

78. Vigny, M., S. Bon, J. Massoulié, and F. Leterrier, "Active-site catalytic efficiency of acetylcholinesterase molecular forms in Electrophorus, Torpedo, rat and chicken," Eur. J. Biochem. Vol. 85 pp. 317-323 (1978).

79. Dudai, Y. and I. Silman, "The effects of solubilization procedures on the release and molecular state of acetylcholinesterase from electric organ tissue," J. Neurochem. Vol. 23 pp. 1177-1187 (1974).

80. McMahan, U. J., J. R. Sanes, and L. M. Marshall, "Cholinesterase is associated with the basal lamina at the neuromuscular junction," Nature Vol. 271 pp. 172-174 (1978).

81. Silman, I., L. Di Giamberardino, J. Lyles, J. Y. Couraud, and E. A. Barnard, "Parallel regulation of acetylcholinesterase and

pseudocholinesterase in normal, denervated and dystrophic chicken skeletal muscle," Nature Vol. 280 pp. 160-162 (1979).

82. Vigny, M., V. Gisiger, and J. Massoulié, " "Nonspecific" cholinesterase and acetylcholinesterase in rat tissues: Molecular forms, structural and catalytic properties, and significance of the two enzyme systems," Proc. Natl. Acad. Sci. USA Vol. 75 pp. 2588-2592 (1978).

83. Rubin, L. L., S. M. Schuetze, C. L. Weill, and G. D. Fischbach, "Regulation of acetylcholinesterase appearance at neuromuscular junctions in vitro ," Nature Vol. 283 pp. 264-267 (1980).

84. Yamamura, H. I., S. J. Enna, and M. J. Kuhar, Neurotransmitter Receptor Binding, Raven, New York (1978).

85. Chang, C. C. and C. Y. Lee, "Isolation of neurotoxins from the venom of Bungarus multicintus and their modes of neuromuscular blocking action," Arch. intern. Pharmacodyn. Vol. 144 pp. 241-257 (1963).

86. Changeux, J.-P., M. Kasal, and C. Y. Lee, "Use of a snake venom toxin to characterize the cholinergic receptor protein," Proc. Nat. Acad. Sci. U.S.A. Vol. 67 pp. 1241-1247 (1970).

87. Bulger, J. E., J. L. Fu, E. F. Hindy, R. L. Silberstein, and G. P. Hess, "Allosteric interactions between membrane-bound acetylcholine receptor and chemical mediators. Kinetic studies," Biochem. Vol. 16 pp. 684-692 (1977).

88. Hunter, W. M. and F. C. Greenwood, "Preparation of iodine-131 labeled human growth hormone of high specific activity," Nature Vol. 194 pp. 495-496 (1962).

89. Chang, C. C., T. F. Chen, and S.-T. Chuang, "N,O-di and N,N,O-tri [^{3}H] acetyl-α-bungarotoxins as specific labelling agents of cholinergic receptors," Br. J. Pharmacol. Vol. 47 pp. 147-160 (1973).

90. Cohen, J. B., M. Weber, M. Huchet, and J.-P. Changeux, "Purification from Torpedo marmorata electric tissue of membrane fragments particularly rich in cholinergic receptor protein," FEBS Lett. Vol. 26 pp. 43-47 (1972).

91. Chin, H. and R. R. Almon, "Fiber-type effects of castration on the cholinergic receptor population in skeletal muscle," J. Pharm. Exp. Therapeutics Vol. 212 pp. 553-559 (1980).

92. Miledi, R. and L. T. Potter, "Acetylcholine receptors in muscle fibres," Nature Vol. 233 pp. 599-603 (1971).

93. Shain, W., L. A. Greene, D. O. Carpenter, A. J. Sytkowski, and Z. Vogel, "Aplysia acetylcholine recetors: blockade by and binding of α-bungarotoxin," Brain Res. Vol. 72 pp. 225-240 (1974).

94. Kohanski, R. A., J. P. Andrews, P. Wins, M. E. Eldefrawi, and G. P. Hess, "A simple quantitative assay of ^{125}I-labeled-α-bungarotoxin binding to soluble and membrane-bound acetylcholine receptor protein," Anal. Biochem. Vol. 80 pp. 531-539 (1977).

95. Miledi, R., P. Molinoff, and L. T. Potter, "Isolation of the cholinergic receptor protein of Torpedo electric tissue," Nature Vol.

229 pp. 554-557 (1971).

96. Almon, R. R., C. G. Andrew, and S. H. Appel, "Acetylcholine receptor in normal and denervated slow and fast muscle," Biochem. Vol. 13 pp. 5522-5528 (1974).

97. Meunier, J.-C., R. W. Olsen, A. Menez, P. Fromageot, P. Boquet, and J.-P. Changeux, "Some physical properties of the cholinegic receptor protein from Electrophorus electricus revealed by a tritiated α-toxin from Naja nigricollis venom," Biochem. Vol. 11 pp. 1200-1210 (1972).

98. Akaronov, A., N. Kalderon, I. Silman, and S. Fuchs, "Preparation and immunochemical characterization of a water-soluble acetylcholine receptor fraction from the electric organ tissue of the electric eel," Immunochem. Vol. 12 pp. 765-771 (1975).

99. Schmidt, J. and M. A. Raftery, "A simple assay for the study of solubilized acetylcholine receptors," Anal. Biochem. Vol. 52 pp. 349-354. (1973).

100. Klett, R. P., B. W. Fulpius, D. Cooper, M. Smith, E. Reich, and L. D. Possani, "The acetylcholine receptor: I. Purification and characterization of a macromolecule isolated from Electro phorus electricus," J. Biol. Chem. Vol. 248 pp. 6841-6853. (1973).

101. Devreotes, P. N. and D. M. Fambrough, "Acetylcholine receptor turnover in membranes of developing muscle fibers," J. Cell Biol. Vol. 65 pp. 335-358 (1975).

102. Lukasiewicz, R. J., M. R. Hanley, and E. L. Bennett, "Properties of radiolabeled α-bungarotoxin derivatives and their interaction with nicotinic acetylcholine receptors," Biochem. Vol. 17 pp. 2308-2312 (1978).

103. Brockes, J. P. and Z. W. Hall, "Acetylcholine receptors in normal and denervated rat diaphragm muscle. I. Purification and interaction with [^{125}I]-α-bungarotoxin," Biochem. Vol. 14 pp. 2092-2099 (1975).

104. Colquhoun, D. and H. P. Rang, "Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle," Mol. Pharm. Vol. 12 pp. 519-535 (1976).

105. Chiu, T. H., J. O. Dolly, and E. A. Barnard, "Solubilization from skeletal muscle of two components that specifically bind α-bungarotoxin," Biochem. and Biophys. Res. Comm. Vol. 51 pp. 205-213 (1973).

106. Miledi, R., "Junctional and extra-junctional acetylcholine receptors in skeletal muscle fibres," J. Physiol. Vol. 151 pp. 24-30 (1960).

107. Barnard, E. A., J. Wieckowski, and T. H. Chiu, "Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions," Nature Vol. 234 pp. 207-209 (1971).

108. Fambrough, D. M. and H. C. Hartzell, "Acetylcholine receptors: number and distribution at neuromuscular junctions in rat diaphragm," Science Vol. 176 pp. 189-191 (1972).

117. Linden, D. C. and D. M. Fambrough, "Biosynthesis and degradation of acetylcholine receptors in rat skeletal muscles. Effects of electrical stimulation," Neurosci. Vol. 4 pp. 527-538 (1979).

118. Gutmann, E., V. Hanzlíková, and M. Sobotkova, "Effect of castration on motor endplate structure and function of the levator ani muscle of the rat," Physiologia bohemoslov. Vol. 18 pp. 482-483. (1969).

119. Hanzlíková, V. and E. Gutmann, "Effect of castration and testosterone administration on the neuromuscular junction in the levator ani muscle of the rat," Cell Tiss. Res. Vol. 189 pp. 155-166 (1978).

120. Menniti, F. S. and M. J. Baum, "Differential effects of estrogen and androgen on locomotor activity induced in castrated male rats by amphetamine, a novel environment, or apomorphine," Brain Res. Vol. 216 pp. 89-107 (1981).

121. Tucek, S., D. Kostirová, and E. Gutmann, "Effects of castration, testosterone and immobilization on the activities of choline acetyltransferase and cholinesterase in the rat limb muscles," J. Neurol. Sci. Vol. 27 pp. 363-372 (1976).

122. Robbins, N., A. Olek, S. S. Kelly, P. Takach, and M. Christopher, "Quantitative study of motor endplates in muscle fibres dissociated by a simple procedure," Proc. R. Soc. Lond. B Vol. 209 pp. 555-562 (1980).

123. Karnovsky, M. J., "The localization of cholinesterase activity in rat cardiac muscle by electron microscopy," J. Cell Biol. Vol.

23 pp. 217-232 (1964).

124. Rubin, L. L., S. M. Schuetze, and G. D. Fischbach, "Accumulation of acetylcholinesterase at newly formed nerve-muscle synapses," Dev. Biol. Vol. 69 pp. 46-58 (1979).

125. Rogers, A. W., Techniques of Autoradiography, Elsevier, Amsterdam (1973).

126. Venable, J. H., "Morphology of the cells of normal, testosterone-deprived and testosterone-stimulated levator ani muscles," Am. J. Anat. Vol. 119 pp. 271-302 (1966).

127. Nystrom, B., "Post-natal development of the motor nerve terminals in 'slow red' and 'fast white' cat muscles," Acta. Neurol. Scand. Vol. 44 pp. 363-383 (1968).

128. Kuno, M., S. A. Turkanis, and J. N. Weakly, "Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog," J. Physiol. Vol. 213 pp. 545-556 (1971).

129. Harris, J. B. and R. R. Ribchester, "The relationship between end-plate size and transmitter release in normal and dystrophic muscles of the mouse," J. Physiol. Vol. 296 pp. 245-265 (1979).

130. Barker, D. and M. C. Ip, "Sprouting and degeneration of mammalian motor axons in normal and de-afferented skeletal muscle," Proc. R. Soc. (Lond.) B. Vol. 163 pp. 538-554 (1966).

131. Humason, G. L., Animal Tissue Techniques, Third Edition, Freeman, San Fransisco (1972).

132. Young, W. S. III and M. J. Kuhar, "A new method for receptor autoradiography: [^{3}H]opioid receptors in rat brain," Brain Res. Vol. 179 pp. 255-270 (1979).

133. Fambrough, D. M. and P. N. Devreotes, "Newly synthesized acetylcholine receptors are located in the Golgi apparatus," J. Cell Biol. Vol. 76 pp. 237-244 (1978).

134. Nottebohm, F., "Asymmetries in neural control of vocalization in the canary," in Lateralization in the Nervous System, ed. Mr. Mysterioso,Academic Press, Inc., New York (1977).

135. Hess, A., "Vertebrate slow muscle fibers," Physiol. Rev. Vol. 50 pp. 40-62 (1970).

136. Chamberlain, F. W., Atlas of Avian Anatomy: Osteology, Arthrology, Myology, Hallenbeck, Lansing, Mich. (1943).

137. Ginsborg, B. L. and B. Mackay, "A histochemical demonstration of two types of motor innervation in avian skeletal muscle," Bibl. Anat. Vol. 2 pp. 174-181 (1961).

138. Vrbová, G., T. Gordon, and R. Jones, Nerve-Muscle Interaction, Chapman and Hall, London (1978).

139. Fedde, M. R., "Electrical properties and acetylcholine sensitivity of singly and multiply innervated avian muscle fibers," J. Gen. Physiol. Vol. 53 pp. 624-637 (1969).

140. Ko, P. K., M. J. Anderson, and M. W. Cohen, "Denervated skeletal muscle fibers develope discrete patches of high acetylcholine

receptor density," Science Vol. 196 pp. 540-542 (1977).

141. Schiaffino, S., S. P. Bormioli, and M. Aloisi, "Fiber branching and formation of new fibers during compensatory muscle hypertrophy," pp. 177-188 in Muscle Regeneration, ed. A. Mauro,Raven, New York (1979).

142. Vaughan, H. S. and G. Goldspink, "Fibre number and fibre size in a surgically overloaded muscle," J. Anat. Vol. 129 pp. 293-303 (1979).

143. Bradley, W. G., "Muscle fiber splitting," pp. 215-232 in Muscle Regeneration, ed. A. Mauro,Raven, New York (1979).

144. Mayr, R., "Structure and distribution of fibre types in the external eye muscles of the rat," Tissue and Cell Vol. 3 pp. 433-462 (1971).

145. Ashmore, C. R., T. Kikuchi, and L. Doerr, "Some observations on the innervation pattern of different fiber types of chick muscle," Exp. Neurol. Vol. 58 pp. 272-284 (1978).

146. Gordon, T., R. Perry, T. Srihari, and G. Vrbová, "Differentiation of slow and fast muscles in chickens," Cell and Tissue Res. Vol. 180 pp. 211-222 (1977).

147. Lentz, T. L., J. E. Mazurkiewicz, and J. Rosenthal, "Cytochemical localization of acetylcholine receptors at the neuromuscular junction by means of horseradish peroxidase-labeled α-bungarotaoxin," Brain Res. Vol. 132 pp. 423-442 (1977).

148. Koenig, J. and M. Vigny, "Influence of neurones and contractile activity on AChE and AChR in muscle cell culture," Progress in Brain Res. Vol. 49 p. 484 (1979).

149. Präve, E., "Der Einflus physiologischer Kastration auf sexuelle Verhaltensweisen von männlichen Zebrafinken," J. Ornithol. Vol. 116 pp. 345-346 (1975).

150. Guth, L., W. C. Brown, and J. D. Ziemnowicz, "Changes in cholinesterase activity of rat muscle during growth and hypertrophy," Amer. J. Physiol. Vol. 211 pp. 1113-1116 (1966).

151. Guth, L., "Effect of immobilization on sole-plate and background cholinesterase of rat skeletal muscle," Exp. Neurol. Vol. 24 pp. 508-513 (1969).

152. Snyder, D. H., D. H. Rifenberick, and S. R. Max, "Effects of neuromuscular activity on choline acetyltransferase and acetylcholinesterase," Exp. Neurol. Vol. 40 pp. 36-42 (1973).

153. Lomo, T. and J. Rosenthal, "Control of acetylcholine sensitivity by muscle activity in the rat," J. Physiol. Vol. 221 pp. 493-513 (1972).

154. Lomo, T. and R. H. Westgaard, "Further studies on the control of acetylcholine sensitivity by muscle activity in the rat," J. Physiol. Vol. 252 pp. 603-626 (1975).

155. Kelley, D. B., "Auditory and vocal nuclei in the frog brain concentrate sex hormones," Science Vol. 207 pp. 553-556 (1980).

156. Luine, V., D. Park, T. Joh, D. Reis, and B. McEwen, "Immunochemical demonstration of increased choline acetyltransferase concentration in rat preoptic area after estradiol administration," Brain Res. Vol. 191 pp. 273-277 (1980).

157. Fernandez, H. L., M. J. Duell, and B. W. Festoff, "Bidirectional axonal transport of 16S acetylcholinesterase in rat sciatic nerve," J. Neurobiol. Vol. 11 pp. 31-39 (1980).

158. Jessel, T. M., R. E. Siegel, and G. D. Fischbach, "Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord," Proc. Natl. Acad. Sci. USA Vol. 76 pp. 5397-5401 (1979).

159. Podleski, T. R., D. Axelrod, P. Ravdin, I. Greenburg, M. M. Johnson, and M. M. Salpeter, "Nerve extract induces increase and redistribution of acetylcholine receptors on cloned muscle cells," Proc. Natl. Acad. Sci. USA Vol. 75 pp. 2035-2039 (1978).

160. Christian, C. N., M. P. Daniels, H. Sugiyama, Z. Vogel, L. Jacques, and P. G. Nelson, "A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells," Proc. Natl. Acad. Sci. USA Vol. 75 pp. 4011-4015 (1978).

161. Buresová, M., E. Gutmann, and M. Klicpera, "Effect of tension upon rate of incorporation of amino acids into proteins of cross-striated muscle," Experientia Vol. 25 pp. 144-145 (1969).

162. Buresová, M. and E. Gutmann, "Effect of testosterone on protein synthesis and contractility of the levator ani muscle of the rat,"

J. Endocrin. Vol. 50 pp. 643-651 (1971).

163. Hanzlikovâ, V. and E. Gutmann, "The absence of androgen-sensitivity in the grafted soleus muscle innervated by the pudendal nerve," Cell. Tiss. Res. Vol. 145 pp. 121-129 (1974).

164. Carlson, B. M., A. A. Herbrychov, and E. Gutmann, "Retention of hormonal sensitivity in free grafts of the levator ani muscle," Exp. Neurol. Vol. 63 pp. 94-107 (1979).

165. Parker, M. G. and W. I. P. Mainwaring, "Effects of androgens on the complexity of poly (A) RNA from rat prostate," Cell Vol. 12 pp. 401-407 (1977).

166. Parker, M. G. and G. T. Scrace, "Regulation of protein synthesis in rat ventral prostrate: Cell-free translation of mRNA," Proc. Natl. Acad. Sci. Vol. 76 pp. 1580-1584 (1979).

167. Holtz, A., R. G. Brennan, D. Battista, and C. Terner, "Androgen control of an inhibitory modulator of phosphodiesterase in rat epididymis and prostate," Endocrin. Vol. 108 (4) pp. 1538-1544 (1981).

168. Cohen, S., B. W. O'Malley, and M. Stastny, "Estrogenic induction of ornithine decarboxylase in vivo and in vitro," Science Vol. 170 pp. 336-338 (1970).

169. Kuroda, Y., W.C. Merrick, and R. K. Sharma, "Polyamines inhibit the protein kinase 380-catalyzed phosphorylation of eukaryotic initiation factor 2-α," Science Vol. 215 pp. 415-416 (1982).

170. Goldstone, A., H. Koenig, and C. Lu, "Testosterone-dependent sexual dimorphism of the mouse kidney is mediated by polyamines," Biochem. and Biophys. Res. Comm. Vol. 104 pp. 165-172 (1982).

171. Kochakian, C. D. and C. Tillotson, "Influence of several C_{19} steroids on the growth of individual muscles of the guinea pig," Endocrinol. Vol. 60 pp. 607-618 (1957).

172. Max, S. R., "Cytosolic androgen receptor in skeletal muscle from normal and testicular feminization mutant (Tfm) rats," Biochem. Biophys. Res. Comm. Vol. 101 pp. 792-799 (1981).

173. Kochakian, C. D. and N. Arimasa, "The metabolism in vitro of anabolic-androgenic steroids by mammalian tissues," pp. 287-359 in Anabolic-Androgenic Steroids, ed. C. D. Kochakian, Springer-Verlag, Berlin (1976).

174. Guth, L., "Trophic" influences of nerve on muscle," Physiol. Rev. Vol. 48 pp. 645-687 (1968).

175. Katz, B. and S. Thesleff, "On the factors which determine the amplitude of the 'miniature end-plate potential'," J. Physiol. Vol. 137 pp. 267-278 (1957).

176. Hodgkin, A. L. and S. Nakajima, "The effect of diameter on the electrical constants of frog skeletal muscle fibres," J. Physiol. Vol. 221 pp. 105-120 (1972).

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