Cell death of motoneurons in the chick embryo spinal cord
XI. Acetylcholine receptors and synaptogenesis in skeletal muscle following the reduction of motoneuron death by neuromuscular blockade

RONALD W. OPPENHEIM, SHERRY BURSZTAJN and DAVID PREVETTE

1Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103, USA
2Department of Neurology, Baylor College of Medicine, Houston, TX 77030, USA

Summary

Treatment of chick embryos with neuromuscular blocking agents such as curare during periods of naturally occurring motoneuron death results in a striking reduction of this normal cell loss. Inactivity-induced changes in motoneuron survival were found to be associated with increased levels of AChRs and AChR-clusters in skeletal muscle and with increased focal sites of AChE that are innervated ('synaptic sites'). Treatment of embryos with curare after the normal cell death period (E12–E15) resulted in no change in motoneuron survival. Although AChR-clusters and focal sites of AChE were increased in these embryos on E16, many of these sites were uninnervated. Treatment of embryos with nicotine or decamethonium (E6–E10) also reduced neuromuscular activity but did not alter motoneuron survival nor did such treatment alter AChRs. The different effects of curare vs nicotine and decamethonium on motoneuron survival and AChRs may be related to the fact that the former is a competitive blocker whereas the latter two drugs are depolarizing blockers. Finally, treatment of embryos (E6–9) with doses of curare (1 mg daily) that allow for the almost complete recovery of neuromuscular activity a few days following treatment (by E16) resulted in the gradual loss of the excess motoneurons that were present on E10, and by E16 the number of remaining AChR clusters and focal sites of AChE were also decreased to levels comparable to control values. Inactivity-induced changes in AChRs or AChR-clusters may be an important factor in the reduced motoneuron death that accompanies neuromuscular blockade during critical stages of development. These receptor changes very likely reflect increased synaptogenesis in the muscles of paralyzed embryos which in turn may act to reduce motoneuron death by providing increased access to muscle-derived neurotrophic molecules.

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; ACh, acetylcholine; NMJ, neuromuscular junction; mdg/mdg, muscular dysgenic homozygous mouse mutant; dTc, d-Tubocurarine; E, embryonic day; LMC, lateral motor column; α-BTX, alpha-bungarotoxin; αCTX, alpha cobratoxin; TTX, tetrodotoxin.

Key words: cell death, motoneuron, acetylcholine receptor, chick embryo, spinal cord, synaptogenesis, skeletal muscle, neuromuscular blockade.

Introduction

The naturally occurring death of motoneurons in the spinal cord and brainstem of amphibian, avian and mammalian embryos is regulated in part by neuromuscular transmission and/or muscle contractions such that chronic neuromuscular blockade reduces, whereas enhanced muscle activity increases, the amount of motoneuron death (Oppenheim, 1981, 1985). In addition to changes in motoneuron survival, neuromuscular blockade also results in a number of other striking changes in neuromuscular development, including muscle atrophy, altered myogenesis, increased branching of peripheral nerves, hyperinnervation of surviving muscle, and alterations in the cholinergic enzymes, acetylcholinesterase (AChE) and choline acetyltransferase (ChAT). The extent to which these changes are primary (i.e. causal) or secondary with regard to the altered motoneuron survival is not yet clear.

In previous publications (Pittman and Oppenheim, 1979; Oppenheim, 1981, 1985; Oppenheim and Chu-Wang, 1983; Hamburger and Oppenheim, 1982), we put forth a working hypothesis that attempted to account for some of these results and their relationship to altered motoneuron survival. The decreased amount of ACh interacting with membrane AChRs following neuromuscular blockade would decrease the extent of membrane depolarization and muscle contraction and thereby
allow muscles to accept additional innervation. Increased innervation would lead to decreased cell death perhaps by allowing more motoneurons to gain access to sufficient amounts of a muscle-derived trophic factor for their maintenance. A part of our working hypothesis included the suggestion that activity-regulated changes in AChRs were involved in this sequence of events. Innervated AChR-clusters may define the site(s) (synapses) at which a muscle-derived trophic factor is released and/or taken up by the presynaptic terminal. Neuromuscular blockade would result in increased numbers of AChRs or AChR-clusters per myofiber (or myotube) which would reflect increased innervation (synaptogenesis) and decreased motoneuron death. In the present paper, we have attempted to examine this hypothesis in more detail by determining whether regimens of neuromuscular blockade that reduce motoneuron death do, in fact, alter synaptogenesis and the occurrence of AChR and receptor clusters in skeletal muscle in a manner consistent with the hypothesis.

It is known that high levels of extrajunctional AChRs are associated with the ability of adult muscle to become hyperinnervated (Jansen et al. 1973; Brookes et al. 1975; Fambrough, 1980). Furthermore, adult muscles exposed to α-CTX ('functional denervation') are also capable of becoming hyperinnervated (Duchen et al. 1975). Direct stimulation of adult denervated muscle can prevent or reverse the development of increased extrajunctional sensitivity to ACh (Lomo and Rosenthal, 1972; Drachman and Witzke, 1972; Jones and Vrbová, 1974; Purves and Sakmann, 1974) and hyperinnervation of adult denervated muscle can be blocked by electrical stimulation (Jansen et al. 1973, 1975). Neuromuscular blockade also alters the innervation of developing muscle (Gordon et al. 1974; Srihari and Vrbová, 1978; Pittman and Oppenheim, 1979; Dahm and Landmesser, 1988a; Ding et al. 1983; Harris and McCaug, 1984; Gordon et al. 1974; Gordon and Vrbová, 1975; Oppenheim et al. 1978); changes the amount and distribution of AChRs (Brathwaite and Harris, 1979; Burden, 1977; Schuetze and Role, 1987; Betz et al. 1980; Ziskind-Conhaim and Bennett, 1982; Harris, 1981) and helps regulate the normal loss of polyneuronal innervation at the embryonic and neonatal NM (Sohal et al. 1979; Thompson, 1983; Srihari and Vrbová, 1978; Benoit and Changeux, 1978; O'Brien et al. 1978; Oppenheim and Chu-Wang, 1983). Finally, inactivity or denervation of muscle also results in increases in mRNA for the α-subunit of the AChR (Goldman et al. 1988; Klarfeld and Changeux, 1985; Merlie et al. 1984). Thus, there is a considerable amount of evidence in support of the argument that neuromuscular activity is involved in regulating the amount and distribution of AChRs and in the development and maintenance of muscle innervation. However, it is not known whether similar changes in innervation and in AChR receptors occur following specific regimens of neuromuscular blockade that are critical for the reduced motoneuron death that accompanies such treatment in the developing chick embryo.

In the mouse mutant, muscular dysgenesis (mdg/mdg), in which there is an absence of muscle contraction in the embryo, virtually all of the same changes that occur in the experimentally immobilized chick embryo are found (see above), including reduced motoneuron death (Oppenheim et al. 1986). Increased motoneuron survival in the dysgenic mouse embryo is associated with branching of peripheral axons, hyperinnervation of muscle and increased numbers of AChRs and AChR-clusters (Rieger and Pincon-Raymond, 1981; Powell et al. 1984). In the present study, we report that similar changes in muscle AChRs and synaptogenesis are also associated with increased motoneuron survival in the chick embryo following neuromuscular blockade. Some of the results were briefly reported previously (Oppenheim and Bursztajn, 1983).

Materials and methods

Chick embryos (Hubbard Pullets no. 664) were used for all experiments. On E5 a window was made in the shell over the embryo, the window was sealed with Parafilm and the embryo returned to the incubator. Beginning on E6, or in one experiment on E12, embryos received daily or twice daily injections of dTc (1 or 2 mg once per day, Sigma), neostigmine methyl sulfate (5 μg twice per day, Sigma), nicotine (75–80 μg twice per day, Aldrich), decamethonium (6 μg once per day, Sigma) or avian Ringer's solution (control). All drugs were dissolved in avian Ringers and administered in volumes of 100–200 μl per injection onto the vascularized chorioallantoic membrane. The effect of the drugs on neuromuscular activity was monitored by daily or twice daily recordings of all trunk and limb movements (motility) as described previously (Pittman and Oppenheim, 1979).

Embryos were sacrificed on E9, E11 or E16. Brachial and lumbar spinal cords were dissected and fixed in Carnoy's solution, processed for paraffin histology, sectioned at 12 μm and stained with thionin. Cell counts of motoneurons in a single LMC (right or left) through the entire brachial or lumbar region were carried out according to previously described methods (Chu-Wang and Oppenheim, 1978; Pittman and Oppenheim, 1979). A correction factor was employed to offset counting errors due to the presence of more than one nucleolus per cell in a proportion of embryonic motoneurons (Clarke et al. 1976; Oppenheim et al. 1982). Muscles from the same embryos that were used for motoneuron counts were also included in the material used for examination of AChRs (see below). In one experiment, the sartorius and pectoralis muscles of control and dTc-treated embryos (1 mg, E6–E9) were removed on E16 and processed en bloc for the histochemical staining of focal sites of AChE (El-Badawi and Schenk, 1967). Individual myofibers were obtained by mechanical dissociation as described below for 125I-aBTX autoradiography, placed on slides in a drop of glycerine, cover-slipped and the number of focal sites of AChE determined as described below for AChR-clusters. A few muscles from E16 control embryos and embryos treated with 2 mg of dTc from either E6–E9 or E12–E15 were also double-stained for AChE and nerve terminals (silver) by the technique of Toop (1976) in order to examine innervation of focal sites of AChE-stained regions. As reported previously (Oppenheim, 1984), this procedure appears to reliably stain nerve terminals at virtually all such sites in embryos older than E13–E14. Furthermore, as reported below, the similarity in the number of AChR-clusters and the number of AChE sites per myofiber indicates that these two features are colocalized (also see Ziskind-Conhaim and Dennis, 1981; De La Porte et
al. 1986; Moody-Corbett et al. 1982). Consequently, it seems reasonable to assume that under most of the conditions studied here clusters of AChR and focal sites of AChE reflect synaptic sites. Using different methods, Dahm and Landmesser (1988a,b) have also recently shown that AChR-clusters are virtually always colocalized with nerve terminals in muscles of chick embryos at the ages used in the present study. By contrast, at earlier stages, AChR clusters often occur in the absence of nerve terminals (Smith and Slater, 1983).

For the examination of AChRs, muscles from chick embryos on E9, E11, and E16 (8 to 12 muscles per developmental stage) were dissected, quickly pinned onto small pieces of cork and fixed in 2% phosphate-buffered formaldehyde for 1h by immersion. For most experiments, the sartorius, pectoralis, peroneus and biceps muscles were examined. In a few experiments, the anterior (ALD) and posterior (PLD) lateral heads of each muscles were also examined. The pectoralis, biceps and PLD are muscles that contain focally innervated myofibers, whereas the sartorius and ALD are primarily multiply innervated muscles. The peroneus is composed of both focally and multiply innervated fibers. After an overnight wash in phosphate-buffered saline (PBS), muscles were incubated with 20 nM of $^{125}$I-a-BTX obtained from New England Nuclear, (specific activity 17–25 Ci/mmol$^{-1}$). Under these conditions, maximal labeling was reached in about 3h after incubation (Burtszian et al. 1984). After 1h washes in PBS, the total aBTX-binding sites in muscles of control and drug-treated tissue were expressed per muscle wet weight. The wet weight of muscles was determined by weighing each muscle on a Mettler balance and then each muscle was counted in a gamma counter. Background counts were determined by incubation of muscle tissue with unlabeled a-BTX (5 x 10$^{-7}$ m) for 1h followed by $^{125}$I-aBTX incubation. All incubations were carried out at 37°C in a 5% CO$_2$ and 95% air incubator. Specific binding was taken as the difference between total $^{125}$I-cyTBX bound and the amount bound with nicotine, neostigmine and decamethonium from E6–E10 treated with 2 mg of dTc daily from E6 to E9 (or E10) had significantly more motoneurons in both the brachial and lumbar spinal cord on E9, E11 and E16 (Fig. 1, Table 1). These increases in motoneuron numbers are comparable to those reported previously following similar regimens of neuromuscular blockade (Pittman and Oppenheim, 1979; Oppenheim and Majors-Willard, 1978; Oppenheim, 1984). As previously reported (Pittman and Oppenheim, 1979), motility in these embryos was also significantly reduced (but not entirely suppressed) from control values at all ages examined (Table 1). By contrast, embryos treated with neostigmine, nicotine or decamethonium from E6–E10 showed little or no change in motoneuron survival on E11 (Fig. 1) despite the fact that these drugs reduced motility to as great, or nearly as great, an extent as dTc (Table 1, Fig. 1). Only the embryos treated with neostigmine were significantly different from controls, having slightly fewer surviving motoneurons on E11. Embryos treated with 2 mg of dTc

![Fig. 1. Motoneuron numbers (E11, A) and motility (E6–11, B) following saline (CON), nicotine (NIC), curare (2 mg, CUR), neostigmine (NEOST) or decamethonium (DEC) treatment on E6–E10 (mean ± s.d.).](image)
from E12 to E15, which is after the period of naturally occurring motoneuron death, had motoneuron counts on E16 that were comparable to controls (Table 1). Embryos treated with 1 mg of curare from E6–E9, had significantly more brachial and lumbar motoneurons on E10, but by E16, following the recovery of motility that occurs after treatment with 1 mg (vs. 2 mg) of curare (Table 1), motoneuron numbers had decreased to control values (Table 1). Examination of the sartorius and pectoralis muscles stained for AChE from these same embryos (1 mg dTc) on E16 revealed no differences in the number of AChE-stained focal sites per unit length of myofiber when compared to controls (Table 2).

Although the sartorius (leg) muscle of E16 embryos treated with 2 mg dTc from E12–E15 had increased numbers of AChE-stained sites per unit length of myofiber (Table 2), when similar material was also stained with silver to exhibit nerve terminals, many of the increased sites of focal AChE were found to be uninnervated (Fig. 2). For instance, in a comparison of single longitudinal sections through roughly the same region of the sartorius muscle, it was found that approximately only 6% of the AChE-stained sites were not associated with nerve endings in controls, compared to 25% in the curare-treated cases (P<0.05). By contrast, embryos treated with 2 mg of dTc from E6–E9 and examined on E16 exhibited no differences in the proportion of innervated AChE sites (control = 93% vs 90% for dTc). That is, in these embryos, in which motoneuron numbers are increased on E16, virtually all AChE sites were innervated.

**Acetylcholine receptors and receptor clusters**

Embryos treated with dTc from E6–E10 (2 mg) or E6–E9 (1 mg, data not shown) had increased numbers of α-BTX-binding sites per mg of muscle wet weight on E11 for all muscles examined (Fig. 3). The embryos treated with 2 mg of dTc (E6–E9 or E6–E10) also had increased α-BTX binding on E16 (Fig. 4), whereas the 1 mg group did not (data not shown). Embryos treated with 2 mg of dTc on E12–E15 also had increased α-BTX binding on E16 (Fig. 5). Embryos treated with neostigmine, nicotine and decamethonium from E6–E10 had levels of α-BTX binding comparable to control values on E11 (Fig. 6).

An analysis of the autoradiograms of dissociated muscle fibers from embryos treated with dTc from E6–E10 (2 mg) shows an increased number of AChR-clusters per unit length of myofiber on both E11 and E16 (Figs 7, 8). By contrast, embryos treated with nicotine, neostigmine or decamethonium (E6–E10) did not differ from controls in the number of AChR-clusters present on E11 (Fig. 9; although not shown, the results for pectoralis, peroneus and biceps muscles were similar to the data reported here for sartorius). Embryos treated with 1 mg of curare from E6–E9 had increased AChR-clusters on E10 (only data for pectoralis is shown) but did not differ from controls on E16.

---

**Table 1. Motoneuron survival and motility following neuromuscular blockade**

<table>
<thead>
<tr>
<th>Treatment and region</th>
<th>Age (days)</th>
<th>N</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>6±1±2±6</td>
<td>10±4±3±3</td>
<td>13±6±4±0</td>
<td>14±8±3±5</td>
<td>16±7±3±7</td>
<td>16±4±4±1</td>
<td>17±0±4±2</td>
<td>16±2±3±6</td>
<td>13±9±3±6</td>
<td>14±1±4±5</td>
<td></td>
</tr>
<tr>
<td>MNs (B)</td>
<td>-</td>
<td>7±1±0±0</td>
<td>10±15±7±7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7±8±6±4±3§</td>
</tr>
<tr>
<td>MNs (L)</td>
<td>-</td>
<td>12±21±0±3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11±12±8±7±9</td>
</tr>
<tr>
<td>Curare 1 mg</td>
<td>8</td>
<td>3±0±1±1*</td>
<td>3±3±1±4*</td>
<td>5±7±1±8*</td>
<td>8±5±2±7*</td>
<td>10±2±3±1*</td>
<td>14±0±5±2</td>
<td>15±7±4±4</td>
<td>16±0±3±2</td>
<td>15±2±3±6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MNs (B)</td>
<td>-</td>
<td>14±8±7±6±4*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8±3±5±5§</td>
</tr>
<tr>
<td>MNs (L)</td>
<td>-</td>
<td>18±4±8±5±5**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10±9±8±6±1</td>
</tr>
<tr>
<td>Curare 2 mg</td>
<td>6</td>
<td>1±4±0±7**</td>
<td>1±2±0±8**</td>
<td>0±7±0±3**</td>
<td>2±8±1±1**</td>
<td>3±6±1±2**</td>
<td>3±0±1±0**</td>
<td>4±5±1±7**</td>
<td>3±8±1±3**</td>
<td>5±2±2±2**</td>
<td>4±6±2±0**</td>
<td>-</td>
</tr>
<tr>
<td>MNs (B)</td>
<td>-</td>
<td>15±7±7±5±3*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14±3±6±4±5**</td>
</tr>
<tr>
<td>MNs (L)</td>
<td>-</td>
<td>18±7±9±4±5**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18±2±4±6±3**</td>
</tr>
<tr>
<td>Curare 2 mg</td>
<td>8</td>
<td>1±4±0±7**</td>
<td>1±2±0±8**</td>
<td>0±7±0±3**</td>
<td>2±8±1±1**</td>
<td>3±6±1±2**</td>
<td>3±0±1±0**</td>
<td>4±5±1±7**</td>
<td>3±8±1±3**</td>
<td>5±2±2±2**</td>
<td>4±6±2±0**</td>
<td>-</td>
</tr>
<tr>
<td>MNs (B)</td>
<td>-</td>
<td>15±7±7±5±3*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14±3±6±4±5**</td>
</tr>
<tr>
<td>MNs (L)</td>
<td>-</td>
<td>18±7±9±4±5**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18±2±4±6±3**</td>
</tr>
</tbody>
</table>

†B, brachial; L, lumbar; MNs, motoneurons.

‡E6–E10 *P<0.01, Mann-Whitney test.

§E12–E15 **P<0.001, Mann-Whitney test.

---

**Table 2. Effects of curare treatment on focal AChE staining in muscle on E16**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of focal AChE sites</th>
<th>µm⁻¹ of fiber (×10⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>Sartorius</td>
<td>0±12±0±06</td>
</tr>
<tr>
<td>Curare (1 mg, E6–9)</td>
<td>-</td>
<td>0±11±0±08</td>
</tr>
<tr>
<td>Curare (2 mg, E12–15)</td>
<td>-</td>
<td>0±34±0±05*</td>
</tr>
</tbody>
</table>

† Sample size = No. of muscle fibers; *, P<0.01 compared to control, t-test.
Motoneuron death and acetylcholine receptors

Fig. 2. Innervation and AChE sites in E16 sartorius muscle following curare treatment (E12-E15, 2 mg). (A,B) control; (C,D) curare. Solid arrows in C indicate innervated regions (In), whereas open arrows indicate uninnervated regions (Uln). D is a higher magnification from an uninnervated region (compare to B).

Fig. 3. $^{125}$I-αBTX binding per wet weight of muscle for E11 control and curare-treated (E6-E10, 2 mg) embryos (mean ± s.d.). *, $P<0.05$, t-test. Values in the bars refer to number of muscles examined.

(Fig. 10). Data obtained for AChR-clusters from sectioned muscles (not shown) was virtually identical to that presented here for dissociated muscle fibers. Autoradiograms of muscles from a few E9 embryos (i.e. at a stage 3 days prior to the cessation of cell death on E12) that were treated with 2 mg dTc from E6-E9 were examined (data not shown) and also found to have an increase in AChR-clusters (Fig. 11). Although it was not possible to determine the relationship between motoneuron survival in specific motor pools and the number of AChR clusters in a particular muscle, a comparison of the data for overall motoneuron survival (Fig. 1) and AChR clusters for E10 embryos (Figs 7A,
Fig. 5. $^{125}$I-αBTX binding per wet weight of muscle for E16 control and curare-treated (E12–E15, 2mg) embryos (mean ± s.d.). *, P < 0.05; **, P < 0.01, t-test.

Fig. 6. $^{125}$I-αBTX binding per wet weight of muscle (sartorius only, see text) for E11 control, curare, neostigmine, nicotine and decamethonium treated embryos (mean ± s.d.). *, P < 0.05 between curare and other groups, t-test.

9, 10A) suggests that there may be a direct relation between these parameters.

Finally, we did not detect any degenerating myotubes in the muscles of either control embryos or embryos treated with nicotine, neostigmine or decamethonium on E10. The muscles of the drug-treated embryos stained with hematoxylin and eosin appeared indistinguishable from controls.

Discussion

A considerable amount of evidence now exists showing that neuromuscular activity in both adult and developing animals plays a role in regulating both AChRs and the pattern and extent of muscle innervation (Schuetze and Role, 1987; Fambrough, 1980). The genetically induced suppression of fetal movements in the mouse mutant mdg/mdg results in an enhanced branching of motor axons in the muscle, hyperinnervation of muscle, increased AChRs and AChR-clusters and a striking reduction of naturally occurring motoneuron death (Rieger and Pincon-Raymond, 1981; Powell et al. 1984; Oppenheim et al. 1986). The pharmacological suppression of embryonic movements in the chick results in an array of neuromuscular changes similar to those seen

Fig. 7. AChR-clusters per unit length of myofiber on E11 (A) and E16 (B) for control and curare-treated (E6–E10, 2mg) embryos (mean ± s.d.). *, P < 0.05 in A and P < 0.01 in B, t-test. Values in the bars refer to number of myofibers examined.

Fig. 8. AChR-clusters per unit length of myofiber on E16 for control and curare-treated (E12–E15, 2mg) embryos (mean ± s.d.). *, P < 0.05; **, P < 0.001, t-test.
Motoneuron death and acetylcholine receptors

in mdg/mdg (Oppenheim and Chu-Wang, 1983; Dahm and Landmesser, 1988b), including reduced motoneuron death. And as we have shown in the present report, changes in AChRs and AChR-clusters can also now be added to this list of inactivity-induced alterations of neuromuscular development in the chick. Thus, the reduction of muscle activity in both the dysgenic mouse embryo and normal chick embryo during the period of naturally occurring motoneuron death results in increased motoneuron survival and an increase in AChRs and AChR-clusters in skeletal muscle. Neuromuscular blockade during the cell death period of trochlear motoneurons in the duck embryo also results in an increase in AChRs in the superior oblique muscle (Creazzo and Sohal, 1983).

Although inactivity-induced increases in AChRs during embryonic development is not a novel finding (e.g. Burden, 1977), the fact that in the present work this increase occurs during a time when motoneuron death is greatly reduced raises the possibility that the two events are somehow causally related. Because neuromuscular blockade from E12–E15 in the chick also resulted in a significant increase in AChRs and AChR-clusters, but no change in motoneuron numbers on E16 (also see Burden, 1977), inactivity-induced changes in AChRs can obviously occur independent of altered motoneuron survival. However, when muscles from these embryos were doubly stained for AChE and visualization of nerve terminals (silver stain), many of the increased sites of focal AChE staining per myofiber, which very likely are colocalized with AChR-clusters (Ziskind-Conhaim and Dennis, 1981; De La Porte et al. 1986; Moody-Corbett et al. 1982; present results), were found to be uninnervated. A similar result was found by Ziskind-Conhaim and Bennett (1982) in rat embryos that were chronically treated with TTX at stages when motoneuron death is nearly over (i.e. the increased numbers of colocalized clusters of AChRs and focal sites of AChE in these rat embryos were uninnervated). By contrast, chick embryos treated with curare (2 mg) from E6–E10 have reduced cell death accompanied by increased AChR-clusters and AChE sites on E16, and in this case virtually all of the increased AChE sites appear to be innervated (present results; Pittman and Oppenheim, 1979; Oppenheim, 1984; Ding et al. 1984). Thus, innervation of the increased focal sites of AChE staining (and the colocalized AChR-clusters) induced by inactivity, and which we interpret as synaptic sites, occurs only when neuromuscular blockade is present during the major period of motoneuron death. Furthermore, the observation that muscles appear to have increased clusters of AChRs on E9 (2 mg dTc, E6–E9), which is three days prior to the complete cessation of motoneuron death, indicates that the effects of inactivity on innervation and AChRs begins already during the normal cell death period and thus is not an independent, secondary effect of inactivity that only becomes manifest sometime following the normal cessation of motoneuron death (cf. Dahm and Landmesser, 1988a). Collectively, these results suggest that when neuromuscular blockade occurs during periods of initial muscle innervation and when neuromuscular function has already begun (limb activity begins on E6), the concomitant increase in AChR-clusters may reflect additional contact (synaptic) sites for motoneurons which may in turn increase the overall survival of motoneurons by providing increased access to target-derived neurotrophic factors (Oppenheim et al. 1988). Furthermore, although our material does not permit a precise quantitative determination of the relationship between AChR clusters and motoneuron numbers, it
Fig. 11. Autoradiograms of AChR-clusters on control (A,B) and curare-treated (C,D; 2 mg, E6–E9) PLD (A,C) and pectoralis (B,D) myofibers on E8.5 (st. 35-). Note the presence of only a single cluster of AChR (asterisks) per myofiber in controls vs multiple clusters per myofiber in curare-treated muscles. The numbers indicate individual myofibers.

would appear that these two parameters may be proportionately related. Finally, it is worth noting that although some previous studies (e.g. Gordon et al. 1974) have reported that chronic neuromuscular blockade in vivo inhibits the formation of focal AChE deposits, in the present study as well as in our earlier papers (Oppenheim et al. 1978; Pittman and Oppenheim, 1979), we were able to demonstrate the appearance of focal AChE deposits after activity blockade. As previously pointed out (Oppenheim et al. 1978), this apparent discrepancy is very likely due to differences in the extent to which activity is blocked in the different studies. With the doses and route of administration used in our studies, a small amount of residual neuromuscular activity remains, which is apparently sufficient for AChE development (see Results).

If the inactivity-induced increase in synaptogenesis, as reflected by the increased clusters of AChR (and innervated focal sites of AChE), is a critical factor in reduced motoneuron death, one might expect that when the neuromuscular blockade is terminated and muscle activity recovers, the excess motoneurons would be lost and that the number of AChR-clusters ('synaptic sites') would then return to normal or near normal levels. In fact, this is exactly what happens. Embryos treated with 1 mg of curare from E6 to E10 recover normal levels of muscle activity by E15–E16 (Pittman and Oppenheim, 1979; Oppenheim, 1984; present data) and the number of motoneurons and AChR-clusters (and innervated AChE sites) also decrease to a level comparable to control values.

Previously we showed that treatment of chick embryos with certain pharmacological agents during the cell death period (E6–E10) reduces muscle activity to an extent comparable to that of dTc or α-BTX but does not increase motoneuron survival (Oppenheim and Maderdrut, 1981; Oppenheim and Chu-Wang, 1983). Although it isn’t clear why the inactivity induced by these agents fails to alter motoneuron survival (but see below), in the present context it was of considerable interest to repeat those experiments in order to dissociate inactivity from increased motoneuron survival and determine whether there were any changes in AChRs or AChR-clusters. If activity-blockade-induced motoneuron survival is dependent upon synaptogenesis (as reflected by increases in AChRs and AChR-clusters) then one might expect that agents that block activity but fail to increase motoneuron survival would also fail to
increase AChRs or AChR-clusters. In fact, we have found that although nicotine and decamethonium* significantly reduce neuromuscular activity, both drugs fail to affect either motoneuron survival or AChR-clusters on E11. Neostigmine produced a small but statistically significant decrease in motoneuron survival but little or no change in AChR-clusters. Although the decreased motoneuron survival following neostigmine treatment could be due to the known myotoxic effects of this agent (Leonard and Salt peter, 1979), we failed to detect any degenerating myotubes in hindlimb muscles of these embryos on E10. Therefore, it seems unlikely that the loss of motoneurons is owing to a reduction in the number of myotubes in target muscles. However, we cannot exclude the possibility that other properties of muscle associated with the survival of motoneurons were altered by neostigmine treatment.

As noted above, the failure of nicotine, neostigmine and decamethonium at the doses used here to increase motoneuron survival is, on the face of it, surprising since these agents blocked activity as effectively as curare. Although these drugs act at different sites at the NMJ, all three agents are known to act as depolarizing blockers (Goodman and Gilman, 1975) and thus have a different mode of action compared to curare or α-BTX which are competitive blockers. This could also explain why higher doses of decamethonium than those used here (see footnote), do, in fact, increase motoneuron survival. High doses of decamethonium are reported to have a curare-like effect at the NMJ (e.g. Zaimis, 1952; Thesleff and Unna, 1954). Accordingly, it seems likely that the different effects of decamethonium, nicotine and neostigmine on motoneuron survival compared to curare and α-BTX may be due to their action as depolarizing agents. Studies are presently in progress to further examine this possibility.

The results of the present series of experiments are consistent with our working hypothesis that inactivity-induced motoneuron survival is associated with increased synaptogenesis (as reflected by an increase in levels of AChRs, and especially AChR-clusters, in skeletal musculature). Treatments that reduce neuromuscular activity and increase motoneuron survival (α-BTX, dTc) also increase AChR-clusters, whereas treatments that reduce neuromuscular activity but do not affect motoneuron survival (nicotine, decamethonium), do not alter AChR-clusters. If treatments are found which reduce motoneuron death but do not affect either AChRs or AChR-clusters (or other indices of increased innervation), this would provide evidence against the critical role of synaptogenesis in this phenomenon. In fact, Sohal et al. (1983) have reported that treatment of duck embryos with Ig from sera of patients with myasthenia gravis during the period of naturally occurring death of trochlear motoneurons reduces this cell loss to as great an extent as curare or α-BTX (Creazzo and Sohal, 1979; Zilles et al. 1981) without altering either neuromuscular activity or AChRs in the superior oblique muscle. However, because AChR-clusters, which may reflect sites of increased innervation and trophic support (see below), were not examined by Creazzo and Sohal, these data are not necessarily inconsistent with the role of AChRs and synaptogenesis in neuronal survival suggested by our results. More importantly, we have not been able to replicate the effect of myasthenia gravis sera on motoneuron survival in the chick embryo spinal cord (Oppenheim and Bursztajn, unpublished). Embryos treated daily (E6–E9) with doses of Ig from the sera of myasthenia gravis patients (concentrated to 17 mg protein ml−1) ranging from 0.06 ml to 0.5 ml (Sohal et al. 1983 used 0.06 ml day−1) had no consistent effect on either neuromuscular activity or motoneuron survival in lumbar spinal cord on E11.

We have suggested previously that increased AChRs or AChR-clusters induced by neuromuscular blockade during the cell death period may reflect a state of hyperinnervation of muscle fibers which then acts to allow motoneurons that would normally die to survive (Pittman and Oppenheim, 1979). Sufficient innervation of muscle may be a necessary condition in vivo for motoneurons to gain access to a muscle-derived neurotrophic factor that is required for their survival. Regions containing AChR-clusters may define contact sites at which this putative neurotrophic factor is released and taken up by innervating neurons.

Independent support for this notion is provided by reports that activity-blockade in the chick embryo results in increased branching and synaptogenesis by intramuscular nerves at even earlier stages than those examined here (Oppenheim and Chu-Wang, 1983; Dahm and Landmesser, 1988a,b). We have previously suggested that the enhanced survival of motoneurons following activity blockade results from the increased availability of a muscle-derived neurotrophic factor (Hamburger and Oppenheim, 1982). Increased availability of a trophic factor could reflect an activity-dependent mechanism, whereby the production of a neurotrophic factor is inversely related to activity. Alternatively, the increased availability of a neurotrophic factor following activity blockade may reflect enhanced uptake owing to increased synaptic sites without inferring that trophic factor production is directly affected. That is, active and inactive muscle may synthesize similar amounts of a neurotrophic factor but because of increased innervation of the inactive muscle, neurons may be better able to gain access to sufficient neurotrophic factor required for survival. Although these alternatives have not yet been critically examined, the present results, together with the recent reports that muscle extracts from inactive muscles are no more

*In a previous study, we found that treatment of chick embryos with a higher dose of decamethonium than used here reduced neuromuscular activity to a greater extent and resulted in a significant increase in motoneuron survival (Oppenheim and Chu-Wang, 1983). The lower dose used in the present study was deliberately chosen because it reduced motility to an extent comparable to curare but did not affect motoneuron survival. As discussed in the text, this difference may reflect depolarizing vs competitive effects of decamethonium at different doses.
effective than muscle extracts from active muscles in maintaining motoneurons in vitro and in vivo (Tanaka, 1987; Houenou et al., 1989), suggest that the increased axonal branching and synaptogenesis observed in activity-blocked muscles may be critical in enhancing motoneuron survival. If the rescue of motoneurons by activity-blockade in vivo is independent of an increased production of a neurotrophic survival factor, then target-derived trophic molecules must be limited by some other mechanism. In view of the effects of activity blockade on branching and synaptogenesis, it is possible that it is these events that are limiting and not trophic factor production or synthesis (Oppenheim, 1989).

This research was supported by NSF Grant BNS-8307324 and by NIH Grants NS-20402, NS 17876 and RCDA 00820. We thank Kenneth Dunner and Phil Zebo for their competent technical assistance, and Lucien Houenou for helpful discussions and suggestions.

References


**Motoneuron death and acetylcholine receptors**

341


(Accepted 20 June 1989)