

The Rescue of Developing Avian Motoneurons from Programmed Cell Death by a Selective Inhibitor of the Fetal Muscle-Specific Nicotinic Acetylcholine Receptor

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ABSTRACT: In an attempt to determine whether the rescue of developing motoneurons (MNS) from programmed cell death (PCD) in the chick embryo following reductions in neuromuscular function involves muscle or neuronal nicotinic acetylcholine receptors (nAChRs), we have employed a novel cone snail toxin α A-OIVA that acts selectively to antagonize the embryonic/fetal form of muscle nAChRs. The results demonstrate that α A-OIVA is nearly as effective as curare or α -bungarotoxin (α -BTX) in reducing neuromuscular function and is equally effective in increasing MN survival and intramuscular

axon branching. Together with previous reports, we also provide evidence consistent with a transition between the embryonic/fetal form to the adult form of muscle nAChRs in chicken that involves the loss of the gamma subunit in the adult receptor. We conclude that selective inhibition of the embryonic/fetal form of the chicken muscle nAChR is sufficient to rescue MNs from PCD without any involvement of neuronal nAChRs. © 2008

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INTRODUCTION

Since its inception more than 50 years ago, considerable evidence has been generated in support of the neurotrophic hypothesis in which the survival of developing neurons is thought to be greatly influenced by competition for target-derived trophic factors (Pettmann and Henderson, 1998). Subsequently, many such factors have been identified and their role

in promoting neuronal survival has been demonstrated by *in vitro* and *in vivo* studies including genetic experiments in which the trophic factors and their receptors have been deleted (Oppenheim and VonBartheld, 2008). More recently, neuronal activity has also been shown to modulate the survival of developing neurons. Synaptic transmission by afferent inputs onto postsynaptic cells can influence postsynaptic cell survival, and the synaptic activation of postsynaptic cells can influence the survival of the presynaptic neurons. For example, afferent inputs to parasympathetic ciliary ganglion (CG) neurons can be either pro- or anti-apoptotic (Wright, 1981; Merinney et al., 1987; Maderdrut et al., 1988; Bunker and Nishi, 2002), whereas synaptic transmission at the neuromuscular junction (NMJ) is pro-apoptotic (Pittman and Oppenheim, 1979; Oppenheim and Nuñez, 1982).

Although considerable progress has been made in understanding the cellular and molecular mechanisms involved in afferent activity-dependent neuronal survival (Hanson et al., 1998; Meyer-Franke et al., 1998; Hruska and Nishi, 2007), the mechanisms involved in the survival of presynaptic neurons by synaptic activation of postsynaptic neurons is less well understood. One hypothesis proposes that increases in postsynaptic activity reduces target-derived neurotrophic factor production and MN survival, whereas decreases in activity promote neurotrophic factor production and MN survival (Tanaka, 1987; Oppenheim, 1989). An alternative hypothesis (Oppenheim, 1989; Landmesser, 1992), based on observations that in the neuromuscular system synaptic activity regulates the intramuscular branching and synaptogenesis of motoneurons (MNs), proposes that branching and synaptogenesis modulate the access of motoneurons to target-derived neurotrophic factors. Therefore, increased activity would decrease branching/synaptogenesis and promote MN PCD, whereas decreased activity would increase branching/synaptogenesis and promote MN survival. Although these two hypotheses are not mutually exclusive, the evidence is more consistent with the access hypothesis (Tanaka, 1987; Houenou et al., 1990; D'Costa et al., 1998; Vernon et al., 2004).

The most common experimental paradigm for testing the role of synaptic activity at the NMJ in regulating MN survival is the chronic blockade of activity *in vivo* by pharmacological agents (e.g., curare) or toxins (e.g., alpha bungarotoxin, α BTX) that act at nicotinic acetylcholine receptors (nAChRs). These agents produce a dose-dependent decrease in neuromuscular activity and an increase in axon branching and MN survival (Oppenheim et al., 2000). Although

it was originally thought that these agents were selective for muscle nAChRs, subsequent studies have argued that they may also act on neuronal nAChRs on MNs in the spinal cord (Renshaw et al., 1993; Hory-Lee and Frank, 1995; Usiak and Landmesser, 1999). Accordingly, in previous studies we have attempted to determine the major site of action of nAChR antagonists in promoting MN survival (Caldero et al., 1998; Oppenheim et al., 2000, 2003) by, for example, using agents that act selectively at neuronal *versus* muscle nAChRs. Although the evidence from these studies is consistent with the selective role of muscle nAChRs in MN survival, reports of the presence of muscle-type nAChRs on chick embryo neurons (Pugh et al., 1995; Keiger et al., 2003) and of neuronal-type nAChRs in chick muscle (Corriveau et al., 1995; Romano et al., 1997) have cast some doubt on whether agents such as curare and α -BTX rescue MNs by a peripheral *versus* CNS site of action (e.g., Hory-Lee and Frank, 1995; Usiak and Landmesser, 1999). For this reason, we have attempted to resolve this issue by the use of a novel cone snail toxin that is highly selective in binding to and antagonizing the embryonic/fetal form of muscle nAChRs (Teichert et al., 2004, 2005, 2006). This peptide toxin, α A-conotoxin OIVA (α A-OIVA), is not only selective for the fetal muscle nAChR (*vs.* the adult muscle nAChR) but has been tested at a high concentration against various neuronal nAChRs and other ligand-gated ion channels without detectable activity (Teichert et al., 2006 and unpublished data). Thus, it is an ideal tool for resolving the issue outlined above. In addition, we have used a toxin from the venom of Wagler's pit viper that is selective for the epsilon (ϵ) form of the adult mammalian nAChR (Tan and Tan, 1989; Lin et al., 1995; McArdle et al., 1999) in an attempt to determine whether the embryonic or adult chicken muscle nAChR expresses the ϵ -subunit.

METHODS

Animals

Fertilized Brown Leghorn chicken eggs were incubated in the laboratory at 37°C and 60% relative humidity until embryonic day (E) 5 (stage 26, Hamburger and Hamilton, 1951) at which time a lateral window (1.5 cm²) was made in the shell over the embryo, the opening sealed with Parafilm, and then the eggs were returned to the incubator. Twenty-four hours later (E6, stage 30), the eggs were removed from the incubator, the Parafilm was removed, and experimental agents were administered in a 0.1 M PBS vehicle onto the highly vascularized chorioallantoic membrane, the window was resealed with Parafilm, and the eggs were returned to the incubator. Curare (D-tubocurarine,

Sigma, St. Louis) was administered once per day at 2.5 mg/egg in 200 μ L; α -BTX was administered once per day at 9.375 nmol/egg (75 μ g/egg) in 100 μ L; alpha (α) A-OIVA at 10–15 nmol/per egg (18.5–27.5 μ g/egg) in 125 μ L twice per day (every 10–12 h); Waglerin-1 (Wag-1) at 10 nmol/egg (25 μ g/egg) in 200 μ L once on E7. Except for Wag-1, the other agents were administered once or twice daily on E6, E7, E8, and E9 and embryos sacrificed on E10.

In addition to embryonic treatment, 1-week-old hatched chickens were given a single intraperitoneal (i.p.) injection of α -BTX at 0.25–0.38 nmol/g (2–3 μ g/g); α A-OIVA at 5–10 nmol/g (9.25–18.5 μ g/g), or Wag-1 at 0.4–0.6 nmol/g (1.5 μ g/g).

Neuromuscular Activity

Embryonic movements (motility) were recorded 2–3 times each day for 5 min through the window in the egg using a reliable, well-established method described previously (Oppenheim et al., 2000). Following the injection of hatched chickens, they were observed every 5 min for up to 2–3 h for signs of paralysis or behavioral dysfunction.

Histology and Cell Counts

Following chronic drug/toxin treatment from E6–E9, embryos were decapitated *in ovo* on E7.5 or E10, removed from the shell and immersed in Bouins fixative for 24 h and processed for paraffin embedding of the thoraco-lumbar trunk and spinal cord region. Paraffin blocks were serially sectioned (6 μ m on E7.5; 12 μ m on E10) and sections placed on microscope slides and stained with thionin. Healthy and pyknotic motoneurons (MNs) were counted in every 10th section through the entire lumbar enlargement as described (Clarke and Oppenheim, 1995). The obtained values were multiplied by 10 to provide an estimate of total healthy and pyknotic MNs. All cell counts were done blind as to treatment. Pyknotic cell counts were done on E7.5, the peak time of MN loss, and compared to the number of surviving MNs on E7.5–8.0.

Intramuscular Nerve Branching

Intramuscular nerve branching analysis was performed in the iliofibularis muscle (IFIB). For this purpose, E9–E10 embryos were fixed in Dent's fixative (4:1 methanol/dimethyl sulfoxide) at -20°C and muscles were dissected and processed for whole-mount immunocytochemistry with a TUJ1 monoclonal antibody (diluted 1/1000, R&D Systems, Minneapolis, MN), which recognizes the neuronal-specific class III β -tubulin, according to our previously described protocol (Oppenheim et al., 2003). Briefly, after progressive rehydration, preparations were incubated for 2 h in PBS containing 0.4% triton X-100 (PBST) and 20% horse serum and, afterwards, with the TUJ1 antibody (1 μ g/mL in PBST-20% horse serum) for 24–48 h at 4°C with gentle agitation. After washes in PBS 0.02% Tween 20, samples were incubated with Alexa 488 conjugated anti-

mouse IgG (diluted 1/500 in PBST-20% horse serum). Samples were mounted in a 1:3 glycerol PBS mixture and observed under a confocal laser scanning microscope FluoView 500 (Olympus, Hamburg, Germany). The density of innervation was measured in digitalized images by using Visilog 6 software (Noesis, Orsay, France). Several parameters were evaluated as follows: (a) the total length of intramuscular nerve branches (length), (b) the total number of terminal branching (end) points, (c) the total number of points with three or more branches (triple points), and (d) the total number of intervals of intramuscular nerves without branches (segments). Five to six muscles per experimental condition were analyzed.

RESULTS

Neuromuscular Activity

Following daily treatment with curare, α -BTX and α A-OIVA there was a chronic and marked reduction of embryonic motility between E6 and E9 (see Fig. 1). Because there were no significant differences between the curare ($n = 5$) vs. α -BTX ($n = 5$) treated embryos ($p > 0.30$) their motility data has been combined. Although there was a small but statistically significant increased loss of motility in the curare/ α -BTX group compared to α A-OIVA, α A-OIVA was

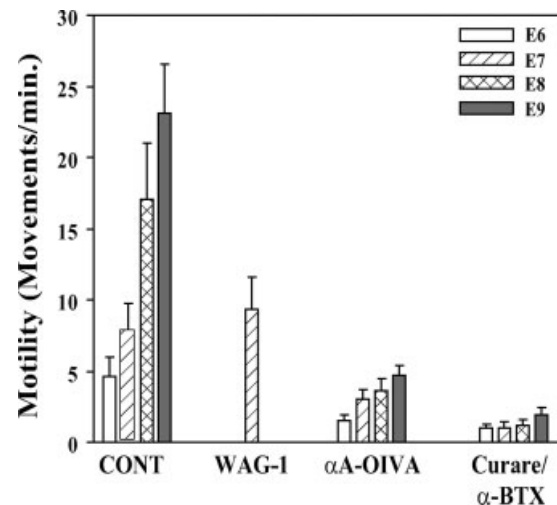


Figure 1 The number (mean \pm SD) of embryo movements (motility) from E6–E9 following daily treatment with α A-OIVA and curare/ α BTX. At all ages motility was reduced compared to controls ($p < 0.001$ for OIVA and $p < 0.0005$ for curare/ α BTX, *t*-tests). As described in the text, because motility was decreased to the same extent in curare and α BTX treated embryos, these data were combined. The motility data for the curare/ α BTX vs. α A-OIVA groups also differed significantly (E6 $p < 0.05$; E7–E9 $p < 0.01$). Sample size at each age: CONT, $n = 11$; WAG-1, $n = 7$; α A-OIVA, $n = 10$; curare/ α BTX, $n = 10$; see text).

nonetheless quite potent in blocking neuromuscular activity. From this we conclude that inhibition by a highly selective antagonist of the embryonic/fetal form of the muscle-specific nAChR is nearly as effective as agents such as curare or α -BTX that are nonselective for the embryonic/fetal *versus* the adult form of the muscle nAChR and that can also inhibit some neuronal nAChRs (Hruska and Nishi, 2007). The failure of Wag-1, a highly selective antagonist of the adult form of the muscle nAChR, to affect neuromuscular activity in the embryo (see Fig. 1) or post-hatched chicken (see later) confirms the complete lack of an avian muscle nAChR subunit that is equivalent to the epsilon subunit of adult mouse muscle. However, because Wag-1 has a low affinity for the rat and human (*vs.* mouse) epsilon subunits (McArdle et al., 1999), we cannot exclude the possibility that there is an avian homolog of a nonmouse epsilon subunit.

To test the selectivity of α A-OIVA for embryonic/fetal *versus* adult muscle nAChRs, we administered it as well as α -BTX and Wag-1 to 1-week-old post-hatched chicks that are developmentally at a stage similar to 3- to 4-week-old weanling mice or rats when Wag-1 but not α A-OIVA blocks neuromuscular activity. Whereas α -BTX (0.25–0.38 nmol/g, 2–3 μ g/g) induced paralysis within 5–10 min (Mean = 8.3 ± 1.7 , $n = 5$), neither α A-OIVA (5–10 nmol/g, 9.25–18.5 μ g/g) nor Wag-1 (0.4–0.6 nmol/g, 1–1.5 μ g/g) had any effect for up to 2–3 h; no signs of muscle weakness or motor dysfunction were observed in either group (OIVA $n = 5$; Wag-1 $n = 6$). Although the lack of effect of α A-OIVA was expected, the Wag-1 results were surprising in that this toxin is selective for the ϵ -subunit of the adult mammalian (mouse) muscle nAChR beginning around postnatal day 10–15. Mice older than this are paralyzed by doses of Wag-1 of 0.5 μ g/g, whereas younger mice are unaffected by doses up to 15 times the LD50 for adult mice (McArdle et al., 1999). As described in the Discussion section, these data suggest that the ϵ -subunit of the nAChR is not expressed in embryonic or adult chicken muscle and that it is the loss of expression of the gamma (γ) subunit in chick muscle between embryonic and posthatch stages that accounts for the developmental selectivity of α A-OIVA.

Motoneuron Survival

As has been previously documented in many studies from different laboratories (Laing and Prestige, 1978; Creazzo and Sohal, 1979; Doi, 1981; Ding et al., 1983; Landmesser, 1992), the inhibition of neuromuscular activity by curare and α -BTX rescues devel-

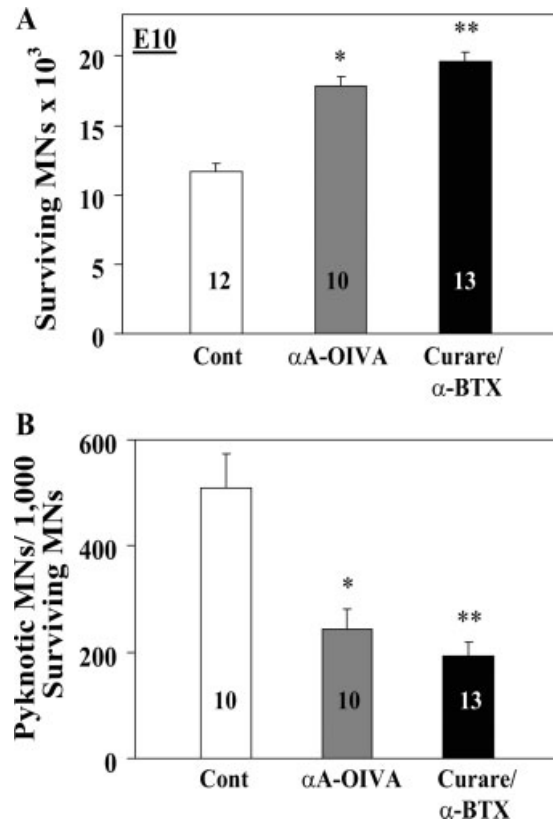


Figure 2 The number (mean \pm SD) of surviving and degenerating (pyknotic) MNs following treatment with α A-OIVA and curare/ α BTX (* $p < 0.002$; ** $p < 0.001$ vs. control, t -test). As described in the text, the MN counts for curare *versus* α BTX did not differ and therefore these data were combined (curare, $n = 6$; α BTX, $n = 7$).

oping MNs from PCD (see Fig. 2). Because the MN counts of curare ($n = 6$) *vs.* α BTX ($n = 7$) treated embryos were statistically indistinguishable ($p > 0.19$), these data were combined for statistical comparisons with controls. We have now demonstrated that activity blockade by a nAChR antagonist, α A-OIVA, which is highly selective for the embryonic/fetal form of the muscle-specific nAChR also rescues MNs from PCD. From these data we conclude that selective inhibition of this embryonic/fetal muscle receptor is sufficient to block neuromuscular activity and rescue developing MNs from PCD.

Intramuscular Axon Branching

Loss of neuromuscular activity in embryonic/fetal chickens and mice has been consistently shown to result in excessive intramuscular branching of MN axons and, in many cases, increases in synapse numbers (Oppenheim et al., 1986, 1997; Dahm and Landmesser, 1988, 1991; Houenou et al., 1990; Banks et al., 2001; Terrado et al., 2001; Misgeld et al.,

2002; Brandon et al., 2003). Whether and to what extent these or other changes following activity blockade are mediated by central (CNS) *versus* peripheral (muscle) events has long been a source of

debate, in large measure because of the lack of selectivity of most previous neuromuscular blocking agents for neuronal *versus* muscle nAChRs (Oppenheim et al., 2000). As shown in Figure 3 and Table 1,

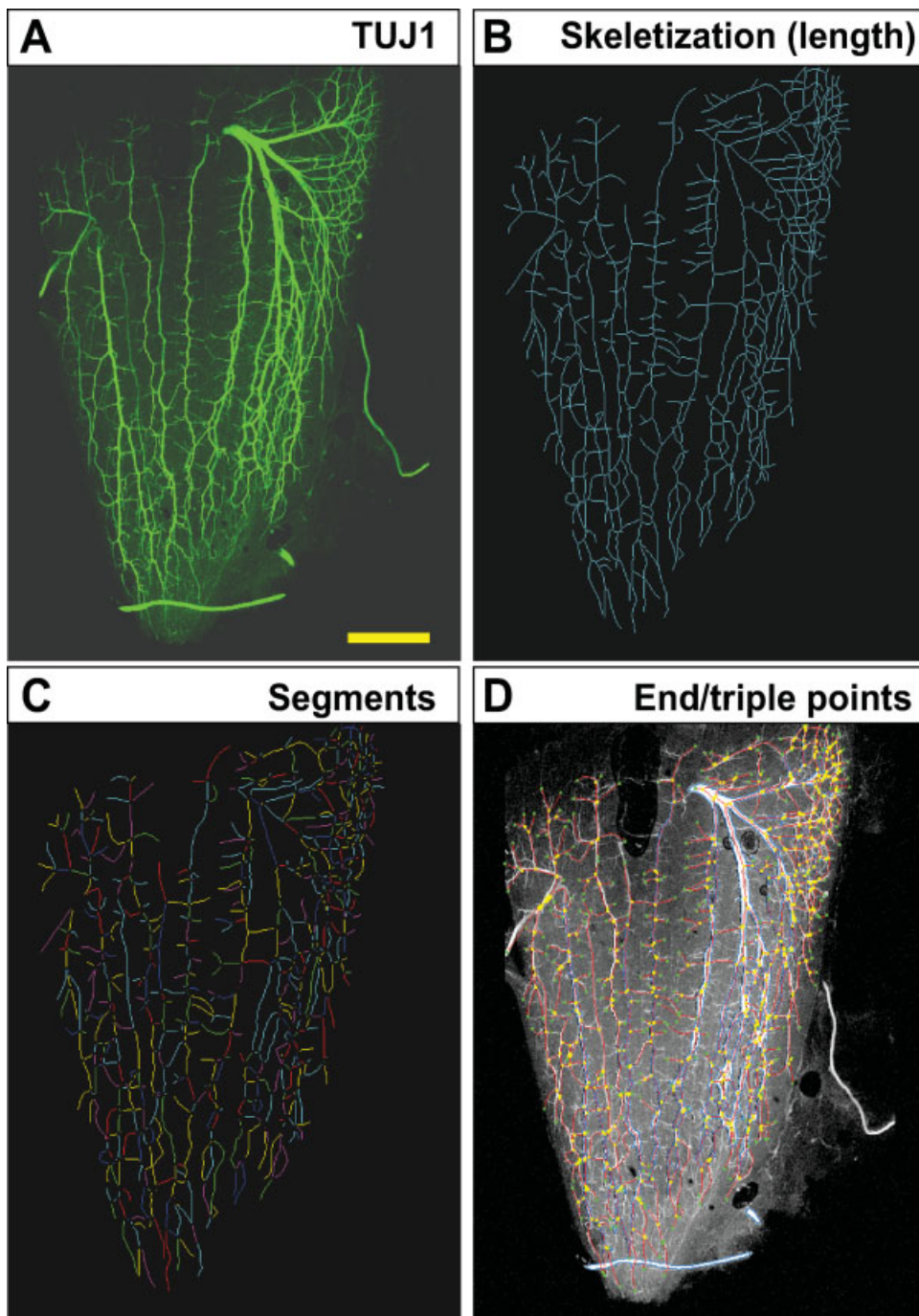


Figure 3 Examples of the intramuscular innervation of E10 chick embryo IFIB muscle observed in whole mounts after β -tubulin immunostaining with TUJ1 antibody. Samples were obtained from embryos treated with saline (control), α A-OIVA, or curare, although only a control muscle is shown. Images obtained by confocal microscopy (A), were subjected to image treatment (skeletonization) and analysis, including length (B), segments (C), and determination of triple branch (yellow) and end points (green) (D). Scale bar = 500 μ m.

Table 1 Parameters (Mean \pm SD) of Intramuscular Axon Branching in the IFIB Muscle After Activity Blockade

| Group | Length (μm) | End Points | Triple Points | Segments |
|---------|--------------------------------|---------------------------------|--------------------------------|---------------------------------|
| Control | 3774.2 \pm 441.6 ($n = 6$) | 292.5 \pm 39.9 ($n = 6$) | 401.3 \pm 72.2 ($n = 6$) | 659.3 \pm 104.3 ($n = 6$) |
| Curare | 3646.7 \pm 289.8 ($n = 5$) | 423.6*** \pm 54.3 ($n = 5$) | 547.4* \pm 85.6 ($n = 5$) | 894.4** \pm 120.8 ($n = 5$) |
| OIVA | 4072.5 \pm 113.9 ($n = 5$) | 426.4*** \pm 27.6 ($n = 5$) | 565.8** \pm 67.4 ($n = 5$) | 942.0** \pm 95.3 ($n = 5$) |

* $p < 0.02$.** $p < 0.01$.*** $p < 0.002$. All comparisons vs. control. *t*-tests with Bonferroni corrections.

however, activity blockade by α A-OIVA was as effective as curare/ α -BTX in increasing intramuscular axon branching in the hindlimb muscle iliofibularis. Branching was analyzed after digital skeletization of TUJ1 immunostained intramuscular nerves in IFIB muscle whole mounts imaged with a confocal microscope. Measured parameters included the following: (a) the total length of intramuscular nerves, (b) the number of segments identified as the intervals without branches, (c) the number of points in which three or more branches arise (triple points), and (d) the number of branch end points. As can be seen in Table 1, segments, triple points, and end points in the IFIB from both OIVA and curare-treated embryos are significantly (about 1.4-fold) higher than in controls. No significant differences were found between OIVA and curare-treated embryos, indicating that branching was increased to a similar extent in both conditions. The increased branching induced by curare and OIVA is not accompanied by a parallel increase in the total length measurements. This is likely due to the previously described muscle atrophy resulting in smaller muscles after activity blockade (Pittman and Oppenheim, 1979; Ding et al., 1983; McLennan, 1983) and the inability of image analysis to detect the thinnest branches at the low magnification required for whole-mount muscle measurements.

DISCUSSION

The Effects of Activity Blockade by α A-OIVA

The loss of neuromuscular activity in avian and mammalian embryos following either genetic perturbation of muscle-specific or NMJ-specific genes or by chronic treatment with neuromuscular blocking agents results in a common phenotype that includes muscle atrophy, increased intramuscular nerve branching and synapse formation and the rescue of MNs from PCD. Although this phenotype has been generally attributed to perturbations at the NMJ or in muscle, in many cases it has been difficult to

entirely exclude the role of deficits involving either neuronal nAChRs or other changes in CNS function (Hory-Lee and Frank, 1995; Usiak and Landmesser, 1999; Oppenheim et al., 2000, 2003). For example, we have previously shown in the chick embryo that drugs and toxins that selectively target muscle nAChRs results in the common phenotype described earlier, whereas agents that target neuronal nAChRs are ineffective (Oppenheim et al., 2000). Although these data are more consistent with a peripheral site of action for generating the common paralytic phenotype, because mRNAs for the adult $\alpha 1$ nAChR have been found in chick embryo neurons (Pugh et al., 1995; Keiger et al., 2003) and because mRNAs for neuronal nAChRs are transiently expressed in chick embryo muscle (Corriveau et al., 1995; Romano et al., 1997; Keiger et al., 2003), we cannot entirely exclude the possibility of either a central site of action on adult muscle-type nAChRs or of a role for neuronal type nAChRs in muscle following treatment with most of the reagents used in previous studies to generate the paralytic phenotype. For example, the conotoxins EIVA, MI, and GI used in our previous study (Oppenheim et al., 2000) do not discriminate between adult *versus* fetal muscle-type nAChRs (Johnson et al., 1995; Jacobsen et al., 1997; Teichert et al., 2005). Unlike α BTX, α A-OIVA does not inhibit neuronal nAChRs nor does it bind the nAChR $\alpha 1$ -subunit alone; rather it requires the assembly of the $\alpha 1/\gamma$ -interface within the fetal muscle nAChR for binding and inhibition. For this reason, the availability of α A-OIVA provides a unique opportunity to more definitively resolve this issue when compared with previous studies.

Chronic daily treatment with α A-OIVA is nearly as effective as curare and α BTX in blocking neuromuscular activity in the chick embryo. All three agents reduced activity by 75–90% compared to saline-treated control embryos. However, despite the small difference in effectiveness of α A-OIVA in blocking activity, it was equally effective as curare and α BTX in increasing intramuscular axon branching and in rescuing MNs from PCD.

The Developmental Transition of Muscle-Specific nAChRs

The results presented here using α A-OIVA are consistent with the role of the muscle-specific embryonic form of the nAChR in mediating the effects of activity blockade on both MN survival and intramuscular axon branching. However, the lack of an effect of Wag-1 on posthatch chickens was unexpected and cannot be explained by our use of an insufficient dose of Wag-1 (Tan and Tan, 1989; Lin et al., 1995; McArdle et al., 1999). In mammals, the switch from the embryonic to the adult form of the α 1 nAChR in muscle involves a change in subunit expression from γ to ε such that the embryonic receptor is composed of α 1 β 1 γ δ -subunits, whereas the adult receptor contains α 1 β 1 ε δ -subunits. Wag-1 is selective for the ε -subunit of the adult mouse receptor, whereas α A-OIVA selectively binds the α 1/ γ -interface of the mouse embryonic receptor. Previous studies have reported the absence of an ε -subunit in embryonic and adult chicken muscle (Schuetze, 1980; Moss et al., 1987; Fischbach and Rosen, 1997) and although it was not a major focus of our study, we have also failed to find evidence for a chicken homolog of the ε -subunit in a BLAST 2 search of the chicken gene data base. Together with our observation that Wag-1 does not block neuromuscular function in posthatch chickens, these data indicate that, in contrast to adult mammals, mature chicken muscle nAChRs lack an ε -subunit homologous to the mouse ε -subunit. Despite the absence of a γ to ε switch in the chicken muscle nAChR, however, the differential effects of α A-OIVA on embryonic *versus* adult muscle receptors is shared by both chicken and mouse (Teichert et al., 2005; present results).

Because the characterization of α A-OIVA specificity is based primarily on mammalian models we have considered the possibility that avian nAChRs may differ from mammalian receptors and thus that the data presented here for α A-OIVA may, in fact, not reflect specific antagonism of the avian fetal muscle nAChR. However, for the following reasons we consider this to be highly unlikely: (1) the γ -subunit in chicken muscle has been identified as the homolog of the mammalian γ -subunit by sequence similarity; (2) the γ -subunit is expressed in both chicken and mammalian embryonic muscle but not in mature chicken or mammalian muscle (i.e., this gene expression pattern is conserved in chicken and mammals); (3) α A-OIVA blocks muscle activity in both avian and mammalian fetal muscle but not in mature muscle; and (4) α A-OIVA has been demonstrated to be highly selective for the γ -subunit in mammals. Taken together, these genetic and functional data are most consistent with a

conserved mechanism of α A-OIVA antagonizing the fetal nAChR subtype in chicken muscle.

As is the case in mammals, embryonic but not adult chicken skeletal muscle nAChRs contain a γ -subunit (Moss et al., 1987; Harris et al., 1988). In addition to the documented loss of the γ -subunit in adult chicken muscle, the fact that α A-OIVA paralyzes embryonic but not posthatch chicken muscle confirms that embryonic chicken muscle expresses a fetal nAChR subtype that is lost in posthatch chickens. This suggests that the adult chicken muscle nAChR may function with only three different subunits, two α 1, two delta, and one β 1 subunit, which form a receptor complex with channel properties nearly identical to the fetal subtype that has a γ -subunit (Moss et al., 1987). Alternatively, the adult chicken muscle nAChR may have a novel subunit that replaces the embryonic γ -subunit. Although the functional significance of the fetal to adult transition from γ to ε in mammals is not fully understood, the fetal receptor subtype has a longer mean open time than the adult receptor (Mishina et al., 1986; Jaramillio et al., 1988) and generates more spontaneous action potentials in developing muscle that could have some developmental significance. For example, in mice, the subunit switch occurs in individual endplates early postnatally when endplates go from multiple to single innervation (Yamoto et al., 2005). Additionally, in newborn mice that lack the γ -subunit following gene deletion, there is an absence of spontaneous action potentials generated from miniature endplate potentials as well as increased intramuscular branching of axons and increased numbers of neuromuscular synapses, similar to the phenotype of the chick embryo described here following treatment with α A-OIVA (Takahasi et al., 2002; Koenen et al., 2005). Although MN survival has not been examined in these mice, we predict that they will, in fact, exhibit increased MN survival.

CONCLUSION

Although there appears to be a difference in subunit composition between chicken and mammalian adult muscle nAChRs, the use of the novel cone snail toxin α A-OIVA, specific for the fetal muscle nAChR subtype, has allowed us to demonstrate for the first time that inhibition of an embryonic form of muscle-specific nAChRs in the chick embryo is sufficient to rescue MNs from PCD and to increase intramuscular axon branching without any involvement of neuronal nAChRs. Together with previous studies in both chicken and mammalian embryos (Oppenheim et al.,

1986, 1997, 2000, 2003; Terrado et al., 2001), these data indicate that the major, if not the sole, site of action of activity-dependent MN survival is the peripheral neuromuscular system (i.e., the NMJ and muscle).

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