Nicotinic antagonists and cell death: an uncoupling of peripheral synaptogenesis from neuronal rescue in the avian ciliary ganglion

by

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ABSTRACT

Although systemic application of nicotinic acetylcholine receptor (nAChR) antagonists has long been known to rescue motor and ciliary ganglion (CG) neurons from developmental cell death, the mechanism of this rescue is unknown. It has been hypothesized that the neuronal rescue is mediated by pharmacological blockade of the postsynaptic nAChRs of the neuromuscular junction. Here, I argue otherwise, demonstrating that neuronal rescue occurs via blockade of neuronal α7-nAChRs. In contrast, axonal sprouting is controlled by the α1-nAChR, the muscle subtype, indicating that axonal sprouting and neuronal rescue are biochemically separate. I used somatostatin immunoreactivity to identify choroid neurons and confirmed with design-based stereology that daily administration of 75 μg/day of the nAChR antagonist, α-bungarotoxin (α-btx), from E7-E14 rescued both populations of ciliary ganglion neurons from cell death. I also found that a lower dose of α-btx, 12.5 mg/day, rescued ciliary, but not choroid neurons. In addition, I found that E8-E14 choroid tissue contained neither α-btx binding sites nor transcripts encoding either α1- or α7-nAChR receptor subunits. Furthermore, α-btx application did not induce nAChR internalization, increase neurotrophic activity in eye extracts, support cultured CG neuron survival, nor potentiate the activity of ciliary neurotrophic factor, glial cell derived neurotrophic factor, fibroblast growth factor, or eye extract, nor did nicotine induce cell death in cultured CG neurons. However, the α7-specific nAChR antagonist, methyllycaconitine (MLA), rescued both ciliary and choroid

neurons from cell death *in vivo*. Taken together, these results indicate that nicotinic antagonists rescue CG neurons from cell death by acting directly on neuronal α7-nAChRs. In addition, synaptogenesis and nerve branching, as measured by immunoreactivity for neurofilament and the synaptic vesicle marker, SV-2, were unchanged in MLA-treated iris, whereas α-btx caused a striking increase in the size of terminals. Alpha-btx-treated choroid neuron terminals, however, were unaffected by α-btx. Thus, in the CG, rescue of neurons was not strictly associated with increased synaptic terminals at the target, showing that, while synaptogenesis is augmented by blockade of α1-nAChRs, neuronal survival is mediated by α7-nAChRs.

INTRODUCTION:

Given that a certain amount of imprecision is bound to exist in any biological system, the fact that some cells die during normal development is perhaps not a wholly unexpected phenomenon. Many factors could cause a cell to die: having a later birth date, slower growth rate, less extensive afferent or efferent contacts, greater need for an exogenous trophic molecule, fewer trophic factor receptors, differential functional activity, or less efficient transport of an exogenous trophic molecule (Oppenheim, 1987). However, when the extent of such cell loss reaches massive proportions, as is the case in programmed cell death, sporadic biological errors that lead to occasional cell deaths no longer provide a sufficient explanation for such a large-scale phenomenon.

Programmed cell death is a nearly ubiquitous phenomenon used by multicellular organisms throughout their lives for a variety of different functions. For example, it can be used as a defensive strategy to remove infected, mutated, or damaged cells. Ongoing cell death in adult organisms maintains the homeostasis of cell number by balancing mitotic cell production. During development, it is involved in the formation of tubes, the separation of the digits, the remodeling of bone, and the involution of the mammary glands. Programmed cell death plays a role in the formation of the inner and outer cell mass in the blastocyst (Coucouvanis, 1995; Brison, 1997). In the haemopoetic system, cells at each stage of development are overproduced, and the excess cells are culled by programmed cell death. During positive selection in the thymus, thymocytes that

recognize self-MHC glycoproteins bound to antigens receive a signal to survive, while other thymocytes undergo cell death in a process that ensures that all mature T-cells carry receptors that can interact with the animal's MHC glycoproteins (Sha *et al.*, 1988; Teh *et al.*, 1988).

Programmed cell death is so pervasive, in fact, that the majority of the cells generated by the human body are destined to die by programmed cell death; it is relatively rare for cells to die through injury or the inability to sustain their own viability (Vaux and Korsmeyer, 1999). The human body eliminates nearly one hundred thousand cells per second by programmed cell death, a number that is balanced by the production of a similar number by mitosis. Most of the cells produced during mammalian embryonic development undergo programmed cell death before the end of the perinatal period. During our lifespan, over 99.9% of our cells will meet with the same fate (Vaux and Korsmeyer, 1999).

Apoptosis:

It took many years for the idea that massive cell loss is a normal part of development to be accepted. Carl Vogt was the first to recognize that cell death occurs in a predictable, "preprogrammed" fashion under physiological circumstances when he identified dying cells in the neuronal system of toad embryos (Vogt, 1842). It was not for more than a hundred years, however, that it became clear that cell death is involved in the process of metamorphosis, both in insects and in amphibians. The phrase "programmed cell death" was coined in

1965 to describe cell deaths in insect metamorphosis that occurred in predictable places at predictable times during development, emphasizing that the deaths are somehow programmed into the developmental plan of the organism (Lockshin and Williams, 1965). Shortly afterwards, it was revealed that the cell death in a tadpole's tail was blockable by cyclohexamide and, therefore, required the expression of endogenous genes (Tata, 1966).

The term "apoptosis" was coined by Kerr *et al.* in 1972 (from the Greek word meaning "leaves falling from a tree") to describe an intrinsic cell suicide program involved in the normal turnover of hepatocytes that involved the condensation of cell contents, breakdown of the nuclear membrane, and the formation of apoptotic bodies, which are small membrane-bound vesicles phagocytosed by neighboring cells (Kerr, 1972). Because apoptotic deaths are so stereotyped, Kerr and his colleagues proposed that these deaths reflect the operation of an active intracellular cell death program that can be activated or inhibited by a variety of environmental stimuli. It took more than ten years, however, for the idea that animal cells have a built-in cellular suicide program to gain general acceptance.

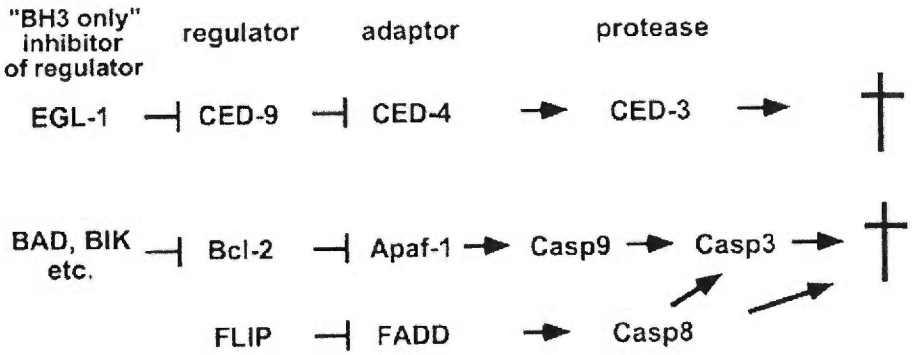
Widespread acknowledgement of apoptosis was aided by genetic studies in the mouse and in the nematode, *Caenorhabditis elegans*. The mammalian gene, *Bcl-2*, a human oncogene overexpressed in follicular lymphoma and the first component of a cell death mechanism to be recognized, was identified in

1982 and found to influence cell apoptotic response (Vaux, 1982; Adams, 1998). Three *C. elegans* genes, *ced3*, *ced4*, and *ced9,* were also shown to be dedicated to the death program and its control (Horvitz, 1982; Ellis, 1986; Ellis, 1991). Human Bcl2 was found to prevent programmed cell death in *C. elegans,* which demonstrated that apoptosis in mammalian cells and programmed cell death in the nematode were the same highly conserved process (Vaux, 1992). Subsequently, it was demonstrated that apoptotic signals converge to activate a group of apoptotic-specific cysteine proteases termed caspases that cleave their substrates after aspartic acid residues (Thornberry, 1998). All of the hallmarks of apoptosis: chromatin condensation, DNA fragmentation into nucleosomal fragments, nuclear membrane breakdown, and the formation of apoptotic bodies, were found to be direct consequences of caspase activation.

A schematic of the apoptosis effector mechanism is outlined briefly in Figure 1 (adapted from Vaux, 1999), which compares homologous proteins in nematode and vertebrate systems.

Cell death in *C. elegans*

Apoptosis is best understood in the nematode, *C. elegans*, in which developmental cell death occurs in neurons, muscle cells, epithelial cells, intestinal cells, and gonadal cells in a highly pre-programmed fashion. Because the organism is transparent, individual cell divisions or deaths can be observed in living nematodes, and the complete pattern of cell divisions and deaths that occurs



from Vaux, D. S. and Korsmeyer, S. J. (1999). Cell death in development. Cell 96, 245-254.

Figure 1. Comparison of apoptosis effector mechanisms in nematodes (top) and vertebrates (bottom). Signals activating the apoptosis effector mechanism may impinge at different levels, such as phosphorylation of BH3-only proteins, induction of BCl2 expression, or multimerization of adaptors such as FADD and Apaf1.

during *C. elegans* development has been determined by direct observation (Kimble and Hirsh, 1979; Sulston, 1983; Sulston and Horvitz, 1977). Development in the nematode involves a highly stereotyped series of cell divisions in which one daughter cell of a given lineage may be programmed to die in order to achieve the correct number of cells for each tissue. Out of an original 1,090 somatic cells, exactly 131 are destined to die during development (Ellis, 1991). Mutation of the genes *egl-2*, *ced-3*, or *ced-4* (see *figure 1*) prevents the deaths of all 131 cells, but the worms' appearance and behavior is essentially normal and their lifespan is unaffected. Most of the 131 cells adopt the fates of their sister cells and function normally. Because double-knockout, *ced-9-/-; ced-3 -/-*, worms survive and are able to reproduce, yet all 131 cells die in *ced-9-/-* animals, it is likely that no deleterious events occur prior to caspase activation in programmed cell death.

Cell death in vertebrates:

Unlike cell death in *C. elegans,* which consists of a pattern of specific, identifiable neurons dying in a highly preprogrammed fashion, programmed cell death in vertebrate nervous systems is much more probabilistic and dependent on extrinsic signals and cellular interactions. In 1906, M. L. Shorey began the first systematic analysis of the role of skeletal muscle targets in the development of spinal cord motor neurons. In 1909, Shorey reported that muscle nerves failed to develop and that there were fewer brachial motor neurons several days after she removed the forelimb bud in the chick embryo prior to peripheral axon outgrowth

(Shorey, 1909). She concluded that "the defects which appear in the nervous system are not due to degeneration, but to the failure of the neuroblasts to develop" (p. 51, *ibid*). Later, V. Hamburger and R. Levi-Montalcini confirmed the massive motor neuron loss following limb bud removal, but ultimately concluded that the loss was, in fact, caused by cellular degeneration, not faulty differentiation (Hamburger, 1934). Additionally, once it was revealed that vertebrate spinal cord motor neurons also undergo a period of cell death during development (Hamburger, 1958; Hamburger, 1975), the effects of target extirpation were reinterpreted as being an exaggeration of this normal programmed cell death.

The simplest interpretation for the finding that naturally occurring cell death can be greatly exacerbated by prior removal of the peripheral target (Hamburger, 1958; Landmesser and Pilar, 1974a; Hamburger, 1975; Prestige, 1976; Landmesser and Pilar, 1976; Chu-Wang and Oppenheim, 1978) is that failure of axons to reach their targets results in normal cell death. However, horseradish peroxidase injection into the chick eye prior to cell death results in the retrograde labeling of virtually all of the neurons supplying efferent innervation to the retina (Cowan and Clarke, 1976) and nearly all of the cells in the ciliary ganglion (Pilar *et al.*, 1980). Similarly, retrograde labeling of motor neurons in the chick spinal cord shows that these cells also reach their targets prior to the phase of cell death (Chu-Wang and Oppenheim, 1978). Therefore, the simple

failure of axons to reach their targets cannot explain normally occurring cell death.

In the mid-1970s M. Hollyday found that, while removing target tissue exacerbates cell death, increasing the amount of target tissue available to a given population of neurons by implanting a supernumerary limb rescues neurons that normally would have died during developmental cell death (Hollyday and Hamburger, 1976; Hollyday *et al.*, 1977). Similarly, manipulations that reduce the number of neurons innervating a given target also rescued neurons from cell death. In the chick ciliary ganglion, neurons reach their targets in the eye by way of three postganglionic nerves. When one or more of these nerves are cut, the total number of ciliary ganglion neurons is reduced before the period of cell death, yet they innervate a full-sized target. The result is that a larger fraction of the remaining neurons survive to maturity (Pilar *et al.*, 1980). Conversely, enlarging the population of neurons innervating a given target before cell death decreases neuronal survival; in the chick visual system, forcing both isthmo-optic nuclei to innervate only one eye results in fewer neurons in the nuclei surviving to maturity (O'Leary and Cowan, 1984). Thus, just as reducing or eliminating the target exacerbates cell death, enlarging the available target attenuates neuronal cell death.

One caveat to some of the target manipulation experiments outlined above is that these manipulations also induce significant numerical and other changes in

the associated dorsal root ganglion (DRG) (Hamburger and Levi-Montalcini, 1949; Hamburger, 1939), which very likely lead to changes in the extent of primary afferent input to interneurons and motor neurons. Thus, it is not entirely clear from these studies that target size is the only critical factor being altered that could affect motor neuron survival; changes in afferents may also be involved. Nevertheless, the target clearly plays a crucial role in neuronal developmental cell death.

Discovery of nerve growth factor:

Though it was clear from these manipulations that the target plays a critical role in neuronal cell death, it was the discovery of nerve growth factor (NGF) that demonstrated that a specific agent is required for neuronal survival. The discovery of NGF began with experiments carried out by E. Bueker in the late 1940s (Bueker, 1948). Bueker implanted mouse tumors into chick embryos to determine whether rapidly growing neoplasms might be an easier way to augment peripheral neuron survival than implanting supernumerary limbs. Three different tumors were implanted: mouse adenocarcinoma, which failed to grow, Rous sarcoma, which induced hemorrhage, and mouse sarcoma "180," which grew well. Bueker found that as the tumor grew, it was invaded by nerve fibers. In addition, the ipsilateral dorsal root ganglia were enlarged, as compared to the contralateral ganglia. By contrast, the spinal motor column was unaffected.

Though the significance of this finding was not immediately understood (Levi-Montalcini *et al.*, 1975), R. Levi-Montalcini and V. Hamburger determined that sensory, as well as sympathetic, ganglia were enlarged by the tumor, and that the effect was mediated by a substance secreted by the tumor. They called this agent "nerve growth factor" (Levi-Montalcini and Hamburger, 1953).

Next, Levi-Montalcini devised a neurite outgrowth assay to measure the effects of NGF (Cohen *et al.*, 1954) and she set about to identify the NGF molecule and evaluate its biological role. During one of the steps of the purification and analysis, Cohen and Levi-Montalcini used snake venom, which they serendipitously found to have the same properties as the tumor agent (Levi-Montalcini and Cohen, 1956; Cohen and Levi-Montalcini, 1956; Cohen, 1959). The fact that NGF was present in snake venom suggested to Cohen that it might also be present in the mammalian analogue of the venom gland, the salivary gland. By chance, the submaxillary gland of male mice contained very large amounts of NGF and provided the large amount of starting material needed to isolate and characterize NGF (Cohen, 1960).

In the late 1950s, the development of an NGF antiserum permitted Levi-Montalcini and Booker to test the hypothesis that NGF is a survival factor for developing sympathetic neurons (Levi-Montalcini and Booker, 1960). They found that after a few days of treatment with the antiserum, sympathetic ganglia in newborn mice and rats virtually disappeared (Levi-Montalcini, 1972).

Conversely, systemic treatment of developing mammals with exogenous NGF caused marked hypertrophy in the peripheral sympathetic system (Angeletti and Vigneti, 1971). *In vitro* studies confirmed that a major effect of NGF is promotion of neuronal survival (Levi-Montalcini, 1963; Varon *et al.,* 1973; Greene, 1977; Chun and Patterson, 1977a; Chun and Patterson, 1977b; Chun and Patterson, 1977c).

Introduction of the Neurotrophic Theory:

Together, the early studies on neuron-target interactions, the emerging recognition of naturally-occurring cell death, and the discovery of NGF led to the development of the Neurotrophic Theory, which proposes that neurons are initially overproduced and then compete with one another for the acquisition of sufficient neurotrophic factor, which is produced in limited amounts by the target tissue (Hamburger *et al.*, 1981; Barde, 1988; Davies, 1988; Barde, 1989; Oppenheim, 1989). The Neurotrophic Theory provides a useful framework for understanding many features of developmental cell death, although several pieces of data are inconsistent with the model. Chief among these is the fact that, though it is often assumed that the relationship between target availability and neuronal survival is linear and proportional (the size-matching hypothesis), in many of the experiments involving alterations in target size, this is not the case. This has been particularly true in experiments in which targets are enlarged. For example, according to the size-matching model, one would predict that doubling the amount of target tissue available to a population of neurons that normally

undergoes a 50% reduction during cell death would rescue nearly all of the neurons. This has not been found to be the case. Generally, less that half of the normal cell loss is prevented by these manipulations (Oppenheim, 1985; Oppenheim *et al.*, 1988; Sperry and Grobstein, 1985; Tanaka and Landmesser, 1986), and in one case cell death was not averted at all (Lamb, 1984).

Similarly, reductions in target size have not always produced the predicted degree of cell death (Lamb, 1980; Tanaka and Landmesser, 1986). Without knowing precisely what the neurons are competing for, it is difficult to interpret these mismatches between target size and neuronal population. Within the bounds of the Neurotrophic Theory, neuronal survival could be related to the number of muscle fibers, myotube or myofiber size, synaptic sites, postsynaptic receptors, trophic factors, non-muscle mesenchyme-derived tissue, molecular components of the cell surface or extracellular matrix, or other unknown features (Oppenheim, 1991). Additionally, there is evidence for the derivation of trophic factors from afferents, glia, and the extracellular matrix (Walicke *et al.*, 1989; Johnson, 1988; Lipton, 1986). Because increases or decreases in overall target size may not correlate with changes in the availability of the critical target-derived factor, they might not be expected to alter neuronal survival in a linear fashion (Sperry and Grobstein, 1985).

Rescue of motor neurons by nAChR antagonists:

Because the Neurotrophic Theory holds that neurons compete with one another for limited amounts of target-derived neurotrophic factor, it was proposed that synaptic activity at the neuromuscular junction might serve as the basis for this competition, perhaps through regulation of the uptake of target-derived factors (Pittman and Oppenheim, 1979). Pittman and Oppenheim tested this hypothesis in 1979 by pharmacologically blocking transmission at the neuromuscular junction during the period of normal motor neuron cell death with *in ovo* application of α-cobratoxin, d-tubocurare, α-bungarotoxin, and botulinum toxin. Botulinum toxin blocks the release of ACh from motoneuron boutons (reviewed in Drachman, 1972) whereas d-tubocurare and the various snake toxins directly block the nicotinic receptors, so all of them would be expected to interrupt neuromuscular transmission. Pittman and Oppenheim predicted that neuromuscular blockade would prevent the neurons from successfully competing for available trophic factors and that cell death would be exacerbated. Paradoxically, they found that rather than increasing cell death, neuromuscular blockade actually reduced it or prevented it entirely (Pittman and Oppenheim, 1979).

The simplest interpretation of this finding is that the drug treatment merely arrests the development of motor neurons before they reach the cell death stage, keeping them incapable of responding to the signals that normally initiate cell death. A measurement of a number of maturational indices, however, including

biochemical, anatomical, and ultrastructural changes, failed to uncover any differences between rescued neurons and their controls (Oppenheim, 1981; Oppenheim, 1984; Oppenheim, 1985; Oppenheim *et al.*, 1989). Thus, the neuronal rescue is not due to an arrested development of the neurons at an early, unresponsive developmental stage.

Another possibility is that the treatment with toxins increased neuronal numbers by changing neuronal proliferation, migration, or cell phenotype. Two facts argue against this interpretation: first, neuromuscular blockade was found to produce a reduction in the number of degenerating neurons in the spinal cord, and second, treatment of embryos with d-tubocurare during the period either before or after cell death did not change motoneuron numbers (Oppenheim, 1981; Oppenheim, 1984; Oppenheim, 1985; Oppenheim *et al.*, 1989). Thus, the increased motoneuron survival seen following systemic application of nAChR antagonists is, in fact, due to a direct reduction of cell death, rather than a change in neuronal proliferation, migration, or cell phenotype.

Another possible explanation for the rescue of neurons by nAChR antagonists is that cholinergic blockers might increase the available target tissue by increasing the number of muscle fibers. Examination of the targets, however, revealed that the various drug and toxin treatments actually resulted in a decreased muscle mass throughout treatment, so this interpretation seems unlikely to be true (Pittman and Oppenheim, 1979). Alternatively, the neuronal rescue

could result from the direct action of the various drugs on the motor neurons of the spinal cord rather than on the neuromuscular junction. Pittman and Oppenheim demonstrated that when they removed the limb bud before cell death, thus removing the source for the target tissue, the pharmacological agents were unable to prevent cell death. They concluded that the cholinergic blockers could not be acting at neuronal receptors. However, another interpretation for these results is that the cholinergic blockers do function at the motor neurons to decrease or fine tune cell death, but the neurons have a basic requirement for target-derived trophic factors for which the toxins cannot compensate.

The avian ciliary ganglion as a model system:

Although much of the work on nAChR antagonists has been carried out in spinal cord motor neurons (Levi-Montalcini, 1947; Hamburger, 1958; Prestige, 1976; Landmesser and Pilar, 1974b; Hamburger, 1975; Hollyday and Hamburger, 1976; Landmesser and Pilar, 1976; Hollyday *et al.*, 1977; Chu-Wang and Oppenheim, 1978; Pittman and Oppenheim, 1979; Oppenheim *et al.*, 2000), avian ciliary ganglion (CG) neurons are also rescued from cell death by the nAChR antagonist, α-bungarotoxin (α-btx), (Meriney *et al.*, 1987). Since the trophic requirements of CG neurons are better defined than those of spinal cord motor neurons, and because the physiology and pharmacology of the target tissues of CGs are well characterized, the avian CG makes an excellent model with which to study the rescue of neurons by nAChR antagonists.

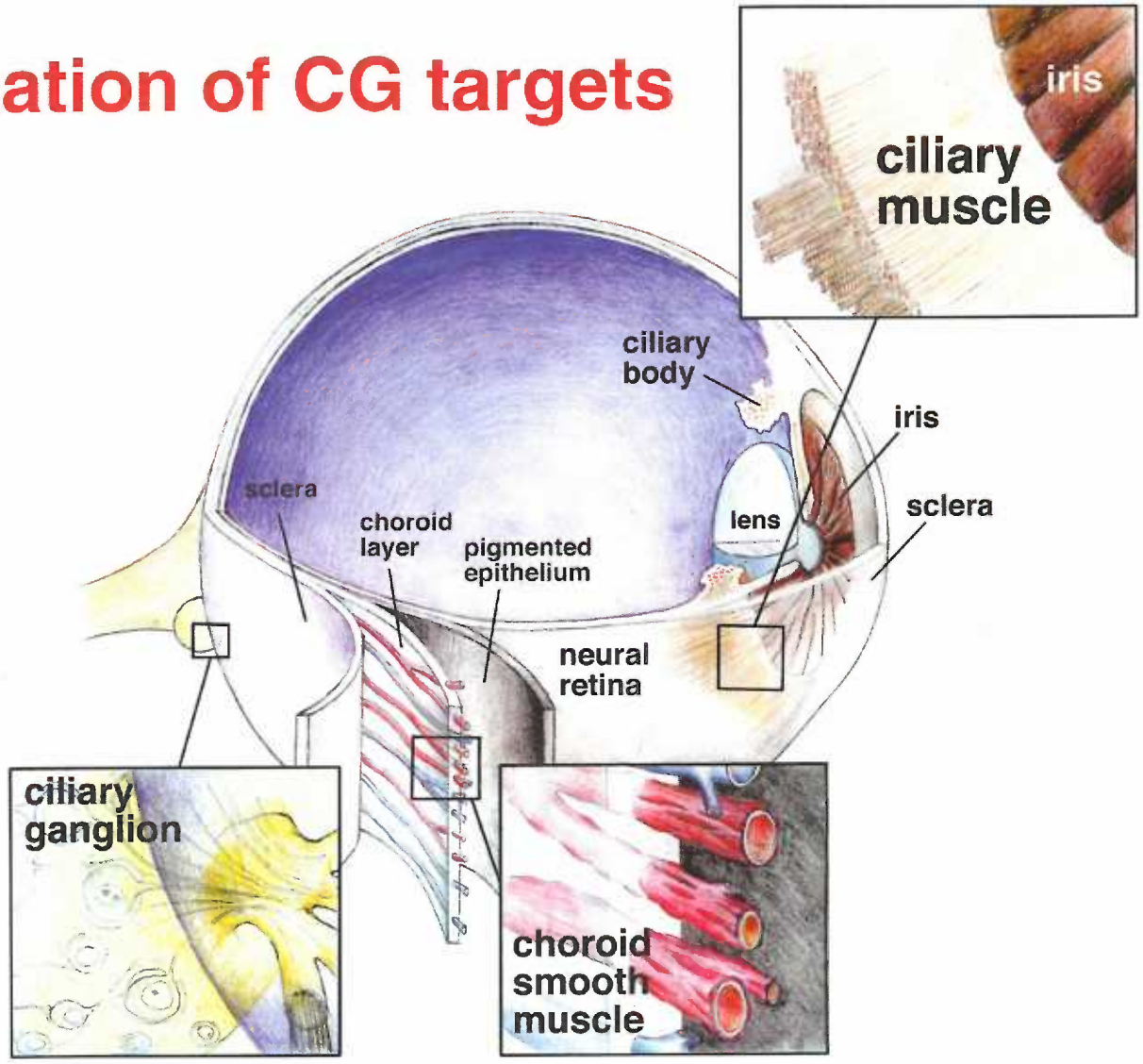
The CG, located in the orbit above the optic nerve, is the only source of parasympathetic motor innervation in the avian eye (*figure 2*). The CG contains two populations of neurons (Marwitt *et al.*, 1971): choroid neurons, which are small, unmyelinated cells that innervate vascular smooth muscle (Meriney and Pilar, 1987), and ciliary neurons, which are large myelinated cells that innervate striated muscle in the iris and ciliary body (Pilar, 1980). In the mature CG, choroid neurons can be distinguished from ciliary neurons by their small size (Landmesser and Pilar, 1974a), peripheral location within the ganglion (Marwitt *et al.*, 1971; Pilar *et al.*, 1980), bouton-like preganglionic innervation (Marwitt *et al.*, 1971; Dryer and Chiappinelli, 1985) and by the expression of the neuropeptide cotransmitter, somatostatin (Coulombe and Nishi, 1991; Distefano, 1993).

Both ciliary and choroid neurons undergo a decrease in number between stage 35 (E9) and stage 40 (E14), when the number of neurons in the ganglion is reduced from around 6,200 to just over 3,000 (Landmesser and Pilar, 1974a; Landmesser and Pilar, 1974b). As is the case for the motor neurons of the spinal cord, this period of naturally occurring cell death corresponds with the time when the neurons form synapses with their targets in the eye (Landmesser and Pilar, 1978; Pilar *et al.*, 1987), and it occurs after all of the ganglion cells have received a functional afferent innervation (Landmesser and Pilar, 1972): preganglionic nerve terminals of the CG project from neurons in the accessory occulomotor nucleus, the avian equivalent of the Edinger-Westphal nucleus, and nerve

Figure 2. The avian ciliary ganglion innervates two structures in the eye.

The CG is formed by cells that migrate from the neural crest of the caudal mesencephalon and rostral mesencephalon (Noden, 1975). Located in the orbit above the optic nerve, the CG is the only source of parasympathetic motor innervation in the avian eye. The CG contains two populations of neurons (Marwitt *et al.*, 1971): choroid neurons, which are small, unmyelinated cells that innervate vascular smooth muscle (Meriney and Pilar, 1987a), and ciliary neurons, which are large myelinated cells that innervate striated muscle in the iris and ciliary body (Pilar *et al.*, 1980). CG neurons, as well as both the iris and choroid muscle, can be grown in culture either alone, or in neuron/target co-cultures.

Location of CG targets



terminals reach the vicinity of the CG by E4. Functional synaptic transmission through the ganglion is detectable by E5, and by E8 all neurons receive a functional synaptic input (Landmesser and Pilar, 1972). CG neurons begin to extend axons as early as E4.5, immediately after formation of the ganglion, and these axons reach the iris and choroid before E6. However, significant numbers of functional synapses with targets are not formed until E8.5 for the iris and E10 for the ciliary body (Narayanan and Narayanan, 1978; Pilar *et al.*, 1987). Synapse formation in both targets is complete by E14 (Landmesser, 1978).

In cell culture, CG neurons depend upon neurotrophic factors such as ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), glial derived neurotrophic factor (GDNF), and neurturin for survival. *In vivo*, CNTF is expressed selectively in CG targets during cell death (Finn and Nishi, 1996a), and overexpression of CNTF in the avian embryo rescues CG neurons from cell death (Finn *et al.*, 1998), so it serves as the best candidate for a target-derived neurotrophic factor in this system.

The differences between iris/ciliary body and choroid muscle pharmacology are particularly useful in the study of the effects of cholinergic antagonists on cell death. Striated muscle is multinucleated and displays a well-organized contractile apparatus. Smooth muscle cells are more diverse in function and morphology, are mononucleated, and do not have banded striations. Choroid neuromuscular activity is mediated by muscarinic nAChRs (Meriney and

Pilar, 1987), while iris and ciliary muscles are also muscarinic at the beginning of cell death, but shortly transition to having a nicotinic phenotype (Pilar *et al.*, 1987). The smooth-to-striated muscle transition of the developing iris has been characterized morphologically, immunohistochemically, and electrophysiologically (Pilar *et al.*, 1987; Scapolo *et al.*, 1988; Volpe *et al.*, 1993; Link and Nishi, 1998b). Because this transition is so well characterized, and because the transitioning iris cells present a physiological contrast to the smooth muscle cells of the choroid, specific questions about the role of neuromuscular blockade in neuronal rescue can be addressed in the avian CG that are not possible in spinal motor neuron populations.

Rescue of CG neurons by nAChR antagonists:

Meriney *et al.* carried out a detailed study of the effect of nAChRs on neuronal survival in the CG, as well as physiological studies of the effect of the drugs on ganglionic and neuromuscular transmission. Using an HRP-backfill technique to label ciliary neurons and a histological stain to count total neurons in the CG, they were able to perform differential counts of ciliary versus choroid neuron numbers after treatment with various agents. They found that treatment with 75μg/day of the nAChR antagonist, α-btx, which blocks both neuronal- and muscle-type nAChRs nearly irreversibly, rescued both ciliary and choroid neurons by 89% and 100%, respectively. Interestingly, a lower dose of the toxin (12.5μg/day) increased ciliary neuron survival by 50% while decreasing choroid neuron survival by 26% (Meriney *et al.*, 1987). The authors were unable to

explain this differential dosage effect. In addition, treatment with curare, a reversible nAChR antagonist that blocks nicotinic receptors in the ganglion, iris, and ciliary body, resulted in a 36% increase in ciliary neuron survival, while causing a 25% decrease in choroid neuron survival. Muscarinic blockade by atropine, which blocks transmission at the choroid neuromuscular junction as well as the early ciliary and iris neuromuscular junctions, increased ciliary neuron survival by 71% while decreasing choroid neuron survival by 25% (Meriney *et al.*, 1987).

Because the counts for ciliary neurons were determined by retrograde filling of the ciliary nerve and the number of choroid neurons was derived by subtracting the number of labeled cells from the total neuronal number, any ciliary neurons that failed to be labeled would be counted as choroid neurons. Thus, this technique could generate an overestimate of the choroid neurons and an underestimate of the ciliary neurons. The total neuronal counts would not be skewed by this situation, however.

Models for the rescue mechanism:

A. nAChR antagonists may increase trophic factor production or secretion:

Because nAChR antagonists rescue neurons from cell death, it has been suggested that synaptic activity at the neuromuscular junction plays a critical role in regulating neuronal number. One means by which nAChR antagonists could influence neuronal survival *in vivo* is by inducing target tissues to produce or

release more trophic factors (Pilar *et al.*, 1988; but see also Tanaka, 1987; Oppenheim *et al.*, 1989). In 1988, Pilar *et al.* reported that *in vivo* application of α-btx produced a fourfold increase in trophic activity in the developing chick eye early in cell death (stage 37) but a 50% reduction in trophic activity by E14. By contrast, two other studies have failed to detect an increase in the production of neurotrophic factor by the targets of spinal cord motor neurons following nAChR antagonist administration (Tanaka, 1987; Oppenheim *et al.*, 1989). None of these studies, however, accounted for the possibility that nAChR antagonists rescue neurons, not by increasing the rate of trophic factor production, but by altering trophic factor secretion or availability. Both possibilities, that nAChR antagonists rescue neurons by increasing neurotrophic factor production or by increasing neurotrophic factor secretion, merit further exploration.

B. nAChR antagonists may regulate cell death via control of synaptogenesis:

An alternative explanation for the rescue of neurons by nAChR antagonists is that the antagonists may regulate nerve branching and synaptogenesis, which may, in turn, reduce cell death by allowing greater access to target-derived trophic factors via increased synaptic sites. This idea is attractive, in part, because in both spinal cord motor neuron populations and CG neurons, the cell death period is coincident with the period of axonal ramification and synapse formation in the target, suggesting that normal cell death results from a failure of some of the neurons to obtain adequate support from the target via synaptic contacts.

In 1988, Dahm *et al.* used immunostaining with a neuron-specific antibody in muscle whole mounts to visualize and quantify parameters of the intramuscular nerve branching pattern following application of d-tubocurare. They observed a rapid defasciculation of the major intramuscular nerve trunks, followed by a dramatic increase in branching and a tripling in the number of synapses (Landmesser *et al.*, 1988). Because the peak rate of increased nerve branching and synaptogenesis occurred prior to the onset of cell death, the increase in nerve branching appeared to be the cause of, rather than the result of, the increased survival (Dahm and Landmesser, 1991). Thus, activity blockade appeared to trigger an increase in nerve branching and synaptogenesis that, it was hypothesized, rescued neurons by allowing them greater access to trophic factors.

Landmesser *et al.* went on to demonstrate that altering branching and synapse formation without altering neuromuscular activity also rescues motor neurons from cell death. Altering the function of adhesion molecules such as the neuronal cell adhesion molecule (N-CAM) by injection of an endosialidase *in ovo* reduced intramuscular branching by 60% (Landmesser *et al.*, 1988; O'Brien *et al.*, 1990). This treatment resulted in a reduction in neuronal number, suggesting that the effects on neuronal survival are mediated by the changes in synaptogenesis and nerve branching, rather than by disruption of neuromuscular signaling (Tang and Landmesser, 1993). Similar correlations between synaptogenesis and neuronal survival have been observed by several other groups (Oppenheim *et al.*,

1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997; Caldero *et al.*, 1998; Usiak and Landmesser, 1999).

A possible inconsistency with this mechanism is that during the period of neuron death there is actually a net increase in the number of neuromuscular junctions (Pilar *et al.*, 1980; Sohal *et al.*, 1979). This trend continues well past the end of cell death, indicating that neurons continue to die despite increasing numbers of synaptic sites. In addition, in some systems, considerable cell death occurs prior to the onset of synaptogenesis (Fox *et al.*, 1985; Carr *et al.*, 1982), while in others cell death occurs in the absence of any synapse formation (Carr *et al.*, 1982; Anderson *et al.*, 1984). So, while synaptogenesis may be important to cell death, it is not clear precisely what role it plays in the process.

Interestingly, in 1995 Hory-Lee and Frank compared the doses of d-tubocurare and α-btx required for paralysis with those needed to prevent cell death (Hory-Lee and Frank, 1995). They found that subparalytic doses of nAChR the antagonists caused small but significant changes in nerve branch number, while higher doses produced a larger effect. In contrast, neuronal survival was already maximal at doses of the cholinergic blockers that produced no visible effect on limb movement. This suggests that blockade of the neuromuscular junction, and perhaps intramuscular nerve branching, may be independent of the mechanism by which cholinergic blockers rescue neurons from cell death.

Instead, both effects may be mediated through neuronal, rather than muscle-type acetylcholine receptors.

C. NAChR antagonists may rescue neurons via neuronal nicotinic receptors:

Another possible mechanism for the rescue of neurons by nAChR antagonists is via neuronal nAChRs, rather than by blocking receptors at the neuromuscular junction. The report by Meriney *et al.* that both ciliary and choroid neurons are rescued by α-btx is intriguing, given that synaptic activation of smooth muscle contraction in the choroid is completely blocked by muscarinic, rather than nicotinic, antagonists (Meriney *et al.*, 1987); therefore, choroid neuromuscular transmission is unaltered by nAChR antagonists. This does not preclude an involvement of α-btx binding sites on the choroid targets, since the binding sites may be expressed at levels sufficient to affect neuronal survival even if they are not physiologically detectable. However, choroid neurons express high levels of α-btx binding sites (Corriveau and Berg, 1994) that may be the true site of action in choroid neuronal rescue.

Alpha-btx is an 8,500 Dalton toxin purified from the venom of *Bungarus multicinctus* which has an extremely high selectivity for binding only two specific alpha subunits of nAChRs in the vertebrate nervous system (Sargent, 1993): α1, which is expressed only in striated muscle, and α7, which is expressed in neurons and many non-neuronal tissues such as the lung (Sekhon *et al.*, 1999). Ciliary ganglion neurons have been shown to contain two distinct populations of nicotinic

acetylcholine receptors: one that is located extrasynaptically, contains the α7 subunit, and binds α-btx, and one that is located synaptically, contains α3, and/or α5, and does not bind α-btx. Both ciliary and choroid neurons express α7-nAChRs, averaging 10^6 per cell at the end of embryogenesis (Chiappinelli and Zigmond, 1978; Corriveau and Berg, 1994).

In addition to the cell bodies of CG neurons, α7-nAChRs are present on the preganglionic terminals, where they can influence neurotransmitter release (Coggan *et al.*, 1997; McGehee and Role, 1995); blockade of these preganglionic α7-nAChRs could conceivably result in neuronal rescue. If this were the case, then one would predict that preganglionic axotomy would produce this same effect. Interestingly, surgical denervation decreases neuronal survival in the CG (Levi-Montalcini, 1949; Furber, 1984). The explanation for the difference between pharmacological receptor blockade and axotomy may reside in the fact that axotomy removes not just afferent input, but also potential trophic molecules from the preganglionic neurons, such as the endogenous opioids that have been shown to affect neuronal number (Meriney *et al.*, 1991). Blockade of the α7-nAChRs, by comparison, may affect afferent input without interrupting trophic support.

Alternatively, α7-nAChRs on the CG neuron cell bodies could mediate the α-btx neuronal rescue. Alpha 7-nAChRs are required in a population of ciliary neurons for reliable synaptic transmission and for tightly synchronized firing early

in development (Chang and Berg, 1999). They have also been shown to have a very high permeability to calcium (Seguela *et al.*, 1993). Blocking Ca^{2+} entry via α7-nAChRs could distrupt signal transduction pathways that ultimately lead to apoptosis.

Summary

Despite the focus that these questions have received over the past two decades, a number of questions remain unanswered concerning the rescue of neurons by nAChR antagonists. Where do nAChRs exert their effects? Do they act the neuromuscular junction? At neuronal nAChRs? Or do they act centrally, via a hormonal change? The mechanism of this neuronal rescue is also unclear. Is blockade of the neuromuscular junction critical to the rescue? Do nAChR antagonists cause an increase in the synthesis or release of target-derived neurotrophic factors? Do they act by increasing local access to limited amounts of neurotrophic factors via increased nerve branching and synaptogenesis? Do they rescue neurons by altering afferent input?

In this study we confirm the efficacy of α-btx in rescuing both populations of CG neurons, ciliary and choroid. We then examine the mechanism of the α-btx rescue by testing whether α-btx supports survival of CG neurons in cell culture or potentiates the activity of other trophic factors, and whether nicotinic agonists induce cell death *in vitro*. In addition, we test the hypothesis that the

neuronal rescue is produced, not by α-btx, itself, but by a minor contaminant of the α-btx preparation.

Next, we determine whether α-btx administration influences neurotrophic factor production or release in the CG targets and whether α-btx increases synaptogenesis in the CG targets. We also test the hypothesis that α-btx acts by blocking neuromuscular transmission by examining the presence of nAChRs in the two targets of the CG, the iris/ciliary body and the choroid. Additionally, we examine whether nAChR antagonists could be acting directly on CG neurons by determining whether a neuronal-nAChR-selective antagonist, methyllycaconotine (MLA), rescues CG neurons from cell death. Finally, we examine the effects of α-btx on nerve branching and synaptogenesis in the targets of the CG.

The sum of our results suggests that, while the increased synaptogenesis that correlates with neuronal rescue occurs via α1-nAChRs, nicotinic antagonists rescue CG neurons from cell death by acting directly on neuronal α7-nAChRs.

METHODS

Drug administration *in vivo*:

White Leghorn X New Hampshire Red (Animal Sciences Dept., Oregon State University, Corvallis, OR) eggs were incubated at 38° C in Roll-Ex egg incubators (Lyon Electric Co., Chula Vista, CA). At E3 eggs were placed on their sides and a 1cm window was cut into each shell directly above the embryo. The windows were immediately sealed with Scotch tape (3M, St. Paul, MN). Drugs were dissolved in sterile isotonic saline and administered daily in 100ml volumes to the chorioallantoic membrane of the egg on E7-14. The efficacy of the neuromuscular antagonists was measured by monitoring the spontaneous frequency of discrete hindlimb kicks during a 3-minute viewing period approximately one hour after drug administration. Ciliary ganglia were dissected at E14 as previously described (Nishi, 1996).

Tissue preparation and Somatostatin/Islet-1 immunocytochemisty for stereology:

CG were fixed in Zamboni's fixative (4% paraformaldehyde, 15% picric acid in 0.1M sodium phosphate-buffered saline solution (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4)) overnight to 48 hours at 4° C. After rinsing three times in PBS, tissues were allowed to equilibrate in 30% sucrose in PBS overnight at 4° C. Serial sections were cut at 35 μm on a Leica Jung Frigocut cryostat at -25° C and collected on gelatin/poly-L-lysine subbed slides. Slides were air dried for 5 minutes and post-fixed for 30 minutes in Zamboni's. Slides were washed 3 times in PBS and incubated overnight at 4° C in blocking solution

(10% (v/v) horse serum, 0.5% (v/v) triton X-100 and 0.2% (w/v) sodium azide in PBS). Sections were then incubated with anti-somatostatin using a rat monoclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) diluted 1:100 in blocking solution overnight at 4° C. Following 3 washes with PBS and 0.5% (v/v) TX-100 (PBST) and inactivation of endogenous peroxidase with 0.5% H_2O_2, slides were incubated for 1.5 hours at room temperature with a biotinylated goat anti-rat antibody (1:500, Vector Labs), followed by 3 washes with PBST. Finally, sections were incubated for 1.5 hours at RT in Vectastain ABC-HRP solution (1:500, Vector Labs). Somatostatin immunoreactivity was visualized by nickel/cobalt enhanced diaminobenzidine (NiDAB) solution (0.5 mg/ml DAB (Sigma), 0.1% $NiCl_2$, 0.1 % CoCl2 and 0.01% H_2O_2 in PBS). After color development, slides were rinsed three times in PBS and incubated in blocking solution overnight at 4° C. Sections were then incubated in anti-islet-1/2 (1:100 hybridoma supernatant, clone 39.405, Developmental Studies Hybridoma Bank, University of Iowa) in blocking buffer overnight at 4° C. Slides were treated with secondary antibody and ABC-HRP as described above, using a biotinylated goat anti-mouse antibody (1:500, Vector Labs). Anti-islet 1/2 immunoreactivity was detected by reaction with 0.5 mg/ml DAB without nickel/cobalt enhancement.

Cell counting:

We counted islet-1- and somatostatin- positive cells with design-based stereology (West *et al.*, 1991) using an optical fractionator probe (Stereo

Investigator, Microbrightfield, Inc.). In order to use this method it was necessary to minimize tissue shrinkage during processing. CG were mounted in groups of 3-7 and then serially sectioned and collected on slides for immunohistochemistry as described above. Section thickness was consistently found to be 27-32 μm after staining, demonstrating that our processing method preserves tissue integrity. In addition, islet-1-positive nuclei were observed throughout the z-axis of the sections, confirming that the immunohistochemical reagents penetrated the full thickness of the section.

The total number of neurons was determined by counting the number of islet-1-positive nuclei, and the number of choroid neurons was determined by counting the number of islet-1 positive nuclei surrounded by a somatostatin-positive cytoplasm. Cells were counted with a 100x oil immersion objective on a Nikon Optiphot microscope equipped with an X, Y, Z stage drive, position transducer, video camera, and computer (Micron Millenia) running the stereology software package. Spacing between sampling sites (grid size) was set such that 13-15 sampling sites per section were counted, which yielded counts of 300-400 objects for each ganglion. In addition, an upper guard zone of 4 μM and a lower guard zone of 7 μM were used to avoid sectioning artifacts. To establish the reliability of our counting method, we first counted the same sections three times using the same sampling sites, then again with different sampling sites. The difference in counts obtained with these tests was less than 5%.

Cell cultures:

CG neuronal cultures were prepared with E8 CG as described (Eckenstein *et al.*, 1990; Nishi and Berg, 1981a) and were grown in triplicate with and without α-btx (1μg/ml, Sigma and Calbiochem). Neurons were cultured in Eagles Minimum Essential Medium (MEM; Gibco-BRL, Grand Island, NY) containing 10% (v/v) heat-inactivated horse serum (Gibco-BRL) with 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine. Trophic support was provided by chicken ciliary neurotrophic factor (chCNTF), basic fibroblast growth factor (bFGF), glial cell line derived neurotrophic factor (GDNF), or chick eye extract as noted. Neuronal survival was determined 4 days after plating by counting the number of large, phase bright cells with processes greater that three cell diameters in each field of view across the diameter of each well. Reported values are the average of three cultures from one experiment.

Cultures for nicotine exposure and TUNEL assay were grown in MEM or Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL) containing 10% (v/v) heat-inactivated horse serum (Gibco-BRL), 2% FCS, 2% CEE, with 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine. Trophic support was provided by 20 ng/ml chCNTF, or 15 ng/ml bFGF plus 10 ng/ml GDNF. Some cultures also included 12.5 ng/ml neuregulin EGF domain (kindly provided by David Stern). Cultures were grown for 24-48 hours before exposure for 2-36 hours to 0.1-0.5 μM nicotine.

E12 chick irises were isolated as described (Link and Nishi, 1998b). Iris cells were grown for 48 hours on collagen-coated plates in L15 with 10% horse serum. Myotubes were then allowed to differentiate by replacing the serum-containing medium with serum-free medium, supplemented with 2.5 mg/ml bovine serum albumin, 25 mg/ml ovotransferrin, 30nM selenium, and 2.5 mg/ml insulin (Sigma chemical Co., St. Louis, MO). Both serum-containing and serum-free L15 medium were supplemented with 6 mg/ml glucose, 20 U/ml penicillin, 2 mM glutamine, and 2 mg/ml streptomycin (Gibco-BRL).

Choroid cells were isolated as previously described (Coulombe and Nishi, 1991). Cells were plated on tissue culture dishes coated with rat tail collagen and grown in modified L15 medium supplemented with 10% chick serum and penicillin/streptomycin/glucose, as described above, for 2 days. Cells were then transferred to serum-free conditions supplemented as described for iris cultures.

For the collection of conditioned medium, confluent E11 iris or choroid cultures were grown in serum-free medium for 3 days in the presence of 1 μg/ml α-btx. Cultures were then rinsed twice with EBSS, fed with fresh serum-free medium without α-btx, and conditioned for 48h. This medium was collected and stored at –80°C for subsequent bioassay.

For neuron/muscle co-cultures, E11 iris or pectoral muscle was grown as described above, except that some cultures were exposed to 1 μg/ml α-btx. After

3 days in serum-free medium, the cultures were switched to MEM containing 10% (v/v) heat-inactivated horse serum with 50 U/ml penicillin, 50mg/ml streptomycin, and 2 mM glutamine. Muscle cultures were allowed to condition this medium for 12-24 hours before E8 CG neurons were plated on top of the muscle cells. No exogenous trophic factors were added to supplement the medium. Neuronal survival was determined 4 days after plating by counting the number of large, phase bright cells with processes greater that three cell diameters in each field of view across the diameter of each well. Reported values are the average of three to six cultures from one experiment.

Detection of dying cells with TUNEL:

Cultures were fixed in 4% paraformaldehyde containing 1mM EDTA to inhibit Dnase activity. After fixation, sells were blocked in 2% BSA, 2% sheep serum in PBST overnight at 4°C. TUNEL assays were performed using the In Situ Cell Death Detection, POD kit (Roche Biochemicals) which incorporates fluorescein isothiocyanate (FITC) labeled nucleotides into the free 3'OH ends of DNA and amplifies the labeled DNA with an antibody against FITC that is conjugated to horseradish peroxidase (POD). The following modifications were made to the manufacturer's protocol: 1) the nucleotide labeling mix was diluted 1:1 and the reaction was run for 3 hours; 2) the anti-fluorescein POD was diluted 1:5 and incubated for 2.5-3 hours; 3) TUNEL labeling was visualized by incubating cells in NiDAB solution.

Receptor internalization:

E8 CG were dissociated and allowed to adhere to glass coverslips coated with Poly-d-lysine (1mg/ml) and laminin for 4-6 hours in MEM containing 10% (v/v) heat-inactivated horse serum with 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine. Subsequently, the cells were washed twice with fresh medium and incubated in 10 ng/ml rhodaminated α-btx for 30 minutes at 37° C to label neuronal nAChRs. A subset of the coverslips was fixed at this point as a control. The remaining coverslips were then washed twice in fresh medium and incubated in 1 μg/ml α-btx for 30 minutes at 37° C, rinsed and fixed in 4% Zamboni's fixative for 30 minutes at 4°C. Another group of control coverslips were incubated first in 1 μg/ml α-btx for 30 minutes at 37° C, follwed by 10 ng/ml rhodaminated α-btx for 30 minutes at 37° C.

Some cultures were then processed for Mab-35 immunoreactivity as follows: fixed cells were rinsed 3 times in PBS and incubated in blocking solution overnight at 4°C. A subset of the slides was processed without permeabilization by omitting the Triton X-100 from the blocking buffer and from subsequent wash steps. Coverslips were then incubated for 3 hours in MAb-35 (1:100 hybridoma supernatant, Developmental Studies Hybridoma Bank, University of Iowa) at room temperature. Samples were then rinsed 3 times in PBST and incubated in goat anti-mouse alexa-488 (1:750, Molecular Probes) for 3 hours at room temperature. Tissue was then rinsed 5 times in PBST before mounting in Permafluor.

Deconvolution images were obtained with the Applied Precision Deltavision image restoration system. This includes the API chassis with precision motorized XYZ stage, a Nikon TE200 inverted fluorescent microscope with standard filter sets, halogen illumination with API light homogenizer, a CH35OL Camera (500KHz, 12-bit, 2 Mp, KAF 1400GL, 1317x1035, liquid cooled), and Deltavision software. Z-axis sections were taken every 0.25-0.4 microns and deconvolution used the iterative constrained algorithm of Sedat and Agard and additional image processing was performed on an SGI Octane workstation.

Preparation of extracts for bioassay:

Alpha-btx was administered *in vivo* from E7-14 as outlined above. Eyes from α-btx-treated and control animals were isolated at E11 and E14 and the vitreous humor was removed. Tissue was immediately frozen on dry ice and stored at -80°C prior to processing. Extracts were prepared by grinding the thawed tissue in a Tekmar homogenizer in 10 mM sodium 3-(N-morpholino)-propane-sulfonic acid (NaMOPS), 5 mM EDTA, pH 7.2, in a cocktail of protease inhibitors followed by sonication with a Cole-Parmer 4710 probe sonicator for two minutes on ice. All subsequent steps were performed at 4°C. The extract was centrifuged at 26,000g for 60 minutes and the supernatant was incubated with heparin-agarose (BioRad) for one hour. All samples were adjusted for equal protein concentration, 0.2 μm filter sterilized, and stored at -80°C until use.

Immunohistochemistry of iris and choroid whole-mounts:

Eggs were windowed at E3 as described above and 75 μg/day α-btx was administered from E7-E14. For rhodaminated α-btx staining, choroid muscle was dissected from the same quadrant of each eye, pinned down in a Sylgard dish, and incubated for 30 minutes at 37°C in 10 ng/ml rhodaminated α-btx (Sigma and Molecular Probes). Tissue was then rinsed three times in warm PBS and fixed in 4% Zamboni's solution for 30 minutes at room temperature. Tissue was rinsed three times in PBS and mounted in Permafluor (Immunotech, Marseilles, France).

For α7-nAChR immunohistochemistry, choroid muscle was dissected from the same quadrant of each eye, pinned down in a Sylgard dish, and fixed for 30 minutes at room temperature. Tissue was then rinsed three times in PBS, incubated in blocking solution overnight at 4°C, and incubated for 3 hours in mouse anti-α7-nAChR (1:1000, Sigma/RBI) at room temperature. Samples were then rinsed 3 times in PBST and incubated in goat anti-mouse alexa-488 (1:750, Molecular Probes) for 3 hours at room temperature. Tissue was then rinsed 5 times in PBST before mounting in Permafluor.

For SV-2 immunohistochemistry, whole eyes were fixed in 4% Zamboni's solution at 4°C overnight and rinsed 3 times in PBS. Iris and choroid muscle was dissected and pinned down in a Sylgard dish for processing. Tissue was incubated in blocking solution overnight at 4°C and for 3 hours in mouse anti-

synaptic vesicle protein-2 antibody (1:20; culture supernatant containing mouse anti-synaptic vesicle protein 2 (SV-2) kindly provided by Dr. Bruce Patton, Oregon Health Sciences University) at room temperature. Samples were then rinsed 3 times in PBST and incubated in goat anti-mouse alexa-488 (1:750, Molecular Probes) for 3 hours at room temperature. Tissue was then rinsed 5 times in PBST before mounting in Permafluor.

For neurofiliament immunohistochemistry, whole eyes were fixed in 4% Zamboni's solution at 4°C overnight and rinsed 3 times in PBS. Iris and choroid muscle was dissected and pinned down in a Sylgard dish for processing. Tissue was incubated in blocking solution overnight at 4°C and for 3 hours at room temperature in rabbit anti-neurofilament (1:1000; Sigma Chemical Company, St. Louis, MO). Samples were then rinsed 3 times in PBST and incubated in goat anti-rabbit texas red (1:750, Molecular Probes) for 3 hours at room temperature. Tissue was then rinsed 5 times in PBST before mounting in Permafluor.

Confocal microscopy and analysis:

Confocal microscopy was performed with a Leica TCS SP confocal microscope system (Leica Microsystems, Heidelberg, Germany) equipped with a 361 nm Ar UV laser, a 488 nm Ar laser, a 568 nm Kr laser, and a 633 nm HeNe laser using a 25x PL Fluotar NA 0.75 objective on tissue from three embryos in each drug treatment group. A separate series of confocal images for each fluorophore was obtained simultaneously with Z intervals of 2.0 μm. The control

samples were examined first and used to adjust the channel gain and offset controls for optimal detection of the neurofilament and SV-2 antibodies. The drug-treated samples were then analyzed without further adjustment.

An average projection image for each of the image stacks was obtained using Metamorph software. A minimum threshold intensity was set and the theresholded area, standard deviation, and percent thresholded area were generated by Metamorph. The projections were subsequently imported into Photoshop where they were image processed and merged.

PCR:

A fragment of the nAChR α1 subunit of 252 bp was obtained by RT-PCR using the primer pair based on the published sequence (Nef *et al.*, 1988): forward, 1046-1065, and reverse, 1297-1276. A fragment of nAChR α7 subunit of 530 bp was obtained by RT-PCR using the primer pair based on the published sequence (Couturier *et al.*, 1990): forward, 314-335, and reverse, 843-822. A fragment of chick ribosomal protein S17 (CHRPS) of 361 bp was obtained by RT-PCR using the primer pair based on the published sequence (Trueb *et al.*, 1988) forward, 49-67, and reverse, 389-409. All PCR reactions used cycling conditions of 95°C (melting), 54 °C (annealing), and 72° (extension), for 1 minute each (30 cycles) with 1U of Taq DNA polymerase per reaction (Promega, Madison, WI).

RESULTS:

A. Alpha-bungarotoxin rescues both ciliary and choroid neurons from cell death *in vivo*:

Introduction:

We chose to use the avian ciliary ganglion (CG) as a model system for our examination of the mechanism of the neuronal rescue by nicotinic acetylcholine receptor (nAChR) antagonists because the CG has a relatively simple neuronal makeup, its neurotrophic requirements are better defined than those of spinal cord motor neurons, and because the physiology and pharmacology of its two target tissues are well characterized. Our first step was to confirm the findings of Meriney *et al.*, that 75 μg/day of α-btx rescues both populations of CG neurons from cell death, whereas 12.5 μg/day α-btx rescues ciliary, but not choroid neurons (Meriney *et al.*, 1987).

Results:

Alpha-btx rescues both ciliary and choroid neurons:

Alpha-btx was administered daily from E7-14 (*figure 3*). We identified all CG neurons using an antibody to the early motor neuron transcription factor, islet-1 (Ericson *et al.*, 1992; Tsuchida *et al.*, 1994), which labels both ciliary and choroid neurons (Lee *et al.*, 2001). Choroid neurons were co-labeled with an antibody to the specific choroid neuron marker, somatostatin, a neuropeptide

cotransmitter (DeStefano *et al.*, 1993; Epstein *et al.*, 1988). Anti-somatostatin staining was punctate and cytoplasmic, while anti-islet-1 staining was nuclear. We scored neurons as "ciliary" if they stained for anti-islet-1 but not anti-somatostatin. Neurons were scored as "choroid" if they expressed both markers (*figure 4A*). In confirmation of previous results (Finn *et al.*, 1998; Furber *et al.*, 1987; Landmesser and Pilar, 1974b), our stereological counts of the neurons revealed a 47% reduction in the number of neurons between E8 (St. 33/34; 18542+/-591) and E14 (St. 40; 9847+/-276, $p<0.01$, Student's t-test). Differential counting of ciliary vs. choroid neurons confirmed that application of α-btx (75 μg/day) from E7-14 rescued both ciliary (7172+/-237 vs. a control value of 5498+/-410, $p<0.01$, Student's t-test) and choroid (8251+/-229 vs. a control value of 4349+/-303; $p<0.001$, Student's t-test) neurons from cell death *in vivo* (*figure 4B)*. This represents a 30% increase in the number of ciliary neurons and a 90% increase in the number of choroid neurons (*figure 4C*).

We also confirmed that a lower dose of α-btx rescues ciliary (8805 +/-350 vs. a control value of 5498 +/- 410, $p<0.001$, Student's t-test), but not choroid neurons (4505 +/- 533 vs. a control value of 4349 +/- 303), from cell death *in vivo* (*figure 5A*). This represents a 60% increase in ciliary neuron survival and a 4% increase in choroid neurons (*figure 5B*). Quantification of the number of spontaneous discrete hindlimb kicks revealed that, while the higher dose of α-btx produced nearly complete paralysis, the lower dose produced only a partial paralysis of the embryos (*figure 5C*).

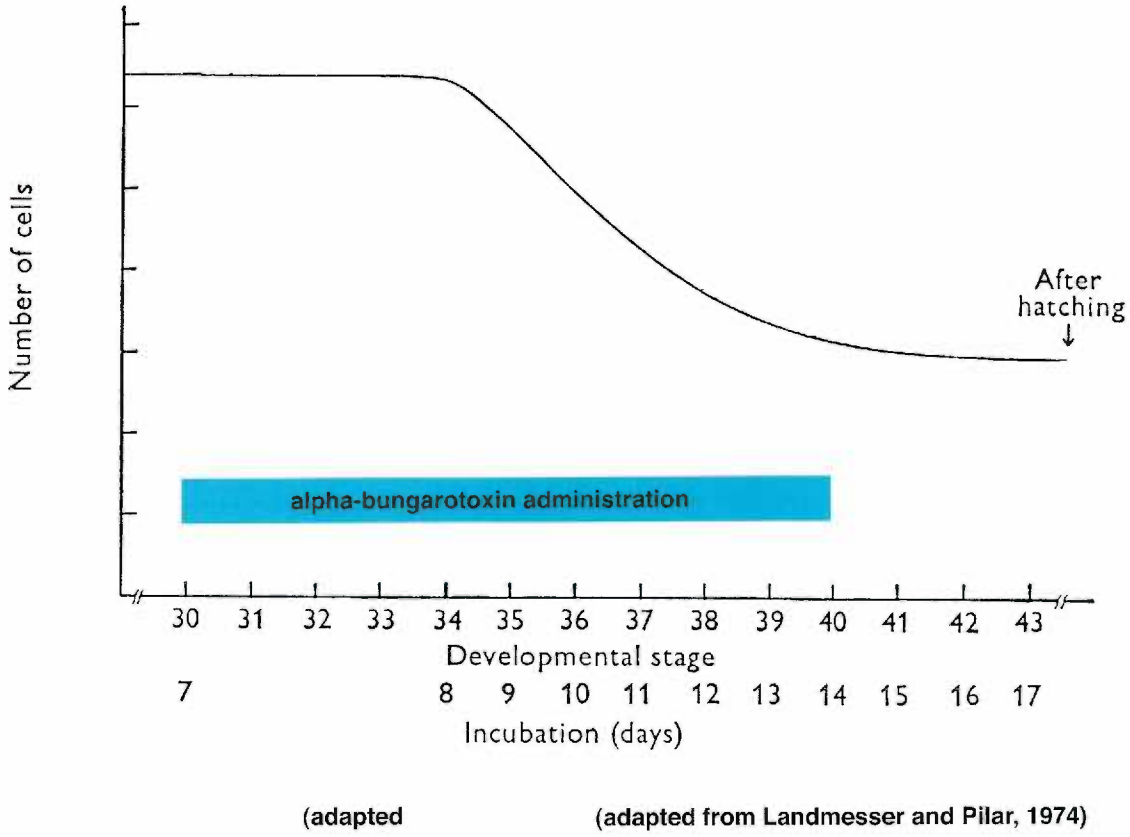
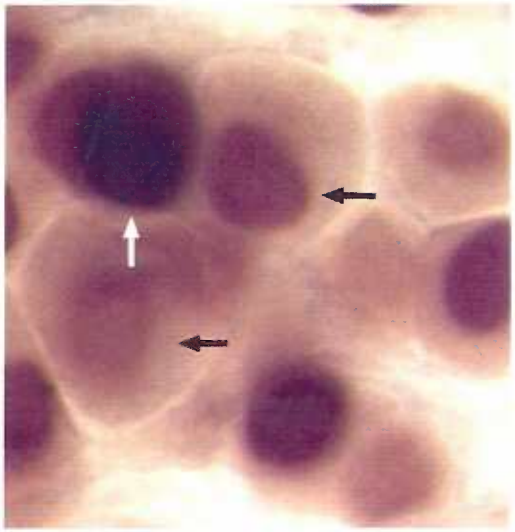


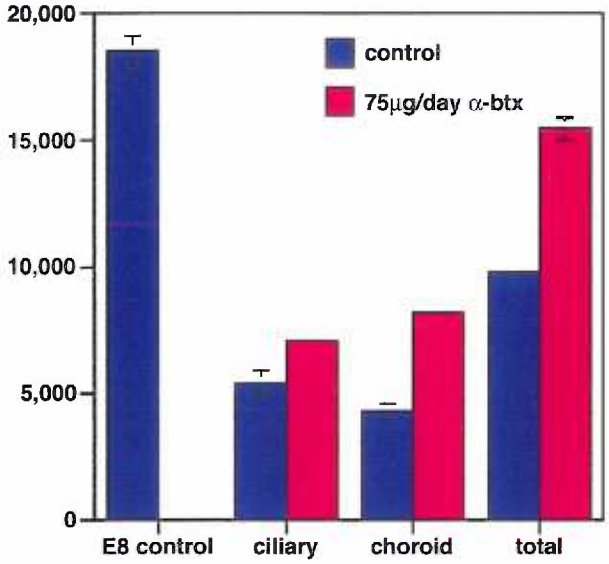
Figure 3. Alpha-bungarotoxin was administered daily throughout the cell death period. Between stage 35 and stage 40 (E9-E14), the number of neurons in the CG is reduced by 50% (Landmesser ans Pilar, 1974b). This reduction in neuronal number coincides with peripheral synaptogenesis, changes in neuronal ion channels, induction of choline acetyltransferase activity, expression of somatostatin in choroid neurons, and gliogenesis (Dryer, 1994; Nishi, 1994). We applied α-btx daily from E7-E14 by dripping onto the chorioallantoic membrane of windowed eggs.

Figure 4. Daily application of 75 μg α-bungarotoxin rescues both ciliary and choroid neurons from cell death. CG were collected and processed for islet-1 and somatostatin immunohistochemistry at E14 after seven days of *in ovo* administration of α-btx or saline. A) Both populations of neurons were identified by islet-1 immunoreactivity, which is brown and nuclear (black arrows). Choroid neurons were distinguished from ciliary neurons by the presence of black, cytoplasmic somatostatin immunoreactivity (white arrow). B) Differential neuronal counts were obtained using design-based stereology and their values represented as histograms. Stereological counts revealed a 47% reduction in the number of neurons between E8 (St. 33/34; 18542+/-591; n=3) and E14 (St. 40; 9847+/-276; n=7; $p<0.01$, Student's t-test). Daily application of 75 μg/day of α-btx onto the chorioallantioc membrane from E7 through E14 rescued both ciliary (7172+/-237; n=5 vs. a control value of 5498+/-410; n=7; $p<0.01$, Student's t-test) and choroid (8251+/-229; n=5 vs. a control value of 4349+/-303; n=7; $p<0.001$, Student's t-test) neurons from cell death *in vivo*. The numbers represent the mean and standard error obtained from at 3-7 independent determinations. C) This translated into a 30% increase in ciliary neurons, a 90% increase in choroid neurons, and a total neuronal increase of 57%.

A



B


20,000
15,000
10,000
5,000
0
control
75μg/day α-btx
E8 control
ciliary
choroid
total

C

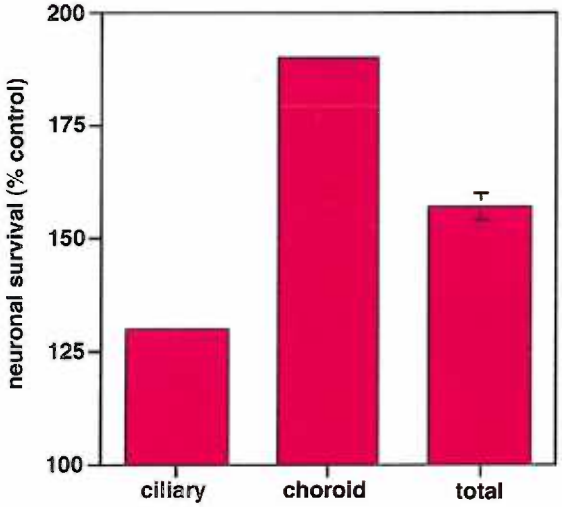
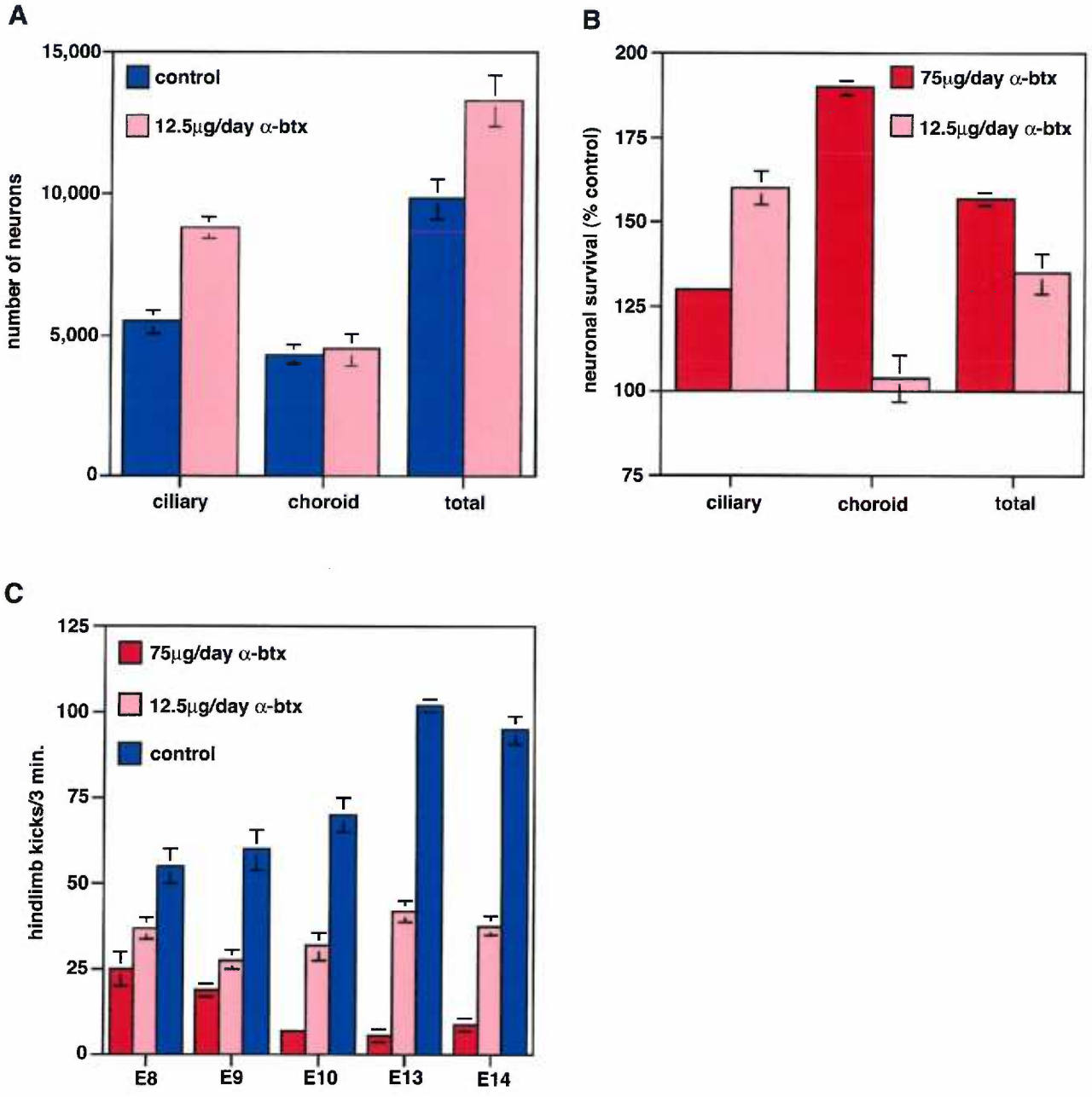

200
175
150
125
100
neuronal survival (% control)
ciliary
choroid
total

Figure 5. Daily application of 12.5 μg α-bungarotoxin rescues ciliary, but not choroid neurons. CG were collected and processed for islet-1 and somatostatin immunohistochemistry at E14 after seven days of *in ovo* administration of α-btx or saline. A) Differential cell counts revealed that 12.5 μg/day α-btx rescued ciliary (8805+/-350; n=3; versus a control value of 5498+/-410; n=7; $p<0.001$, Student's t-test), but not choroid neurons (4505+/-533; n=3; versus a control value of 4349+/-303; n=7). The numbers represent the mean and standard error obtained from 3-7 independent determinations. B) This translates into a 60% increase in ciliary neurons, a 4% increase in choroid neurons, and a total neuronal increase of 35%. C) The number of discrete hindlimb kicks per three-minute viewing window was scored each day approximately 60 minutes after drug delivery. Motility of control embryos increased steadily until it reached a plateau at E13-14. A dose of 75 μg/day α-btx caused nearly complete paralysis. By contrast, 12.5 μg/day α-btx produced only a partial paralysis throughout E7-14.


A
15,000
10,000
5,000
0
number of neurons
control
12.5μg/day α-btx
ciliary
choroid
total
B
200
175
150
125
100
75
neuronal survival (% control)
75μg/day α-btx
12.5μg/day α-btx
ciliary
choroid
total
C
125
100
75
50
25
0
hindlimb kicks/3 min.
75μg/day α-btx
12.5μg/day α-btx
control
E8
E9
E10
E13
E14

B. Alpha-bungarotoxin does not promote survival of CG neuron cultures nor potentiate the action of neurotrophic factors:

Introduction:

One of the advantages of the avian CG as a model system is that both CG neurons and their targets can be grown in culture alone or in combination. We used a bioassay for neuronal survival to test three hypotheses: that α-btx rescues CG neurons by substituting for a neurotrophic factor, that it rescues CG neurons by potentiating the neurotrophic activity of an endogenous target-derived neurotrophic factor, and that activation of neuronal nAChRs with nicotine induces apoptosis in cultured CG neurons, as it does in hippocampal neuronal precursor cells (Berger *et al.*, 1998).

Results:

Alpha-btx does not substitute for a neurotrophic factor:

We tested whether α-btx exerts a direct neurotrophic effect on the CG neurons by comparing CG neurons cultured with α-btx to those cultured with chicken ciliary neurotrophic factor (chCNTF). Nearly 100% of the neurons cultured with α-btx as their source of trophic support died within 48 hours of plating, whereas neurons cultured with saturating chCNTF were phase-bright and had extensive processes at 4 days in culture when they were scored for survival (*figure 6*). Thus, α-btx does not have a direct neurotrophic effect on CG neuron cultures.

Nicotine application fails to induce cell death *in vitro*:

Because nicotine has been shown to induce apoptosis in hippocampal neuronal precursor cells (Berger *et al.*, 1998), we tested whether application of nicotine (0.1-0.5 μM) would induce apoptosis in cultured CG neurons. Dying cells were identified by TUNEL, which labels fragmented DNA. More than 95% of the TUNEL-labeled cells were morphologically identifiable as neurons, and many TUNEL+ nuclei were observed that were comparable in diameter to the nuclei of healthy neurons, indicating that dying neurons could be identified prior to the formation of a pyknotic nucleus. Exposure of neuronal cultures to nicotine for 4-36 hours produced no increase in the number of dying cells (*figure 7*). Thus, nicotine does not induce cell death in CG neuron cultures.

Alpha-btx rescues neurons co-cultured with muscle cells:

The lack of effect on survival of α-btx or nicotine *in vitro* may be due to the failure of the neurons to express normal levels of nAChRs *in vitro.* Because an isoform of neuregulin with a conserved cysteine-rich domain has been shown to augment the levels of expression of neuronal nAChR mRNAs encoding the α3, α5, and α7 subunits in synaptically naïve sympathetic neurons (Yang *et al.*, 1998), we tested whether a target-derived factor is required for α-btx responsiveness in cultured CG neurons. When neurons were co-cultured with iris or pectoral muscle, we found that α-btx produced a 75% increase in neuronal survival (*figure 8*) in five separate trials. Unfortunately, we were unable to

follow up on this line of investigation after a problem with our chicken supplier led to a change in the genetic background of our chickens, and we were unable to replicate this effect (see discussion).

Alpha-btx does not potentiate the neurotrophic activity of chCNTF , bFGF, GDNF, or crude eye extract:

Next, we tested whether α-btx potentiates the ability of neurotrophic factors to support CG neurons in culture. We generated dose-response curves for chCNTF in the presence and absence of α-btx and found that α-btx did not alter the ED50 of chCNTF (*figure 9A*). We also tested whether α-btx would potentiate the neurotrophic activity of basic fibroblast growth factor (bFGF) and glial cell line derived growth factor (GDNF), both of which support CG neurons in culture, as well as crude eye extract, which would contain any as-yet-unidentified target-derived neurotrophic factors. Alpha-btx did not shift the dose-response curves for any of these factors (*figures 9B, C, D*). Thus, α-btx does not rescue CG neurons by augmenting the neurotrophic activity of endogenous neurotrophic factors.

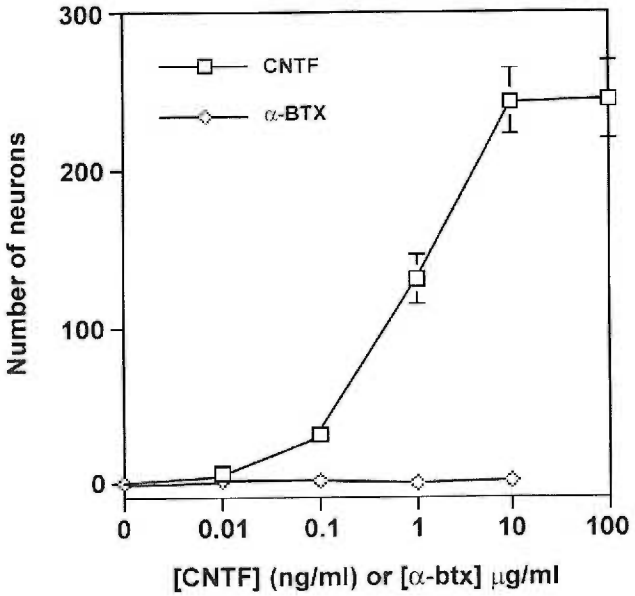
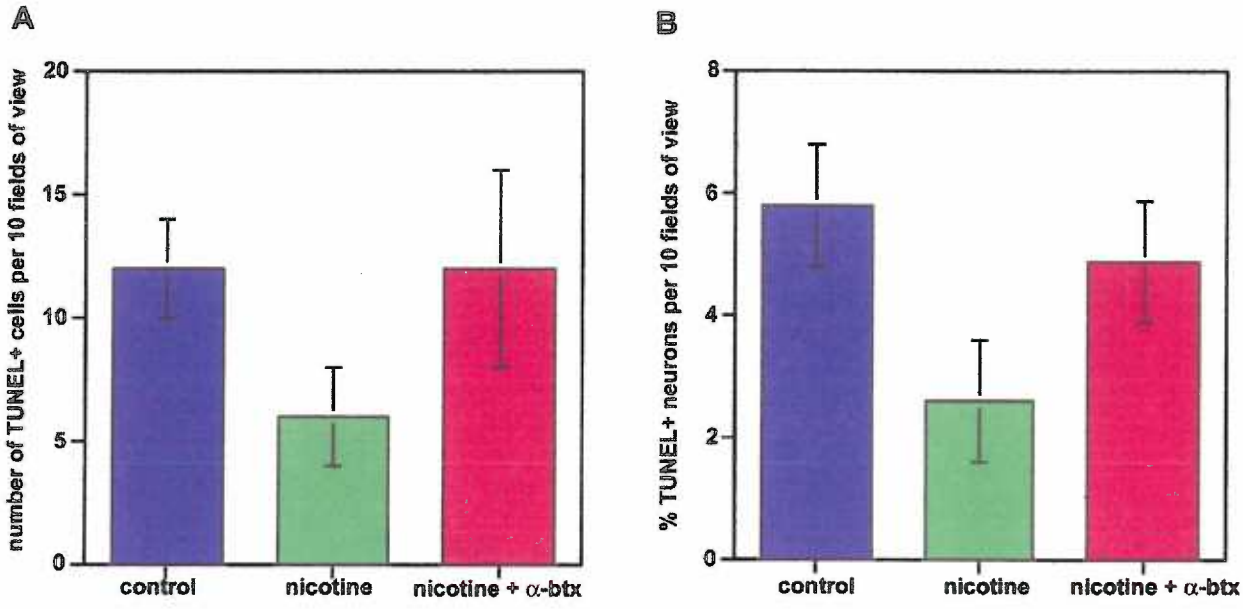


Figure 6. Alpha-btx does not promote the survival of CG neurons in culture. CG neurons were grown with either 0-100 ng/ml chCNTF or 0-10 μg/ml α-btx as the source of neurotrophic support. Neurons were grown in triplicate and scored for survival after 4 days in culture. Error bars represent the standard deviation for one representative experiment. ChCNTF supported CG neurons in culture with an ED50 of approximately 1 ng/ml. By contrast, α-btx did not support neuronal survival at any dose.

Figure 7. Nicotine application fails to induce cell death *in vitro.* E8 CG cultures were grown with 15 ng/ml bFGF and 10 ng/ml GDNF for 48 hours prior to nicotine exposure. Cultures were then exposed to 0.5 μM nicotine or 0.5 μM nicotine plus 0.5 μg/ml α-btx for 8 hours before fixation and processing for TUNEL assay. Neuronal number was determined by counting the number of large, phase-bright cells with processes greater than three cell diameters in ten random fields of view. The number of TUNEL+ cells was determined by counting nuclei that were filled with black HRP reaction product. A) Nicotine exposure did not induce apoptosis in cultured E8 CG neurons. In fact, nicotine exposure caused a slight decrease in the number of TUNEL+ cells (6+/-2 for nicotine, 12+/-2 for control; $p<0.1$, Student s t-test). B) The failure of nicotine to increase the number of TUNEL+ cells is not due to a change in total cell number; the percentage of total neurons that are TUNEL+ does not increase with nicotine exposure. Cultures were grown in triplicate and error bars represent the standard error.


A
number of TUNEL+ cells per 10 fields of view
20
15
10
5
0
control
nicotine
nicotine + α-btx
B
% TUNEL+ neurons per 10 fields of view
8
6
4
2
0
control
nicotine
nicotine + α-btx

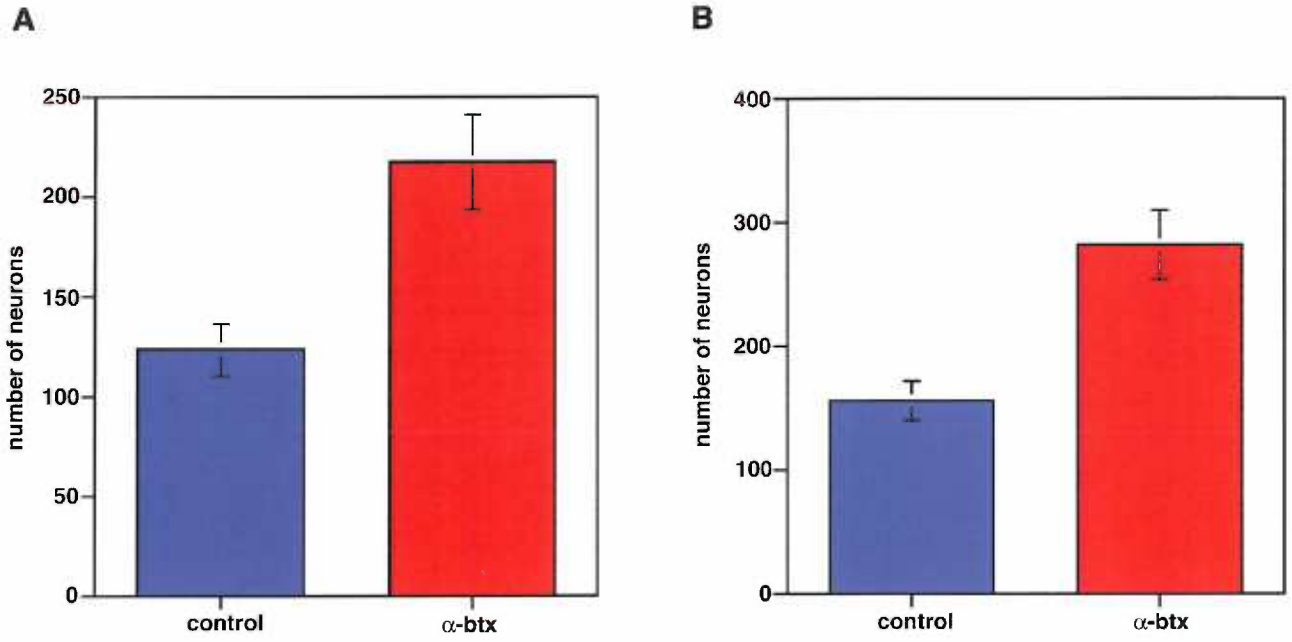
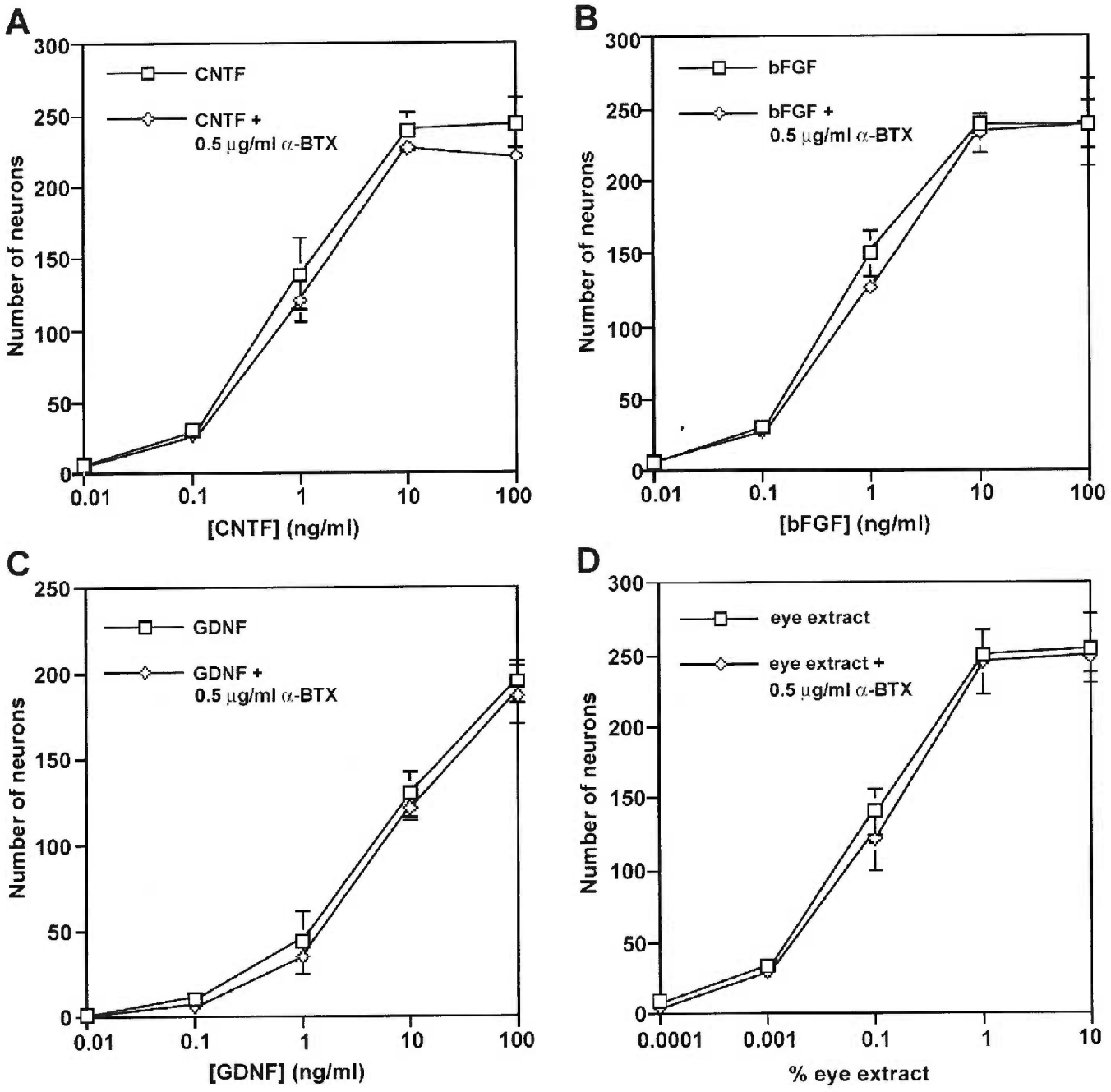


Figure 8. Alpha-bungarotoxin rescues ciliary ganglion neurons when they are co-cultured with iris or pectoral muscle. Iris muscle cultures A), or pectoral muscle cultures B) were grown for three days in serum-free medium before E8 CG neurons were cultured on top of them, with or without 1 μg/ml α-btx. Neuronal survival was scored at 4 days in culture plating by counting the number of large, phase bright cells with processes greater that three cell diameters in each field of view across the diameter of each well. Reported values are the average of six cultures from one experiment and error bars represent standard errors. Both neuron/iris and neuron/pectoral muscle co-cultures showed a 75% increase in neuronal survival with α-btx-treatment (iris co-cultures: 218+/-21 vs. a control value of 124+/-12; $p<0.01$, Student's t-test; pectoral muscle co-cultures: 282+/-29 vs. a control value of 156+/-17; $p<0.01$, Student's t-test).

Figure 9. Alpha-bungarotoxin does not potentiate the neurotrophic activity of chCNTF, bFGF, GDNF, or crude eye extract. E8 CG neurons were cultured in the presence of 0-100 ng/ml chCNTF (A), bFGF (B), or GDNF (C), or 0-10% eye extract, both with and without 0.5 μg/ml α-btx. Neuronal cultures were grown in duplicates and neuronal survival was scored at 4 days (chCNTF, bFGF, and eye extract) or at 2 days (GDNF) in culture by counting the number of large, phase-bright cells with processes greater than 3 cell diameters in a series of non-overlapping fields of view across the center of each well. Alpha-btx did not shift the dose-response curve for A) chCNTF, B) bFGF, C) GDNF, or D) eye extract. One representative experiment is shown in each panel, and error bars represent standard deviations.


A
CNTF
CNTF +
0.5 µg/ml α-BTX
Number of neurons
[CNTF] (ng/ml)
B
bFGF
bFGF +
0.5 µg/ml α-BTX
Number of neurons
[bFGF] (ng/ml)
C
GDNF
GDNF +
0.5 µg/ml α-BTX
Number of neurons
[GDNF] (ng/ml)
D
eye extract
eye extract +
0.5 µg/ml α-BTX
Number of neurons
% eye extract

C. Alpha-bungarotoxin does not rescue neurons via receptor internalization:

Introduction:

The venom of *Bungarus multicinctus* contains several components, including α-bungarotoxin (α-btx) and a protein neurotoxin present as a minor component, Bgt 3.1. Bgt 3.1 can induce a rapid internalization of α-btx bound on the surface of ciliary ganglion (CG) neurons that can be seen with fluorescence microscopy using rhodaminated α-btx (Ravdin *et al.*, 1981). The rapid internalization can be blocked by low temperatures and is not induced by α-btx, itself.

The dose of α-btx needed to produce a full rescue of both ciliary and choroid neurons is high enough (75 μg/day) that a minor contaminant of the α-btx preparation, rather than α-btx, itself, could be the actual agent responsible for the neuronal rescue, if such a contaminant were present. Accordingly, we tested whether α-btx from a second supplier produced the same *in vivo* rescue effect as the first, as well as whether α-btx from the two different sources caused receptor internalization on freshly dissociated CG neurons.

Results:

Alpha-btx from two different sources rescues CG neurons in an identical fashion:

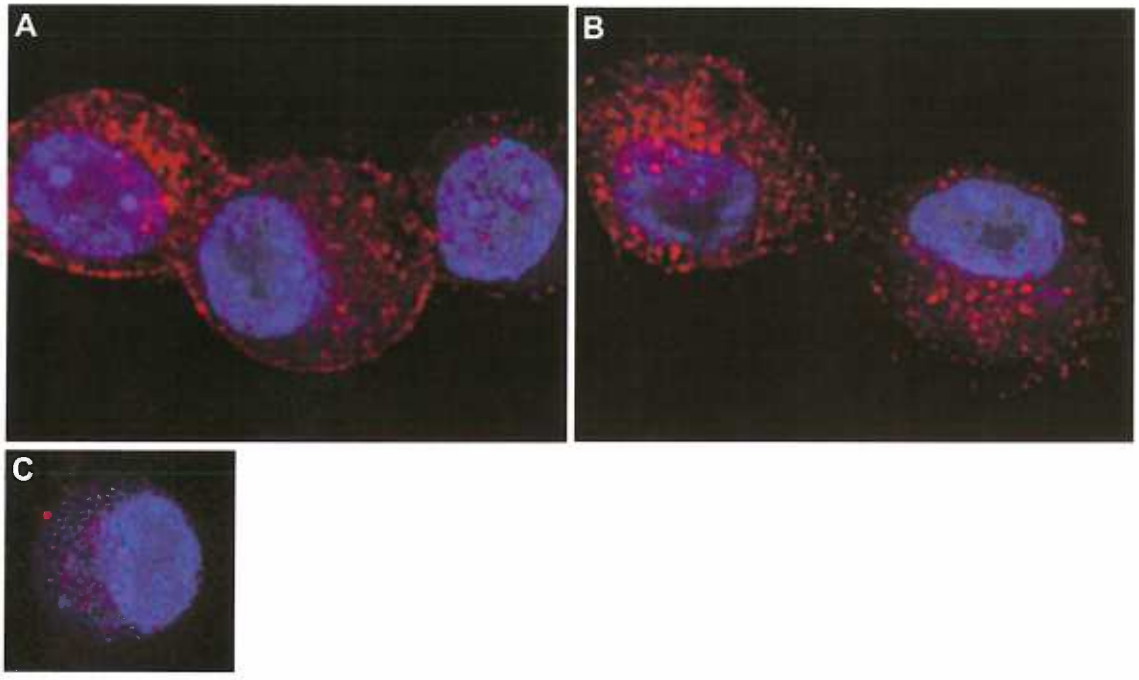
Alpha-btx from two different sources (Sigma and Calbiochem) was administered daily from E7-14 as before (*figure 3*). Neurons were labeled with

islet-1 and somatostatin antibodies, as before, and neurons were scored as "ciliary" if they stained for anti-islet-1, but not anti-somatostatin. "Choroid" neurons were defined as expressing both markers (*figure 4A*). Differential counting of ciliary vs. choroid neurons demonstrated that the rescue of CG neurons by Sigma α-btx was indistinguishable from the rescue by Calbiochem α-btx (data not shown). Thus, it is unlikely that the rescue is produced by a contaminant of the α-btx preparation unless the contaminant is present in α-btx from two different suppliers.

Alpha-btx does not induce receptor internalization:

Next, we tested whether application of α-btx caused internalization of nAChRs. CG were dissociated and allowed to adhere to the culture dish before incubation in 75 ng/ml rhodaminated α-btx to label neuronal nAChRs. Cells were then incubated in 75 μg/day α-btx, rinsed and fixed. Some cultures were then processed for Mab-35 immunoreactivity, which labels non-α-btx-binding nAChRs. Neither source of α-btx produced any change in the distribution of either α-btx-binding sites or MAb-35 immunoreactivity (*figure 10*). Thus, the neuronal rescue is not due to rapid nAChR internalization caused by a minor contaminant of the α-btx preparation.

Figure 10. Alpha-bungarotoxin does not cause receptor internalization. E8 CG neurons were dissociated and allowed 4 hours to adhere to glass coverslips. Panels A-C show deconvolution images of E8 CG neurons that were exposed to: A) 10 ng/ml rhodaminated α-btx to label α-btx binding sites; B) 10 ng/ml rhodaminated α-btx to label α-btx binding sites, followed by 1 μg/ml unlabeled α-btx; or C) 1 μg/ml unlabeled α-btx followed by 10 ng/ml rhodaminated α-btx. There was no change in the distribution of rhodaminated α-btx when neurons were exposed to the higher dose of α-btx, indicating that α-btx does not rescue neurons by driving receptor internalization (this is more apparent when the image stacks are viewed in 3D). Because α-btx binds nearly irreversibly, preincubating the neurons with the higher dose of α-btx occupies most of the α-btx binding sites and prevents the subsequent binding of rhodaminated α-btx (C).


A
B
C

D. Alpha-bungarotoxin does not increase the production or secretion of neurotrophic factor by the targets:

Introduction:

Because nAChR antagonists rescue neurons from cell death, it has been hypothesized that synaptic activity at the neuromuscular junction influences neuronal survival by inducing the target tissue to produce more neurotrophic factor (Tanaka, 1987; Pilar *et al.*, 1988; Oppenheim *et al.*, 1989). We used both *in vivo* and *in vitro* approaches to assess whether α-btx increases either synthesis or release of neurotrophic factors in the targets of the CG.

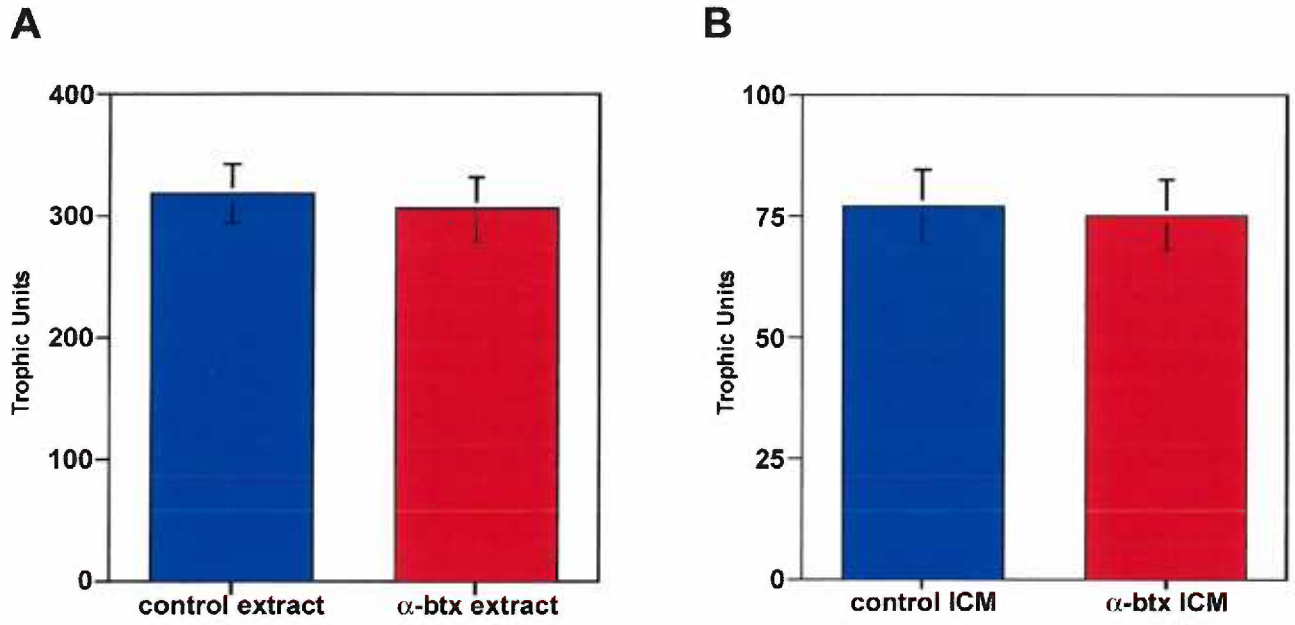
Results:

We tested whether α-btx increases neurotrophic factor production by making eye extracts from α-btx-treated E11 and E14 embryos and measuring the neurotrophic activity of the extracts in a bioassay. One neurotrophic factor that meets many of the criteria for a CG target-derived molecule is chicken ciliary neurotrophic factor (chCNTF) (Finn and Nishi, 1996a; Finn *et al.*, 1998). In order to detect the neurotrophic activity due to chCNTF, we excluded neurotrophic activity due to fibroblast growth factor (FGF) 1 and 2, which are abundant in the embryonic chick eye, but not secreted (Eckenstein *et al.*, 1991; Nishi, unpublished observations) by passing the extracts over a heparin-affinity column. Under these conditions, over 90% of the remaining, non-heparin binding neurotrophic activity can be neutralized with anti-chCNTF antibody (Finn and Nishi, 1996a). We then

compared the ability of the extracts to support CG neurons in culture by performing dose-response curves with the extracts in E8 CG neuron survival assays. We found that the α-btx-treated eye extract contained the same number of trophic units as the control eye extract at E11 (*figure 11a*). There was an increase in the neurotrophic activity of both eye extracts from E11 to E14, but no difference in the activity of α-btx-treated E14 eye extract versus control E14 extract (data not shown).

The lack of increase in the neurotrophic activity of the eye extracts did not preclude the possibility that α-btx may induce the target tissues to release more neurotrophic activity to the neurons. We examined this possibility by growing iris and choroid cultures in the presence of α-btx and testing the conditioned medium with a bioassay for neurotrophic activity. We found no difference in the ability of α-btx-treated iris-conditioned medium (CM, *figure 11b*) or choroid-conditioned medium to support CG neurons in culture (data not shown). However, it was possible that activity was lost during freezing or storage of the CM. Accordingly, we also performed the experiment with fresh CM that was transferred directly from muscle cultures to neurons with no intervening freezing step. We detected no difference in the neurotrophic activity of the fresh α-btx - treated or control-CM (data not shown), which, together with the eye extract experiment, indicates that α-btx does not alter the synthesis or release of neurotrophic activity by target tissues.

Figure 11. Alpha-btx does not increase the production or secretion of trophic factor by the targets. A) Eye extracts were made from five control embryos and five embryos treated with α-btx from E7 through E11. Because FGFs are not normally secreted, but are released with cell lysis (Eckenstein *et al.*, 1994), their presence could mask changes in other, biologically relevant trophic factors. Accordingly, the extracts were passed over heparin affinity columns to remove FGFs. The total amount of trophic activity present in each extract was determined by bioassay on E8 CG neurons. The numbers represent the mean and standard error obtained from four independent determinations. No difference was found in the ability of control versus α-btx-treated eye extract to support neuronal survival *in vitro*. B) Iris cells were cultured in a 24-well plate in serum-free medium for 3 days in the presence of 1μg/ml α-btx. Cultures were then rinsed twice with EBSS, fed with fresh serum-free medium without α-btx, and conditioned for 48h. This medium was collected and stored at –80°C. The total amount of trophic activity in each culture was measured by bioassay on E8 CG neurons. The numbers represent the mean and standard error obtained from three independent determinations. No difference was detected in the ability of conditioned medium from control or α-btx-treated iris cultures to support neuronal survival *in vitro*.


A
400
300
200
100
0
Trophic Units
control extract
α-btx extract
B
100
75
50
25
0
Trophic Units
control ICM
α-btx ICM

E. Alpha-btx rescues CG neurons via neuronal α7-nAChRs:

Introduction:

Because nicotinic acetylcholine receptor (nAChR) antagonists rescue motor neurons from cell death, it has been suggested that synaptic activity at the neuromuscular junction plays a critical role in regulating neuronal number (Tanaka, 1987;Pilar *et al.*, 1988; Oppenheim, 1984; Oppenheim *et al.*, 1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997; Oppenheim *et al.*, 2000). Although this makes sense for spinal cord motor neurons, which innervate striated muscle, we have shown that both ciliary and choroid neurons are rescued by the nAChR, α-btx, and the smooth muscle of the choroid is unlikely to contain α-btx binding sites.

As mentioned previously, α-btx has an extremely high selectivity for binding only two specific alpha subunits of nAChRs in the vertebrate nervous system (Sargent, 1993): α1, which is expressed only in striated muscle, and α7, which is expressed in neurons and many non-neuronal tissues such as the lung (Sekhon *et al.*, 1999). We examined the presence of α-btx binding sites on embryonic CG neurons and targets using two sensitive techniques. In addition, we tested whether an α7-nAChR-specific antagonist can mimic the neuronal rescue of α-btx.

Results:

E8-14 Choroid muscle contains neither α1 nor α7 nAChRs:

Although α-btx binding sites have been described on CG neurons and iris (Corriveau and Berg, 1994), it has not been known whether α-btx binding sites could be found in the choroid layer. To test this, we incubated whole choroid layers in rhodaminated α-btx. No specific binding could be detected; however, this method may not have detected low levels of highly dispersed nAChRs. Therefore, we also used RT-PCR, a highly sensitive method, to determine whether the choroid expressed transcripts encoding α1 or α7 nAChRs, the predominant nAChR subtypes that bind α-btx.

A product of the expected size was amplified from E8 CG and E15 brain cDNA using α7-nAChR specific primers. Low levels of transcripts encoding α7-nAChR were also detected in E8 iris (*figure 12*). Alpha-1 mRNA was not detected in E8 iris cDNA, although it was detectable by E11 with the onset of the smooth-to-striated muscle transition (data not shown). Significantly, neither α1 nor α7 mRNA was present in choroid cDNA from E8, E11, or E14. All samples generated amplification products for chick ribosomal binding protein (CHRPS), a constitutively expressed gene.

MLA rescues both ciliary and choroid neurons from cell death *in vivo*:

Embryonic CG neurons express high levels of neuronal α7-nAChRs, which represent virtually all of the α-btx binding sites in the ganglion (Chang and

Berg, 1999). Accordingly, we tested whether the α7-specific antagonist, methyllycaconitine (MLA), could rescue CG neurons *in vivo*. The dosage of MLA we selected (26 μg/day) caused no paralysis of the embryos, indicating that α1-nAChRs on muscle were not blocked (*figure 13C*). MLA rescued both ciliary (6822+/-196 vs. a control value of 5498+/-410; $p<0.01$, Student's t-test) and choroid neurons (5860+/-191 vs. a control value of 4349+/-303; $p<0.001$, Student's t-test; *figure 13A*). This represents 24% increase in the number of ciliary neurons and a 35% increase in the number of choroid neurons (*figure 13B*). These results suggest that both MLA and α-btx are rescuing CG neurons by blocking the neuronal α7-nAChR.

If MLA is rescuing CG neurons from cell death via the same mechanism used by α-btx, then we would predict that a lower dose of MLA would also rescue ciliary, but not choroid, neurons. We found that a tenfold lower dose of MLA rescued ciliary (8840 +/- 314 vs. a control value of 5498 +/- 410, $p<0.01$, Student's t-test), but not choroid, neurons (3942 +/- 190 vs. a control value of 4349 +/- 303, $p<0.05$, Student's t-test), from cell death *in vivo* (*figure 14A*). This represents a 61% increase in ciliary neurons and 9% decrease in the number of choroid neurons (*figure 14B*). This is further evidence that both MLA and α-btx are rescuing CG neurons by blocking neuronal α7-nAChRs.

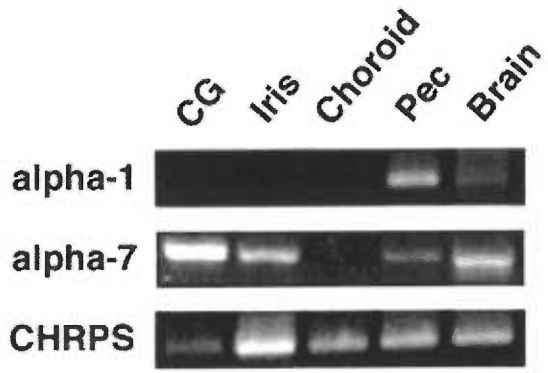
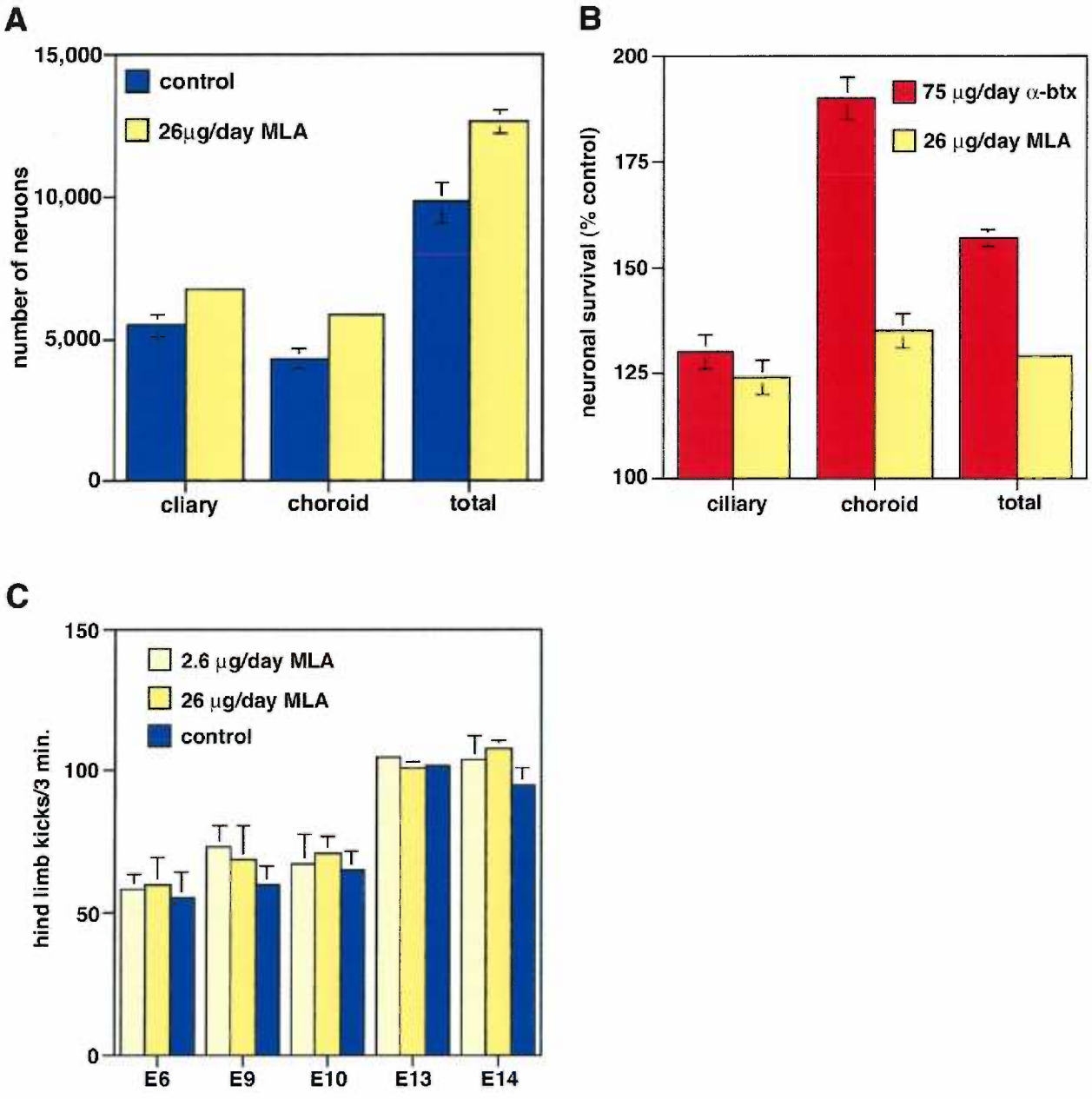


Figure 12. E8 choroid muscle contains neither α1- nor α7-nAChRs. RT-PCR was used to detect transcripts encoding α1- and α7-nAChRs, the predominant nAChR subtypes that bind α-btx, in E8 CG, iris, and choroid. A 252 bp product was amplified from pectoral muscle and brain using α1-nAChR-specific primers based on the published sequence (Nef, *et al.*, 1988): forward, 1046-1065, and reverse, 1297-1276. A 530 bp product was amplified from CG, iris, pectoral muscle and E15 brain using *a*7-nAChR-specific primers based on the published sequence (Couturier *et al.*, 1990): forward, 314-335, and reverse, 843-822. All samples generated amplification products of 361 bp using the primer pair (forward, 49-67, and reverse, 389-409) based on the published sequence for chick ribosomal binding protein (CHRPS), a constitutively expressed gene (Trueb *et al.*, 1988). All PCR reactions used cycling conditions of 95°C (melting), 54 °C (annealing), and 72° (extension), for 1 minute each (30 cycles) with 1U of Taq DNA polymerase per reaction (Promega, Madison, WI). Significantly, neither the α1- nor the α7-nAChR transcript was detected in choroid muscle.

Figure 13. 26 μg/day MLA rescues both ciliary and choroid neurons from cell death. CG were collected and processed for islet-1 and somatostatin immunohistochemistry at E14 after seven days of *in ovo* administration of MLA or saline. A) Differential neuronal counts were obtained using design-based stereology and their values represented as histograms. We applied 26 μg/day MLA, an α7-nAChR-specific antagonist, once a day to the chorioallantioc membrane from E7 through E14. Differential neuronal counts revealed that MLA rescued both ciliary (6822+/-196; n=3vs. a control value of 5498+/-410; n=7; $p<0.01$, Student's t-test) and choroid neurons (5860+/-191; n= 3; vs. a control value of 4349+/-303; n= 7; $p<0.001$, Student's t-test) from cell death *in vivo.* The numbers represent the mean and standard error of the percent change in neuronal number from control as compared to α-btx-treated embryos. Error bars represent standard errors. B) The neuronal rescue produced by MLA application translates into a 24% increase in the number of ciliary neurons and a 35% increase in the number of choroid neurons. The total neuronal number increased by 29% over control. For comparison, 75 μg/day α-btx caused a 30% increase in ciliary neurons, a 90% increase in choroid neurons, and a 57% increase in total neurons. Error bars represent the standard error. C) MLA caused no paralysis of the embryos as measured by the number of discrete hindlimb kicks per three-minute viewing window, indicating that it does not block α1-nAChRs at 26μg/day. Error bars represent standard deviations.


A
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number of neruons
control
26μg/day MLA
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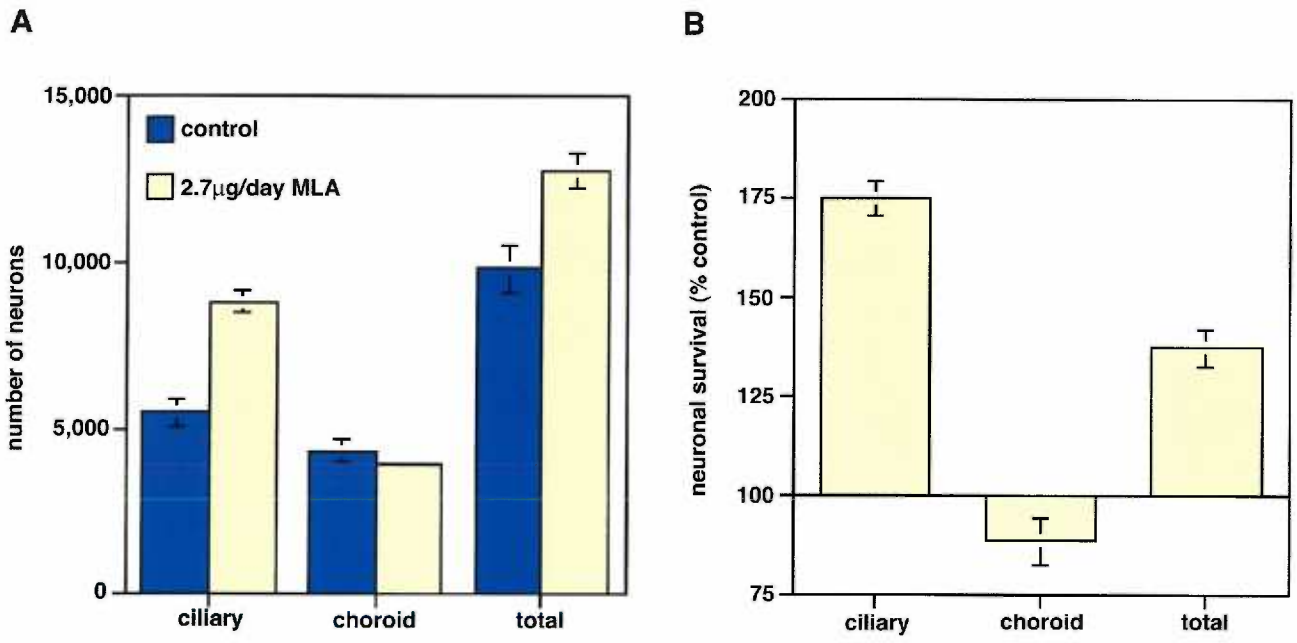
total
B
200
175
150
125
100
neuronal survival (% control)
75 μg/day α-btx
26 μg/day MLA
ciliary
choroid
total
C
150
100
50
0
hind limb kicks/3 min.
2.6 μg/day MLA
26 μg/day MLA
control
E6
E9
E10
E13
E14

Figure 14. A tenfold lower concentration of MLA rescues ciliary, but not choroid neurons. CG were collected and processed for islet-1 and somatostatin immunohistochemistry at E14 after seven days of *in ovo* administration of MLA or saline. A) Differential neuronal counts were obtained using design-based stereology and their values represented as histograms. Daily application of 2.6 μg/day of MLA onto the chorioallantoic membrane from E7 through E14 rescued ciliary (8840+/-704; n=3 vs. a control value of 5498+/-410; n=7; $p<0.01$, Student's t-test) but not choroid (3942+/-337; n=3 vs. a control value of 4349+/-303; n=7) neurons from cell death *in vivo.* The numbers represent the mean and standard error obtained from 3-7 independent determinations. B) This translated into a 61% increase in ciliary neurons, a 9% decrease in choroid neurons, and a total neuronal increase of 30%.


A
15,000
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number of neurons
control
2.7μg/day MLA
ciliary
choroid
total
B
200
175
150
125
100
75
neuronal survival (% control)
ciliary
choroid
total

F. Nerve branching and vesicle accumulation can be uncoupled from cell rescue:

Introduction:

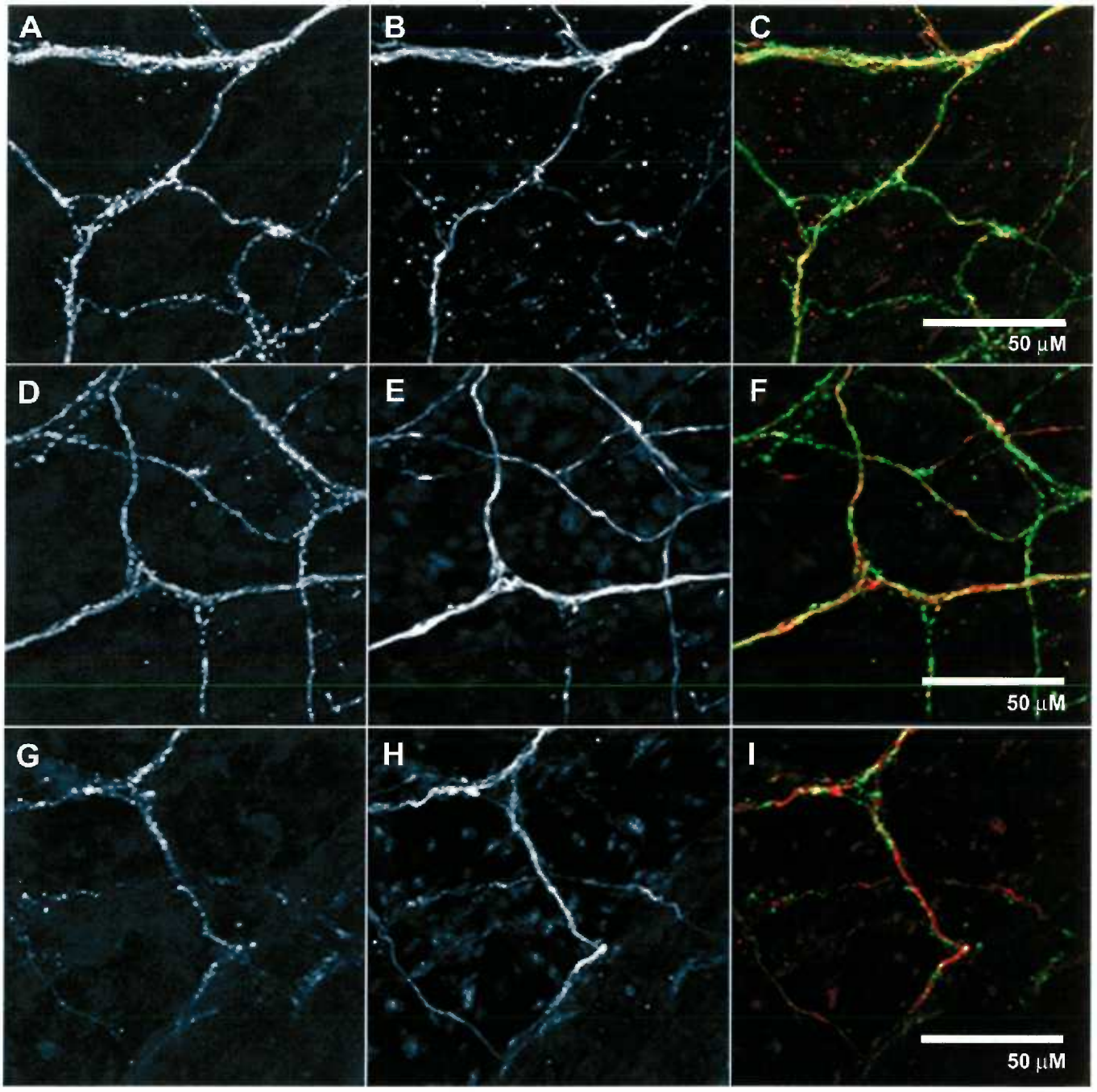
Another mechanism that has been proposed to explain the rescue of neurons by nAChR antagonists is that synaptic activity may regulate the extent of intramuscular nerve branching and synaptogenesis, which may, in turn, reduce cell death by allowing greater access to target-derived neurotrophic factors (Oppenheim, 1984; Oppenheim *et al.*, 1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997). Because this increased nerve branching is hypothesized to occur via blockade of the neuromuscular junction, we tested whether nerve branching and synaptogenesis were altered in either the choroid muscle, which we have shown does not have α-btx-binding receptors, or the iris, which begins to express α-btx binding sites only partway through cell death. In addition, we tested whether the α7-nAChR antagonist, methyllycaconitine (MLA), which we have shown rescues CG neurons from cell death *in vivo*, affects nerve branching or synaptogenesis.

Results:

We monitored nerve branching by staining whole mount preparations of choroid and iris/ciliary body for neurofilament. In addition, we stained for the vesicular marker SV2 as a measure of the frequency and size of synaptic contacts made by CG neurons. Interestingly, we found that α-btx treatment produced no

change in the branching pattern of axons in the choroid layer, nor did α-btx alter the frequency or size of synaptic varicosities (*figure 15A-C, G-I*). In fact, we observed a slight decrease in the number of varicosities at the choroid in a-btx treated embryos. Furthermore, MLA, which also rescues choroid and ciliary neurons, altered neither synaptogenesis nor nerve branching at either choroid (*figure 15D-F*) or iris (*figure 16D-F*). These observations suggest that the rescue of CG neurons by α-btx and MLA are not increased through contacts with the targets, and, thus, it is unlikely to be mediated by an enhanced access of neuronal terminals to a target-derived neurotrophic factor.

In contrast, α-btx treatment produced a marked increase in the size of terminal varicosities containing SV2 staining on the iris muscle, which could be observed at E14 (*table 1, figure 16A-C, G-I*). This effect increased with prolonged paralysis of the embryo with α-btx (data not shown). For example, the varicosities were significantly larger in embyros treated through E18 in comparison to embryos paralyzed until E14. This effect on peripheral synaptogenesis is reminiscent of that previously seen in a variety of skeletal muscles after curare treatment (Oppenheim, 1981). The results indicate that the alteration of presynaptic differentiation at muscle targets produced by nicotinic antagonists is likely to be mediated through a separate, α1-nAChR-mediated mechanism.


A
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50 μM
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50 μM

Figure 16. Alpha-bungarotoxin, but not MLA, increases synaptogenesis in the iris. Embryos were treated with 75 μg/day α-btx or 26 μg/day MLA from E7-14. At E14, eyes were removed and whole-mount iris muscles were processed for neurofilament and SV-2 immunohistochemistry. Panels A, B, and C are from a control embryo. Panels D, E, and F are from an embryo treated with MLA. Panels G, H, and I are from an α-btx-treated embryo. Neither MLA nor α-btx altered nerve branching patterns, as determined by anti-neurofilament staining (panels B, E, and H). Although MLA did not alter the frequency or size of synaptic varicosities, as measured by anti-SV-2 staining (panels A, D), α-btx produced a marked increase in the size of terminal varicosities containing SV-2 (panel G). Panels C, F, and I show the merged confocal images.

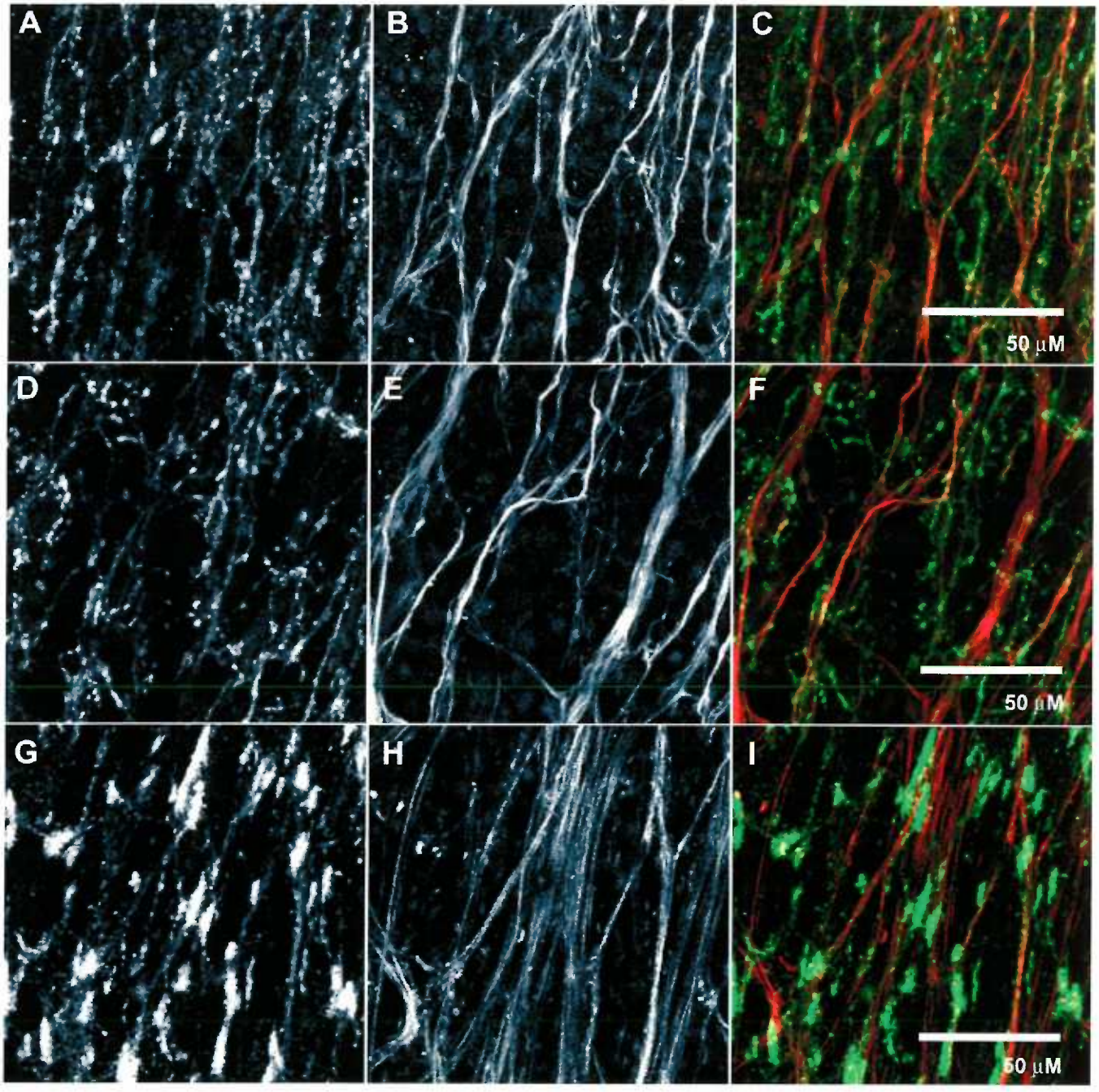
A
B
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Table 1. Percentage of total area occupied by SV-2 staining in the iris

Treatment	percent area	n
Saline	10.2% +/- 1.1	3
MLA	9.7% +/- 2.4	3
α-btx	17.1% +/- 2.6	3

Table 1. Alpha-bungarotoxin, but not MLA, increases the size of presynaptic terminals in iris. Embryos were treated with 75 μg/day α-btx or 26 μg/day MLA from E7-14. At E14, eyes were removed and whole-mount iris muscles were processed for neurofilament and SV-2 immunohistochemistry. Following confocal microscopy, an average projection image for each of the image stacks was obtained using Metamorph software. A minimum threshold intensity was set and the theresholded area, standard deviation, and percent thresholded area were generated by Metamorph. Alpha-btx, but not MLA, produced a dramatic increase in the percent thresholded area of anti-SV-2 staining ($p<0.05$, ANOVA; control vs. α-btx, $p<0.01$, Student's t-test).

DISCUSSION:

Summary:

It has been known for quite some time that the chronic application of nicotinic antagonists is the most effective way to rescue motor neurons from target-dependent cell death. The mechanism by which these agents act, however, has never been resolved. It is particularly curious that the snake toxin, α-btx, rescues choroid neurons of the avian ciliary ganglion in spite of the fact that they do not form functional nicotinic junctions on their vascular smooth muscle targets. Our studies have shown that α-btx does not rescue CG neurons from cell death via an activity-dependent increase in the amount or availability of target-derived neurotrophic activity, nor does it operate by substituting for a trophic factor or potentiating responsiveness to known trophic molecules. Instead, the rescue of choroid neurons despite the lack of α7-nAChR expression in choroid, the rescue of CG neurons by the selective α7-nAChR antagonist, MLA, and the uncoupling of neuronal rescue from synpatogenesis suggest that α-btx and MLA rescue CG neurons through the pharmacological block of neuronal α7-nAChRs.

Counting Methods:

We began our studies by confirming that daily application of the nAChR antagonist, α-btx, rescues both ciliary and choroid neurons from cell death. Our approach to quantifying the two populations of neurons had two important advantages over previous methods. First, we used design-based stereology to count neurons, allowing a rapid, unbiased means of sampling more CGs. Second,

we used the specific choroid neuron marker, somatostatin, to distinguish between the two populations of neurons. Previously, Meriney *et al.* had quantified ciliary and choroid neuron survival by selectively labeling ciliary neurons with HRP (Meriney *et al.*, 1987), and found that both populations of CG neurons are rescued by α-btx. However, since they derived the number of choroid neurons by subtracting the number of HRP-labeled ciliary neurons from the total neuronal number, it is possible that cells counted as choroid neurons were actually unlabeled ciliary neurons. Our complementary counting scheme circumvented this problem; labeling choroid neurons directly gave us a more reliable measure of the number of choroid neurons rescued by α-btx.

Importantly, our counting method also showed that the rescued neurons express the expected neurochemical phenotype, rather than failing to differentiate appropriately. Because the acquisition of somatostatin expression is target-regulated (Coulombe *et al.*, 1993; Darland *et al.*, 1995; Darland and Nishi, 1998), somatostatin expression in the rescued neurons suggests that they projected to the appropriate target.

Toxin Dose Effects:

Our finding that a lower dose of α-btx rescues ciliary neurons but not choroid neurons differs slightly from previous reports of a small decrease in choroid neuron survival following application of 12.5 μg/day of α-btx (Meriney and Pilar, 1987). By contrast, our choroid neuron number did not drop below

control levels. This difference could be due to any of several factors: the different methodologies employed for distinguishing the two populations of neurons, the different means of counting the cells, or differences in the genetic backgrounds of the chicks. In particular, because the drug was applied by dripping onto the chorioallantoic membrane and became diluted into the total egg volume, the size of the eggs could skew the doses of α-btx the embryos were exposed to; Meriney *et al.* reported that they used a dose of 12.5 μg/day, which translates to a dose of around 50 μM for the eggs we used. Egg volume can vary considerably, however, which would alter the final dilution of the drug. Therefore, we could have been using a different effective dose of α-btx than was originally reported, which might explain our different results.

The differential effects of this lower dose of α-btx on the two populations of neurons may have its root in the structural differences between ciliary and choroid neurons. Over 90% of the peak synaptic current in ciliary neurons is generated by α7-nAChRs (Zhang *et al.*, 1996). While both ciliary and choroid neurons express high levels of α7-nAChRs, averaging 10^6 per cell at the end of embryogenesis (Chiappinelli and Zigmond, 1978; Corriveau and Berg, 1994), on ciliary neurons the α7-nAChRs are concentrated on groups of folded somatic spines, or "pseudodendrites" (Blumenthal *et al.*, 1999). The spines are engulfed by a single, large presynaptic calyx packed with synaptic vesicles. By contrast, choroid neurons are innervated at multiple boutons. Because α7-nAChRs have a high permeability to calcium (Seguela *et al.*, 1993), their physical arrangement on

somatic spines may serve to concentrate the calcium influx and confer a greater sensitivity to the effects of α7-nAChR activation to the ciliary neurons, as compared to the choroid neurons.

In addition to their different spatial locations on ciliary and choroid neurons, α7-nAChRs may play different roles on the two populations of neurons. Neurons maintain multiple classes of nicotinic AchRs that are distinguished by their different subunit compositions (Conroy and Berg, 1995); the relative densities of α3-, α5-, and α7-nAChRs are different on ciliary and choroid neurons (Corriveau and Berg, 1994). Also, α7-nAChRs carry more than 90% of the peak synaptic current in ciliary neurons, whereas they contribute far less to current generation in choroid neurons (Zhang *et al.*, 1996). This suggests that, particularly in choroid neurons, α7-nAChRs play other, perhaps modulatory or signaling, roles. These different roles may explain the apparent differential sensitivity of the two populations of neurons to α-btx and MLA.

Though the choroid neurons were not rescued by the lower dose of α-btx, it is worth noting that at the higher dose they showed a 90% increase over control levels, as compared to a 30% increase in ciliary neuron survival. This suggests that, though a higher dose of the toxin is necessary to completely block α7-nAChRs on choroid neurons, once the receptors are occupied, choroid neurons behave in a very homogeneous fashion. The ciliary neurons, by contrast, may be a more heterogeneous population, as evidenced by the smaller overall reduction in

cell death. This is consistent with the fact that the ciliary neurons innervate two separate targets: the iris and ciliary muscle. In addition, α7-nAChRs are required in a population of about 67% of the ciliary neurons for reliable synaptic transmission and for tightly synchronized firing early in development (Chang and Berg, 1999). When stimulated synaptically at 25Hz, about 33% are still able to fire without failures with their α7-nAChRs blocked. This implies that α7-nAChRs may serve different roles, not only in ciliary versus choroid neurons, but in different populations of ciliary neurons, as well.

Also worth noting is the fact that, whereas the higher dose of α-btx rescued only 30% of ciliary neurons, the lower dose rescued 60%. This suggests that there is a bell-shaped curve for the neuronal rescue effect of α-btx, a high dose of the drug having a deleterious effect on neuronal survival. The same may be true for choroid neurons, only they may require an even higher dose than the ciliary neurons do to produce this detrimental effect. This hypothesis is consistent with the fact that choroid neurons did not respond to the lower dose of α-btx, and it would be of interest to determine whether a dose of α-btx greater than 75 μg/day would prove deleterious to choroid neurons.

Pre- vs. Postsynaptic Target for the Toxin:

The simplest way for α-btx to rescue CG neurons via neuronal nAChRs would be for α-btx to substitute for a neurotrophic factor, much the way that elevated K+ concentrations have been found to promote neuronal survival in the

absence of trophic support in a number of culture systems (Lasher and Zagon, 1972; Phillipson and Sandler, 1975; Scott, 1977; Chalazonitis and Fischbach, 1980), including CG neurons (Bennett and White, 1979; Nishi and Berg, 1981a). We found, however, that α-btx did not support CG neuron survival *in vitro* when neurons were cultured alone. Similarly, α-btx did not rescue neurons by potentiating the trophic activity of a variety of endogenous target-derived neurotrophic factors, including chCNTF, GDNF, and FGF-2. Conversely, application of the nicotine at low concentrations (0.1-0.5μM), which has been shown to induce apoptosis in hippocampal neuronal precursor cells (Berger *et al.*, 1998), failed to induce apoptosis in cultured CG neurons.

One possibility for the lack of efficacy of α-btx or nicotine *in vitro* may be the failure of the neurons to express normal levels of nAChRs *in vitro.* The number of α-btx binding sites per ciliary ganglion is about three times higher *in vivo* than it is in cell culture (Corriveau and Berg, 1994), so the number of receptors present in culture may be insufficient to produce the effect they do *in vivo*. In addition, pre- or post-synaptically-derived molecules can influence receptor expression. For instance, an isoform of neuregulin with a conserved cysteine-rich domain augments the levels of expression of neuronal nAChR mRNAs encoding the α3, α5, and α7 subunits in synaptically naïve sympathetic neurons (Yang *et al.*, 1998). This, or some other factor from preganglionic neurons, glia, or target tissue may be required for the *in vitro* expression of normal *in vivo* levels of α-btx-binding receptors.

That α-btx rescued CG neurons grown in co-culture with iris or pectoral muscle argues that a target-derived factor is necessary for neuronal responsiveness to the toxin. Though the most straightforward interpretation of the co-culture rescue is that α-btx acts not at neuronal receptors, but at muscle receptors, our finding that CM from α-btx-treated iris cultures rescues no more neurons than control CM suggests that muscle nAChRs do not transduce the α-btx rescue effect. Therefore, an alternate explanation for the lack of efficacy of α-btx in rescuing neurons grown alone is insufficient *in vitro* expression of α7-nAChRs.

Unfortunately, after our initial finding that α-btx rescues neurons grown in co-culture, a problem with our chicken supplier led to a change in the strain of chickens we were receiving. After this time, we were unable to reproduce the co-culture rescue, although it was very reliable prior to the change (n=3 for neuron/iris co-cultures and n=5 for neuron/pectoral muscle co-cultures). Subsequent assays revealed a much smaller (10-20%) and more labile rescue that was only discernable with exhaustive counting schemes (i.e. sampling >70% the cells). At least two other reliable, unrelated bioassays stopped working in the lab at this same time as well, and, although we tried to reproduce the co-culture rescue in a number of different chicken strains, we were not able to return to the F1 hybrid chicken strain we originally used, nor were we able to duplicate the effect in other strains of chickens.

Does the fact that our co-culture rescue effect was so fleeting mean that it is of limited relevance? Possibly. However, this sort of strain-specific difference is not unprecedented; numerous strain-specific differences have been found in mice. For example, the methylation of the pAd2E2AL-CAT (7-1A) transgene is regulated in a strain-specific manner in mice; transmission of the 7-1A transgene into an inbred DBA/2, 129/sv, or FVB/N genetic background leads to a significant loss of methylation in the transgene, whereas C57BL/6, CB20, and Balb/c backgrounds favor the *de novo* methylation in very specific patterns (Schumacher *et al.*, 2000. See also Ingram and Jucker for a review of strain-specific differences in mouse models of aging). If an exogenous factor is necessary for the full expression of nAChRs on CG neurons, as we hypothesize, it may normally be generated by preganglionic neurons, glia, or targets *in vivo*. While the target tissue of our original chickens may have produced enough of the putative factor to induce α-btx responsiveness in the neurons, its distribution in other populations of chickens may be different. The residual survival effect seen in the more recent cultures may occur via lower levels of nAChRs, or they may be due to a secondary rescue mechanism, such as increased synapse formation, that was previously masked by the larger rescue. In any case, the *in vivo* rescue of CG neurons by α-btx remains unchanged in the new line of chickens.

Several questions remain unanswered as a result of our inability to continue this line of investigation. Ideally, we would have liked to repeat the

nicotine experiment under co-culture conditions to determine whether the failure of nicotine to induce cell death is due to a lack of nAChRs on cultured CG neurons, as we suspect. Additionally, it would be interesting to determine the identity of the putative muscle factor and whether it is secreted. Ultimately, we would have liked to use the co-culture assay to investigate the mechanism of the α-btx rescue (Does it involve Ca^{2+}? What signal transduction pathways are involved?). Without an *in vitro* assay to tie our manipulations to a reduction in cell death, however, we were unable to do this. Instead, we focused on testing several long-standing hypotheses and using our *in vivo* model to determine where and how α-btx rescues CG neurons.

The fact that a relatively high dose of α-btx (75 μg/day) was required to rescue both populations of neurons raised the concern that the rescue effect was, in fact, produced by a minor contaminant of the α-btx preparation, rather than by the nAChR antagonist. A previous report that certain commercial lots of α-btx blocked all nicotinic transmission through the ganglion used micromolar concentrations of the toxin; later it was discovered that the blockade resulted from a contaminant, κ-btx, that blocked both α-btx-sensitive and α-btx-insensitive nAChRs (Chiappinelli and Zigmond, 1978). We controlled for the possibility of a minor contaminant by repeating the *in vivo* experiment with α-btx from a second supplier (Calbiochem). The rescue effect produced by the new preparation of α-btx was indistinguishable from the first (data not shown), suggesting that either

the preparation of α-btx from both suppliers is pure, or they both contain the same contaminant.

Another protein neurotoxin present as a minor component in the venom of *Bungarus multicinctus* is Bgt 3.1, which can induce a rapid internalization of α-btx bound on the surface of CG (Ravdin *et al.*, 1981). Because of the dose of α-btx we used, if Bgt 3.1 were present in the α-btx preparation, even as a 1% contaminant, it could be available in sufficient levels to be responsible for the neuronal rescue. Our results suggest, however, that Bgt 3.1 is not present in our α-btx preparation because no nAChR internalization occurs on CG neurons as a result of α-btx exposure.

Our finding that α-btx caused neither an increase in the total content nor rate of release of trophic activity in CG confirms what has been found in targets of spinal cord motor neurons (Tanaka, 1987; Oppenheim *et al.*, 1989), though it contradicts what Pilar *et al.* found in the CG: that α-btx produced a fourfold increase in trophic activity in the chick eye at E11 followed by a 50% decrease in trophic activity at E14 (Pilar *et al.*, 1988). Because this report was published in abstract form, we have limited information on how the eye extract experiment was carried out. However, a crucial difference between our methods and that of Pilar *et al.* may be that they did not exclude FGFs from their extracts, and FGF1 and 2 are not normally secreted (Eckenstein *et al.*, 1994).

In contrast, our heparin affinity step excluded FGF-1 and FGF-2, which are only released by cell lysis, and so cannot act as true target-derived neurotrophic factors (Eckenstein *et al.*, 1994). In addition, although we have not formally excluded the contribution of GDNF and neurturin from the biological activity we observed, affinity purified antibodies against chCNTF have been shown to deplete more than 90% of the remaining trophic activity (Finn and Nishi, 1996a), so the contributions of the other trophic molecules appear to be minimal. Furthermore, chCNTF is the only trophic molecule that has been shown to be expressed in CG targets during the period of cell death (Finn and Nishi, 1996a). Thus, the neurotrophic activity we measured was largely due to chCNTF, while Pilar, *et al.* may have been measuring neurotrophic activity that is not physiologically relevant in controlling neuronal cell death.

Although choroid neurons activate vascular smooth muscle contraction via muscarinic, rather than nicotinic, receptors, it was still possible that nicotinic receptors that bind α-btx could be present, but unable to mediate a functional response in the target. For example, a variety of non-neural tissues, such as lung and skeletal muscle, have been reported to express α7-nAChRs (Corriveau *et al.*, 1995; Sekhon *et al.*, 1999). We confirmed the lack of α-btx binding sites in the choroid using two methods that did not involve monitoring smooth muscle contractility: rhodaminated α-btx binding and RT-PCR. Since even a highly sensitive technique such as RT-PCR was unable detect an amplification product from the choroid layer with α7-specific primers, it is highly unlikely that α-btx

could be rescuing choroid neurons via a direct interaction with the smooth muscle target. In spite of this lack of α-btx binding sites in the choroid, the higher dose of α-btx rescued significantly more choroid than ciliary neurons.

In contrast to choroid neurons, the rescue of ciliary neurons could be effected by α-btx at the iris and ciliary muscle, which undergoes a transition from a muscarinically controlled smooth muscle to a nicotinically activated striated muscle (Pilar *et al.*, 1987; Link and Nishi, 1998b). However, ciliary neurons also express extremely high levels of α7-nAChRs early in development, and in the mature CG, >90% of the synaptically activated acetylcholine-induced current can be blocked by α-btx (Zhang *et al.*, 1996). Thus, α-btx could be acting either directly on neuronal nAChRs or by altering transmission between ciliary neurons and their muscle targets.

The strongest evidence that α-btx rescues CG neurons via neuronal nAChRs comes from the observation that the α7-specific antagonist, MLA, rescued both ciliary and choroid neurons from cell death *in vivo*. Because interpretation of this rescue could be difficult if the dose of MLA exceeded the specific range for the drug, we were careful to select a dose of MLA that caused no paralysis of the embryos, indicating that α1-nAChRs on muscles were not blocked.

Though stereological counts of ciliary and choroid neurons revealed that MLA rescues both ciliary and choroid neurons, the α7-nAChR-specific toxin produced a less robust rescue than that seen with α-btx. This difference may be due to the fact that MLA is a reversible antagonist, whereas α-btx blocks nAChRs nearly irreversibly, possibly occupying all of the receptors at a lower dose than MLA can. In addition, MLA is considerably less effective at blocking homomeric α7 receptors than α-btx (Tsetlin, 1999). Nevertheless, the fact that MLA rescues both ciliary and choroid neurons from cell death strongly suggests that both MLA and α-btx rescue CG neurons via the α7-nAChR. Further confirmation of this hypothesis comes from our finding that a tenfold lower dose of MLA, like α-btx, also rescues ciliary, but not choroid neurons.

The rescue of CG neurons differs from that which is seen in spinal cord motor neurons, which are not rescued by MLA (Oppenheim *et al.*, 2000). This is not the first instance of a toxin having a different effect in CG neurons than it has in spinal cord motor neurons, however: D-tubocurare, which produces just as robust a rescue in spinal cord motor neurons as does α-btx, does not rescue CG neurons (Meriney *et al.*, 1987). This differential drug sensitivity likely stems from the fundamental differences between the two populations of neurons. For example, though α-btx binding sites have been described in the chick spinal cord (Renshaw *et al.*, 1993; Renshaw, 1994), afferent input to spinal cord motor neurons is primarily mediated by glutamate and γ-aminobutyric acid (GABA). Transmission in CG neurons, by contrast, uses ACh as the major neurotransmitter,

with substance P, enkephalin, and GABA exerting modulatory influences (Boyd *et al.*, 1988; Dryer and Chiappinelli, 1985; McEachern *et al.*, 1985; Margiotta and Gurantz, 1989; Role, 1988). Indeed, over 90% of the peak synaptic current in ciliary neurons is generated by α7-nAChRs (Zhang *et al.*, 1996). Thus, α7-nAChRs likely play very different roles in the two populations of neurons: mediating synaptic transmission in CG neurons, and serving a modulatory role in motor neurons.

Additionally, the different responses of CG neurons and motor neurons to nAChR antagonists may be rooted in the different embryonic origins of the two populations of neurons. Motor neurons arise in the ventral portion of the neural tube from multipotential progenitors that also give rise to interneurons and glial cells (Leber *et al.*, 1990). Motor axons exit the central nervous system through ventral roots (or cranial nerves), then run long distances through peripheral nerves to muscles.

By contrast, both neural and nonneural cells of the chick ciliary ganglion arise from precursor cells migrating out of the posterior and anterior portions of the mesencephalic neural crest (Hammond and Yntema, 1958; Narayan and Narayan, 1978b; Noden, 1978). These cells reach their final position behind the eye and coalesce into a recognizable structure by the fourth day of embryogenesis (stage 24, Dryer, 1994). The final round of cell division occurs over a relatively narrow window of time, and is completed by stage 25 (Landmesser and Pilar,

1974a). Ciliary ganglion cells do not express neural specific marker proteins until after terminal mitosis (Rohrer and Thoenen, 1987), which differs from other chick autonomic ganglia and motor neurons, which may express neural markers prior to the terminal mitosis, and in which cell division extends for a much longer period of time (Rohrer and Thoenen, 1987).

Thus, there are marked differences between spinal cord motor neurons and ciliary ganglion neurons, both in terms of the role and subunit composition of nAChRs, and in terms of developmental origin. Any or all of these differences could contribute to the different responses of the two populations of neurons to nAChR antagonists. Though it appears that the primary mechanism of rescue may be different in these two populations of neurons (*i.e.* neuronal branching in motor neurons versus a direct effect on signalling in ciliary ganglion neurons), the fact that nAChR antagonists rescue both types of neurons from cell death argues the importance of nAChRs in the regulation of cell death.

That α-btx failed to increase nerve branching or synaptogenesis on choroid muscle also contrasts with what is seen when spinal cord motor neurons are chronically exposed to nicotinic antagonists (Oppenheim *et al.*, 1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997). In fact, there was a decrease in terminal area visualized in the choroid using the synaptic vesicle marker, SV-2, when embryos were chronically exposed to concentrations of α-btx that rescued neurons from dying.

This is consistent with previous reports that α-btx inhibits process outgrowth of CG neurons (Pugh and Berg, 1994). Thus, α-btx does not rescue choroid neurons by inducing an increase in cell surface exposed to target-derived trophic factors.

The fact that MLA rescues ciliary neurons without increasing nerve branching or synaptogenesis in the iris is further confirmation that the rescue occurs via neuronal α7-nAChRs. This is consistent with previous reports that sub-paralytic doses of nAChR antagonists rescue motor neurons from cell death without affecting nerve branching (Hory-Lee and Frank, 1995), though it contrasts with reports that show a clear correlation between spinal cord motor neuron survival and the extent of nerve branching (Oppenheim, 1984; Oppenheim *et al.*, 1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997). That α-btx, which binds to α1-nAChRs in addition to α7-nAChRs, causes a dramatic increase in synaptogenesis in the iris, as is seen in the targets of spinal cord motor neurons (Oppenheim, 1984; Oppenheim et al., 1989; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993; Oppenheim *et al.*, 1997), suggests that the effect on synaptogenesis occurs through a separate, α1-nAChR-mediated mechanism that may or may not enhance neuronal survival, as well.

These discrepancies between what is seen in motor neurons and what is seen in CG neurons (the MLA rescue effect, synaptogenesis and nerve branching) suggest that not only are there multiple ways to influence cell death, but that

different mechanisms of regulating cell death are weighted differently in importance in different populations of neurons. For example, the finding that 90% of choroid neurons are rescued by MLA suggests that activation of α7-nAChRs is the primary means of regulating choroid neuron cell death. By comparison, MLA does not rescue motor neurons (Oppenheim *et al.*, 2000), but synaptogenesis is critically important in regulating cell death in motor neurons (Landmesser *et al.*, 1988; Oppenheim *et al.*, 1989; Landmesser *et al.*, 1988; Houenou *et al.*, 1991; Landmesser, 1992; Tang and Landmesser, 1993, Oppenheim *et al.*, 1997; Caldero *et al.*, 1998; Usiak and Landmesser, 1999). Therefore, it appears that cell death is predominantly controlled by α7-nAChRs in choroid neurons and by *a*1-nAChRs in motor neurons.

Regulation of cell death is more complex in ciliary neurons, where both receptor types may play roles. Clearly cell death in ciliary neurons is largely dependent on activation of α7-nAChRs, since MLA rescued 61% of ciliary neurons, but what about the other 39%? Though α-btx produces a dramatic increase in presynaptic terminal size, it did not rescue any more ciliary neurons than MLA did, so synapse size does not appear to limit neuronal survival. However, we are unable to exclude the possibility that both α7- and α1-nAChRs are important in regulating cell death in ciliary neurons. There are a number of other factors that may be involved in the regulation of cell death, as well, including preganglionic input (Levi-Montalcini, 1949; Furber, 1984; Meriney *et al.*, 1987; Oppenheim, 1991; Linden, 1994), endogenous opioids (Meriney *et al.*,

1991), and other, unknown trophic, glial, or hormonal influences. Though we did not examine the roles of these other factors, they may also be involved in controlling developmental cell death in both ciliary and choroid neurons, as well as motor neurons.

Pre- vs. Postganglionic Target for the Toxin:

If α-btx acts on neuronal α7-nAChRs when it rescues CG neurons from cell death, what is the mechanism of this rescue? In addition to the cell bodies of CG neurons, α7-nAChRs are present on the preganglionic terminals where they can influence neurotransmitter release (Coggan *et al.*, 1997). One could propose a different mechanism for each of these two α7-nAChR sites. If α7-nAChRs on preganglionic neurons are the site of the α-btx rescue, then one would expect preganglionic axotomy to produce this same effect. Interestingly, surgical denervation decreases neuronal survival in the CG (Furber, 1984; Levi-Montalcini, 1949). The difference between these two findings may be due to the fact that axotomy removes not just afferent input, but also potential trophic molecules from the preganglionic neurons, such as the endogenous opioids that have been shown to affect neuronal number (Meriney *et al.*, 1991). Blockade of the α7-nAChRs, by comparison, may affect afferent input without interrupting trophic support.

A preganglionic site of action for α-btx would provide an alternate explanation for why α-btx did not rescue neurons cultured alone: CG neurons

were grown in the absence of the preganglionic neurons from the accessory oculomotor nucleus. The rescue of CG neurons by α-btx when co-cultured with target tissue, however, is not consistent with a preganglionic site of action. Although we were unable to reproduce this result with a different chicken strain, we saw the rescue effect reliably in our original F1 hybrids. Because we were able to rescue large numbers of neurons in the absence of preganglionic neurons, it seems unlikely that preganglionic α7-nACgRs are the site of the α-btx rescue.

Alternatively, α7-nAChRs on the CG neuron cell bodies could be the site of the α-btx rescue. Alpha 7-nAChRs are required in a population of ciliary neurons for reliable synaptic transmission and for tightly synchronized firing early in development (Chang and Berg, 1999). They have also been shown to have a very high permeability to calcium (Seguela *et al.*, 1993); calcium entry via α7-nAChRs may induce cell death. Although a certain range of intracellular calcium concentrations is compatible with neuronal survival, the precise range needed for survival may vary between cell types and in a given cell type at different developmental stages (Collins *et al.*, 1991). Although increased intracellular calcium induced by elevated potassium keeps CG neurons alive *in vitro* (Collins *et al.*, 1991), other neurons are killed by a similar increase in intracellular calcium when it is induced by excitatory amino acids (Collins *et al.*, 1991; Murphy *et al.*, 1987; Rothman *et al.*, 1987; Kater *et al.*, 1989) or by the HIV viral coat protein, gp 120 (Dreyer *et al.*, 1990).

In addition, arachidonic acid is released in a calcium-dependent fashion by phospholipase A_2 and by phospholipase C followed by diacylglycerol lipase in response to activation of CG nAChRs (Vijayaraghavan *et al.*, 1995). This compound and its metabolites can act as membrane-permeant second messengers and have been proposed as trans-synaptic modulators of neurotransmitter release as well as regulators of several ionic currents (Vijayaraghavan *et al.*, 1995; Kim and Clapham, 1989; Ordway *et al.*, 1989; Ordway *et al.*, 1991; Keyser and Alger, 1990; Piomelli and Greengard, 1990; Schweitzer *et al.*, 1990; Harish and Poo, 1992; Shimada and Somlyo, 1992; Fraser *et al.*, 1993).

Furthermore, activation of α7-nAChRs may result in transcriptional changes ultimately leading to apoptosis. For example, stimulation of E14 CG at 1Hz via the preganglionic nerve produces calcium transients confined to ciliary dendritic spines, whereas long-term stimulation at 50 Hz results in sustained phosphorylation of CREB (Shoop *et al.*, 2001, personal communication). Blocking Ca^{2+} entry via α7-nAChRs could, therefore, have profound effects on gene expression in CG neurons.

Future Directions:

How could we test whether CG neurons are rescued via blockade of preganglionic versus ganglionic nAChRs? We could use calcium imaging with ratiometric dyes to examine how activation and blockade of α7-nAChRs influences intracellular calcium flux, but without a way to tie these results to cell

death, it would be difficult to draw any meaningful conclusions. One approach would be to deliver ribozymes or antisense oligonucleotides to selectively knock out α7-nAChRs in either the accessory occulomotor nucleus or in the CG. Achieving a sufficient reduction in receptor numbers to see an effect with either of these techniques might be difficult, but either of these approaches, perhaps coupled with an α7-nAChR overexpression, might give the desired results. Alternatively, we could look for candidate genes that are upregulated or downregulated with stimulation of α7-nAChRs *in vitro*, then determine whether they are also altered in α-btx-treated embryos. Finally, we would manipulate these signal transduction molecules *in vitro* and *in vivo* to determine whether they rescue neurons or exacerbate cell death, as well as to see if they block the α-btx rescue.

In other future studies, it would be interesting to ask what causes the dramatic increase in SV-2 staining we saw in the iris following α-btx administration. The simplest explanation for the effect of α-btx on presynaptic terminal size is that the lack of receptor activation on the muscle α1-nAChRs mimics lack of innervation. Conceivably, the muscle may normally secrete a factor that promotes synaptogenesis. Innervation by the nerve would cause this factor to be downregulated, which normally would end the period of synapse growth. Blocking α1-nAChRs would keep this factor from being downregulated and result in large clusters of synaptic vesicles. Conversely, the opposite could be true instead, that innervation causes a release of a factor that limits synapse size.

To identify this putative factor we would need a bioassay to detect its effect and, ideally, an automated way to screen for the effect. Then, since α-btx is reported to increase nerve branching and synaptogenesis in skeletal muscle, it may be possible to make a cDNA library from a large source of skeletal muscle, such as pectoral muscle. A library in a viral vector would permit infection of cultures plated near clonal density, and individual cultures would be used in our bioassay and screened for the SV-2 effect. Finally, a subtractive PCR approach would allow us to identify transcripts present in control- but not α-btx-treated tissue and vice versa.

In summary, these studies eliminate several long-standing hypotheses about the regulation of cell death in the avian CG: that the rescue by nAChR antagonists is mediated through receptors at the neuromuscular junction, that this rescue is caused by an increase in the synthesis and/or release of endogenous neurotrophic factors, and that the rescue is driven by an increase in nerve branching and synaptogenesis at the target that allows greater uptake of target-derived trophic factors. Instead, our studies reveal that the rescue is mediated by neuronal α7-nAChRs. It will be of interest to determine what role these receptors play in regulating normal cell death, as well as to determine whether similar mechanisms are in operation in other neuronal populations.

REFERENCES

Anderson, M. J., Waxman, S. G., and Tadlock, C. H. (1984). Cell death of asynaptic neurons in regenerating spinal cord. Dev Biol *103*, 443-55.

Angeletti, P., and Vigneti, E. (1971). Assay of nerve growth factor (NGF) in subcellular fractions of peripheral tissues by micro complement fixation. Brain Res *33*, 601-604.

Barde, Y.-A. (1989). Trophic factors and neuronal survival. Neuron *2*, 1525-1534.

Barde, Y. A. (1988). What, if anything, is a neurotrophic factor? Trends Neurosci *11*, 343-6.

Bennett, M. R., and White, W. (1979). The survival and development of cholinergic neurons in potassium-enriched media. Brain Res. *173*, 549-553.

Berger, F., Gage, F. H., and Vijayaraghavan, S. (1998). Nicotinic receptor-induced apoptotic cell death of hippocampal progenitor cells. J Neurosci *18*, 6871-81.

Blumenthal, E. M., Shoop, R. D., and Berg, D. K. (1999). Developmental changes in the nicotinic responses of ciliary ganglion neurons. J Neurophysiol *81*, 111-20.

Boyd, R. T., Jacob, M. H., Couturier, S., Ballivet, M., and Berg, D. K. (1988). Expression and regulation of neuronal acetylcholine receptor mRNA in chick ciliary ganglia. Neuron *1*, 495-502.

Bueker, E. D. (1948). Implantations of tumors in the hind limb field of embryonic chick and the developmental response of the lombosacral nervous system. Anat. Rec. *102*, 369-390.

Caldero, J., Prevette, D., Mei, X., Oakley, R. A., Li, L., Milligan, C., Houenou, L., Burek, M., and Oppenheim, R. W. (1998). Peripheral target regulation of the development and survival of spinal sensory and motor neurons in the chick embryo. J Neurosci *18*, 356-70.

Carr, C. E., Maler, L., and Sas, E. (1982). Peripheral organization and central projections of the electrosensory nerves in gymnotiform fish. J Comp Neurol *211*, 139-53.

Chalazonitis, A., and Fischbach, G. D. (1980). Elevated potassium induces morphological differentiation of dorsal root ganglionic neurons in dissociated cell culture. Dev Biol *78*, 173-83.

Chang, K. T., and Berg, D. K. (1999). Nicotinic acetylcholine receptors containing alpha7 subunits are required for reliable synaptic transmission in situ. J Neurosci *19*, 3701-3710.

Chiappinelli, V. A., and Zigmond, R. E. (1978). Alpha-bungarotoxin blocks nicotinic transmission in the avian ciliary ganglion. Proc. Natl. Acad. Sci. USA *75*, 2999-3003.

Chu-Wang, I.-W., and Oppenheim, R. W. (1978). Cell death of motoneurons in the chick embryo spinal cord: I. A light and electron microscopic study of naturally ocurring and induced cell loss during development. J. Comp. Neurol. *177*, 33-58.

Chun, L. L., and Patterson, P. H. (1977a). Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production. J Cell Biol *75*, 694-704.

Chun, L. L., and Patterson, P. H. (1977b). Role of nerve growth factor in the development of rat sympathetic neurons in vitro. II. Developmental studies. J Cell Biol *75*, 705-11.

Chun, L. L., and Patterson, P. H. (1977c). Role of nerve growth factor in the development of rat sympathetic neurons in vitro. III. Effect on acetylcholine production. J Cell Biol *75*, 712-8.

Coggan, J. S., Paysan, J., Conroy, W. G., and Berg, D. K. (1997). Direct recording of nicotinic responses in presynaptic nerve terminals [In Process Citation]. J Neurosci *17*, 5798-806.

Cohen, S. (1960). Purification and metabolic effects of a nerve growth-promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. Proc. Nat. Acad. Sci. (USA) *46*, 302-311.

Cohen, S. (1959). Purification and metabolic effects of a nerve-growth promoting protein from snake venom. J. Biol. Chem. *234*, 1129-1137.

Cohen, S., and Levi-Montalcini, R. (1956). A nerve growth stimulating factor isolated from snake venom. Proc. Natl. Acad. Sci. USA *42*, 571-574.

Cohen, S., Levi-Montalcini, R., and Hamburger, V. (1954). A nerve-growth stimulating factor isolated from sarcomas 37 and 180. Proc. Nat. Acad. Sci. (USA) *40*, 1014-1018.

Collins, F., Schmidt, M. F., Guthrie, P. B., and Kater, S. B. (1991). Sustained increase in intracellular calcium promotes neuronal survival. J Neurosci *11*, 2582-7.

Corriveau, R. A., and Berg, D. K. (1994). Neurons in culture maintain acetylcholine receptor levels with far fewer transcripts than in vivo. J Neurobiol *25*, 1579-92.

Corriveau, R. A., Romano, S. J., Conroy, W. G., Oliva, L., and Berg, D. K. (1995). Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. J Neurosci *15*, 1372-83.

Coulombe, J. N., and Nishi, R. (1991). Stimulation of somatostatin expression in developing ciliary ganglion neurons by cells of the choroid layer. J. Neurosci. *11*, 553-562.

Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P., and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid cell-conditioned medium. Neuron *10*, 899-906.

Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Ballivet, M. (1990). A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron *5*, 847-56.

Cowan, W., and Clarke, P. (1976). The development of the isthmo-optic nucleus. Brain Behav Evol *13*, 345-375.

Dahm, L. M., and Landmesser, L. T. (1991). The regulation of synaptogenesis during normal development and following activity blockade. J Neurosci *11*, 238-55.

Darland, D. C., Link, B. A., and Nishi, R. (1995). Activin A and follistatin expression in developing targets of ciliary ganglion neurons suggests a role in regulating neurotransmitter phenotype. Neuron *15*, 857-866.

Darland, D. C., and Nishi, R. (1998). Activin A and follistatin influence expression of somatostatin in the ciliary ganglion in vivo. Dev Biol *202*, 293-303.

Davies, A. M. (1988). Role of neurotrophic factors in development. Trends Gen. *4*, 139-143.

DeStefano, M., Ciofi, L. A., and Mugnaini, E. (1993). Neuronal ultrastructure and somatostatin immunolocalization in the ciliary ganglion of chicken and quail. J Neurocytol *22*, 868-92.

Distefano, P. S. (1993). Neurotrophic Factors in the Treatment of Motor Neuron Disease and Trauma. Exp Neurol *124*, 56-59.

Drachman, D. (1972). Neurotrophic regulation of muscle cholinesterase: effects of botulinum toxin and denervation. J Physiol *226*, 619-27.

Dreyer, E. B., Kaiser, P. K., Offermann, J. T., and Lipton, S. A. (1990). HIV-1 coat protein neurotoxicity prevented by calcium channel antagonists. Science *248*, 364-7.

Dryer, S. E., and Chiappinelli, V. A. (1985). Properties of choroid and ciliary neurons in the avian ciliary ganglion and evidence for substance P as a neurotransmitter. J Neurosci *5*, 2654-61.

Eckenstein, F. P., Esch, F., Holbert, T., Blacher, R. W., and Nishi, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. Neuron *4*, 623- 631.

Eckenstein, F. P., Kuzis, K., Nishi, R., Woodward, W. R., Meshul, C., Sherman, L., and Ciment, G. (1994). Cellular Distribution, Subcellular Localization and Possible Functions of Basic and Acidic Fibroblast Growth Factors. Biochem Pharmacol *47*, 103-110.

Eckenstein, F. P., Shipley, G. D., and Nishi, R. (1991). Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve. J. Neurosci. *11*, 412-419.

Epstein, M. L., Davis, J. P., Gellman, L. E., Lamb, J. R., and Dahl, J. L. (1988). Cholinergic neurons of the chicken ciliary ganglion contain somatostatin. Neuroscience *25*, 1053-1060.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M., and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. Science *256*, 1555-60.

Finn, T. P., Kim, S., and Nishi, R. (1998). Overexpression of ciliary neurotrophic factor in vivo rescues chick ciliary ganglion neurons from cell death. J Neurobiol *34*, 283-93.

Finn, T. P., and Nishi, R. (1996a). Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell-death phase. J Comp Neurol *366*, 559-571.

Fox, G. Q., Richardson, G. P., and Kirk, C. (1985). Torpedo electromotor system development: neuronal cell death and electric organ development in the fourth branchial arch. J Comp Neurol *236*, 274-81.

Fraser, D. D., Hoehn, K., Weiss, S., and MacVicar, B. A. (1993). Arachidonic acid inhibits sodium currents and synaptic transmission in cultured striatal neurons. Neuron *11*, 633-44.

Furber, S., Oppenheim, R. W., and Prevette, D. (1987). Naturally-occurring neuron death in the ciliary ganglion of the chick embryo following removal of preganglionic input: evidence for the role of afferents in ganglion cell survival. J Neurosci *7*, 1816-32.

Furber, S. E. (1984). A Golgi study of the development of the inferior olivary nuclear complex in the chicken. J Comp Neurol *225*, 244-58.

Greene, L. A. (1977). Quantitative *in vitro* studies on the nerve growth factor (NGF) requirement of neurons
I. Sympathetic Neurons. Dev. Biol. *58*, 96-105.

Hamburger, V. (1975). Cell death in the development of the lateral motor column of the chick embryo. J Comp Neurol *160*, 535-46.

Hamburger, V. (1934). The effects of wing bud extirpation on the development of the central nervous system in chick embryos. J. Exp. Zool. *68*, 449-494.

Hamburger, V. (1939). Motor and sensory hyperplasia following limb-bud transplantations in chick embryos. J. Exp. Zool. *80*, 347-389.

Hamburger, V. (1958). Regression versus peripheral control of differentiation in motor hypoplasia. A. J. Anat. *102*, 365-409.

Hamburger, V., Brunso-Bechtold, J. K., and Yip, J. W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J. Neurosci. *1*, 60-71.

Hamburger, V., and Levi-Montalcini, R. (1949). Proliferation, differentiation, and degeneration in the spinal ganglia of the chick embryo under normal and experimantal conditions. J. Exper. Zool. *111*, 457-501.

Harish, O. E., and Poo, M. M. (1992). Retrograde modulation at developing neuromuscular synapses: involvement of G protein and arachidonic acid cascade. Neuron *9*, 1201-9.

Hollyday, M., and Hamburger, V. (1976). Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. J Comp Neurol *170*, 311-20.

Hollyday, M., Hamburger, V., and Farris, J. M. (1977). Localization of motor neuron pools supplying identified muscles in normal and supernumerary legs of chick embryo. Proc Natl Acad Sci U S A *74*, 3582-6.

Hory-Lee, F., and Frank, E. (1995). The nicotinic blocking agents d-tubocurare and alpha-bungarotoxin save motoneurons from naturally occurring death in the absence of neuromuscular blockade. J Neurosci *15*, 6453-60.

Houenou, L. J., Haverkamp, L. J., McManaman, J. L., and Oppenheim, R. W. (1991). The regulation of motoneuron survival and differentiation by putative muscle-derived neurotrophic agents: neuromuscular activity and innervation. Development *2*, 149-55.

Kater, S. B., Mattson, M. P., and Guthrie, P. B. (1989). Calcium-induced neuronal degeneration: a normal growth cone regulating signal gone awry (?). Ann N Y Acad Sci *568*, 252-61.

Keyser, D. O., and Alger, B. E. (1990). Arachidonic acid modulates hippocampal calcium current via protein kinase C and oxygen radicals. Neuron *5*, 545-53.

Kim, D., and Clapham, D. E. (1989). Potassium channels in cardiac cells activated by arachidonic acid and phospholipids. Science *244*, 1174-6.

Kimble, J., and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Dev Biol *70*, 396-417.

Lamb, A. H. (1984). Motoneuron death in the embryo. Crc Crit Rev Clin Neurobiol *1*, 141-79.

Lamb, A. H. (1980). Motoneurone counts in Xenopus frogs reared with one bilaterally-innervated hindlimb. Nature *284*, 347-350.

Landmesser, L. (1978). The development of motor projection patterns in the chick hind limb. J Physiol (Lond) *284*, 391-414.

Landmesser, L. (1992). The relationship of intramuscular nerve branching and synaptogenesis to motoneuron survival. J Neurobiol *23*, 1131-9.

Landmesser, L., Dahm, L., Schultz, K., and Rutishauser, U. (1988). Distinct roles for adhesion molecules during innervation of embryonic chick muscle. Dev Biol *130*, 645-70.

Landmesser, L., and Pilar, G. (1976). Fate of ganglionic synapses and ganglion cell axons during normal and induced cell death. J Cell Biol *68*, 357-74.

Landmesser, L., and Pilar, G. (1978). Interactions between neurons and their targets during in vivo synaptogenesis. Fed Proc *37*, 2016-2022.

Landmesser, L., and Pilar, G. (1972). The onset and development of transmission in the chick ciliary ganglion. J Physiol (Lond) *222*, 691-713.

Landmesser, L., and Pilar, G. (1974a). Synapse formation during embryogenesis on ganglion cells lacking a periphery. J. Physiol. *241*, 715-736.

Landmesser, L., and Pilar, G. (1974b). Synaptic transmission and cell death during normal ganglionic development. J. Physiol. *241*, 737-749.

Lasher, J., and Zagon, R. (1972). The effect of potassium on neuronal differentiation in cutlures of dissociated newbon rat cerebellum. Brain Res. *41*, 482-488.

Lee, V. M., Smiley, G. G., and Nishi, R. (2001). Cell death and neuronal replacement during formation of the avian ciliary ganglion. Dev Biol *233*, 437-48.

Levi-Montalcini, R. (1949). The development of the acoustico-vestibular centers in the chick embryo in the absence of afferent root fiber and of descending fiber tracts. J. Comp. Neurol. *91*, 209-241.

Levi-Montalcini, R. (1972). The morphological effects of immunosympathectomy. In Immunosympathectomy, G. Steiner and E. Schonbaum, eds. (Amsterdam: Elsevier), pp. 55-78.

Levi-Montalcini, R. (1947). Regressione secondaria del ganglio ciliare dopo aportazione della vesicola mesencefalica in embrione di pollo. R Acad Naz Lincei *3*, 144-146.

Levi-Montalcini, R., Aloe, L., Mugnaini, E., Oesch, F., and Thoenen, H. (1975). Nerve growth factor induces volume increase and enhances tyrosine hydroxylase synthesis in chemically axotomized sympathetic ganglia of newborn rats. Proc Natl Acad Sci U S A *72*, 595-599.

Levi-Montalcini, R., and Booker, B. (1960). Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals. Proc. Natl. Acad. Sci. USA *85*, 324-341.

Levi-Montalcini, R., and Cohen, S. (1956). In vitro and in vivo effects of a nerve growth-stimulating agent isolated from snake venom. Proc. Natl. Acad. Sci. USA *42*, 695-699.

Levi-Montalcini, R., and Hamburger, V. (1953). Effects of mouse tumor transplantation in the nervous system. Ann. NY Acad. Sci. *55*, 330-343.

Linden, R. (1994). The survival of developing neurons: a review of afferent control. Neuroscience *58*, 671-82.

Link, B. L., and Nishi, R. (1998b). Development of the avian iris and ciliary body: mechanisms of cellular differentiation during the smooth-to-striated muscle transition. Devel. Biol. *203*, 163-176.

Lipton, S. A. (1986). Blockade of electrical activity promotes the death of mammalian retinal ganglion cells in culture. Proc Natl Acad Sci U S A *83*, 9774-8.

Lockshin, R., and Williams, C. (1965). Programmed cell death. II. endocrine potentiation of the breakdown of the intersegmental muscles of silkworms. J. Insect Physiol. *11*, 803-809.

Margiotta, J. F., and Gurantz, D. (1989). Changes in the number, function, and regulation of nicotinic acetylcholine receptors during neuronal development. Dev Biol *135*, 326-39.

Marwitt, G. R., Pilar, G., and Weakly, J. N. (1971). Characterization of two cell populations in the avian ciliary ganglion. Brain Res. *25*, 317-334.

McEachern, A. E., Margiotta, J. F., and K., B. D. (1985). Gamma-aminobutyric acid receptors on chick ciliary ganglion neurons *in vivo* and in cell culture. J. Neurosci. *5*, 2690-2695.

McGehee, D. S., and Role, L. W. (1995). Physiological diversityof nicotinic acetylcholine receptors expressed by vertebrate neurons. [Review]. Ann Rev Physiol *57*, 521-546.

Meriney, S. D., Ford, M. J., Oliva, D., and Pilar, G. (1991). Endogenous opioids modulate neuronal survival in the developing avian ciliary ganglion. J Neurosci *11*, 3705-3717.

Meriney, S. D., and Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid coat of the chick eye and its development. J. Neurosci. *7*, 3827-3839.

Meriney, S. D., and Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid coat of the chick eye and its development. J Neurosci *7*, 3827-3839.

Meriney, S. D., Pilar, G., Ogawa, M., and Nunez, R. (1987). Differential neuronal survival in the avian ciliary ganglion after chronic acetylcholine receptor blockade. J. Neurosci. *7*, 3840-3849.

Murphy, S. N., Thayer, S. A., and Miller, R. J. (1987). The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons in vitro. J Neurosci *7*, 4145-58.

Narayanan, C. H., and Narayanan, Y. (1978). Neuronal adjustments in developing nuclear centers of the chick embryo following transplantation of an additional optic primordium. J. Embryol. exp. Morph. *44*, 53-70.

Nishi, R. (1996). Autonomic and sensory neuron cultures. Methods Cell Biol *51*, 249-263.

Nishi, R., and Berg, D. K. (1981a). Effects of high K+ concentrations on the growth and development of ciliary ganglion neurons in cell culture. Dev. Biol. *87*, 301-307.

O'Brien, M. K., Landmesser, L., and Oppenheim, R. W. (1990). Development and survival of thoracic motoneurons and hindlimb musculature following transplantation of the thoracic neural tube to the lumbar region in the chick embryo: functional aspects. J Neurobiol *21*, 341-55.

O'Leary, D., and Cowan, W. (1984). Survival of isthmo-optic neurons after early removal of one eye. Brain Res *314*, 293-310.

Oppenheim, R. (1987). Muscle activity and motor neuron death in the spinal cord of the chick embryo. Ciba Found Symp *126*, 96-112.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. Annu Rev Neurosci *14*, 453-501.

Oppenheim, R. W. (1981). Cell death of motoneurons in the chick embryo spinal cord. V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. J Neurosci *1*, 141-51.

Oppenheim, R. W. (1984). Cell death of motoneurons in the chick embryo spinal cord. VIII. Motoneurons prevented from dying in the embryo persist after hatching. Dev Biol *101*, 35-9.

Oppenheim, R. W. (1985). Naturally occurring cell death during neural development. Trends Neurosci. *8*, 487-493.

Oppenheim, R. W. (1981). Neuronal cell death and some related regressive phenomena during neurogenesis: a selective historical review and progress report. In Studies in Developmental Neurobiology, W. M. Cowan, ed. (Oxford: Oxford University Press).

Oppenheim, R. W. (1989). The neurotrophic theory and naturally ocurring motoneuron death. Trends Neurosci *12*, 252-255.

Oppenheim, R. W., Bursztajn, S., and Prevette, D. (1989). 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. Development *107*, 331-41.

Oppenheim, R. W., Haverkamp, L. J., Prevette, D., McManaman, J. L., and Appel, S. H. (1988). Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor. Science *240*, 919-22.

Oppenheim, R. W., Prevette, D., D'Costa, A., Wang, S., Houenou, L. J., and McIntosh, J. M. (2000). Reduction of neuromuscular activity is required for the rescue of motoneurons from naturally occurring cell death by nicotinic-blocking agents. J Neurosci *20*, 6117-6124.

Oppenheim, R. W., Prevette, D., Houenou, L. J., Pincon-Raymond, M., Dimitriadou, V., Donevan, A., O'Donovan, M., Wenner. P, Mckemy, D. D., and Allen, P. D. (1997). Neuromuscular development in the avian paralytic mutant crooked neck dwarf (cn/cn): further evidence for the role of neuromuscular activity in motoneuron survival. J Comp Neurol 1997 *381*, 353-372.

Ordway, R. W., Singer, J. J., and Walsh, J. V., Jr. (1991). Direct regulation of ion channels by fatty acids. Trends Neurosci *14*, 96-100.

Ordway, R. W., Walsh, J. V., Jr., and Singer, J. J. (1989). Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells. Science *244*, 1176-9.

Phillipson, O. T., and Sandler, M. (1975). The influence of nerve growth factor, potassium depolarization and dibutyryl (cyclic) adenosine 3',5'-monophosphate on explant cultures of chick embryo sympathetic ganglia. Brain Res *90*, 273-81.

Pilar, G., Freyer, H. J., Meriney, S. D., and Ford, M. J. (1988). The regulation of neuronal number during embryonic chick development. P R Health Sci J *7*, 149-55.

Pilar, G., Landmesser, L., and Burstein, L. (1980). Competition for survival among developing ciliary ganglion cells. J. Neurophysiol. *43*, 233-254.

Pilar, G., Nunez, R., McLennan, I. S., and Meriney, S. D. (1987). Muscarinic and nicotinic synaptic activation of the developing chicken iris. J Neurosci *7*, 3813-26.

Piomelli, D., and Greengard, P. (1990). Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling. Trends Pharmacol Sci *11*, 367-73.

Pittman, R., and Oppenheim, R. W. (1979). Cell death of motoneurons in the chick embryo spinal cord. IV. Evidence that a functional neuromuscular interaction is involved in the regulation of naturally occurring cell death and the stabilization of synapses. J Comp Neurol *187*, 425-46.

Prestige, M. C. (1976). Evidence that at least some of the motor nerve cells that die during development have first made peripheral connections. J Comp Neurol *170*, 123-33.

Pugh, P. C., and Berg, D. K. (1994). Neuronal acetylcholine receptors that bind alpha-bungarotoxin mediate neurite retraction in a calcium-dependent manner. J Neurosci *14*, 889-96.

Ravdin, P. M., Nitkin, R. M., and Berg, D. K. (1981). Internalization of alpha-bungarotoxin on neurons induced by a neurotoxin that blocks neuronal acetylcholine sensitivity. J Neurosci *1*, 849-61.

Renshaw, G., Rigby, P., Self, G., Lamb, A., and Goldie, R. (1993). Exogenously administered alpha-bungarotoxin binds to embryonic chick spinal cord: implications for the toxin-induced arrest of naturally occurring motoneuron death. Neuroscience *53*, 1163-72.

Renshaw, G. M. (1994). [125I]-alpha-bungarotoxin binding co-varies with motoneurone density during apoptosis. Neuroreport *5*, 1949-52.

Role, L. W. (1988). Neural regulation of acetylcholine sensitivity in embryonic sympathetic neurons. Proc Natl Acad Sci U S A *85*, 2825-9.

Rothman, S. M., Thurston, J. H., and Hauhart, R. E. (1987). Delayed neurotoxicity of excitatory amino acids in vitro. Neuroscience *22*, 471-80.

Sargent, P. B. (1993). The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci *16*, 403-43.

Scapolo, P. A., Peirone, S. M., Filogamo, G., and Veggetti, A. (1988). Histochemical, immunohistochemical, and ultrastructural observations on the iris muscles of Gallus gallus. Anat Rec *221*, 687-99.

Schweitzer, P., Madamba, S., and Siggins, G. R. (1990). Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current. Nature *346*, 464-7.

Scott, B. S. (1977). The effect of elevated potassium on the time course of neuron survival in cultures of dissociated dorsal root ganglia. J Cell Physiol *91*, 305-16.

Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J. A., and Patrick, J. W. (1993). Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci *13*, 596-604.

Sekhon, H. S., Jia, Y., Raab, R., Kuryatov, A., Pankow, J. F., Whitsett, J. A., Lindstrom, J., and Spindel, E. R. (1999). Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. J Clin Invest *103*, 637-47.

Sha, W. C., Nelson, C. A., Newberry, R. D., and Kranz, D. M. (1988). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature, 73-76.

Shimada, T., and Somlyo, A. P. (1992). Modulation of voltage-dependent Ca channel current by arachidonic acid and other long-chain fatty acids in rabbit intestinal smooth muscle. J Gen Physiol *100*, 27-44.

Shoop, R., Chang, K., Ellisman, M., and Berg, D. (2001). Synaptically driven calcium transients via nicotinic receptors on somatic spines. J Neurosci *21*, 771-81.

Shorey, M. L. (1909). The effect of the destruction of peripheral areas on the differentiation of the neuroblasts. J. Exp. Zool. *7*, 25-63.

Sohal, G. S., Creazzo, T. L., and Oblak, T. G. (1979). Effects of chronic paralysis with alpha-bungarotoxin on development of innervation. Exp Neurol *66*, 619-28.

Sperry, D. G., and Grobstein, P. (1985). Regulation of neuron numbers in Xenopus laevis: effects of hormonal manipulation altering size at metamorphosis. J Comp Neurol *232*, 287-98.

Sulston, J. (1983). Neuronal cell lineages in the nematode Caenorhabditis elegans. Cold Spring Harb Symp Quant Biol *48*, 443-52.

Sulston, J., and Horvitz, H. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol *56*, 110-56.

Tanaka, H. (1987). Chronic application of curare does not increase the level of motoneuron survival-promoting activity in limb muscle extracts during the naturally occurring motoneuron cell death period. Dev Biol *124*, 347-57.

Tanaka, H., and Landmesser, L. T. (1986). Cell death of lumbosacral motoneurons in chick, quail, and chick-quail chimera embryos: a test of the quantitative matching hypothesis of neuronal cell death. J Neurosci *6*, 2889-99.

Tang, J., and Landmesser, L. (1993). Reduction of intramuscular nerve branching and synaptogenesis is correlated with decreased motoneuron survival. J Neurosci *13*, 3095-103.

Tata, J. R. (1966). Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev. Biol. *13*, 77-94.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., and Uematsu, Y. (1988). Thymic major histocompatability antigens and the a-b T cell receptor determine the CD4/CD8 phenotype of T cells. Nature, 229-35.

Trueb, B., Schreier, T., Winterhalter, K. H., and Strehler, E. E. (1988). Sequence of a cDNA clone encoding chicken ribosomal protein S17. Nucleic Acids Res *16*, 4723.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M., and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes [see comments]. Cell *79*, 957-70.

Usiak, M. F., and Landmesser, L. T. (1999). Neuromuscular activity blockade induced by muscimol and d-tubocurarine differentially affects the survival of embryonic chick motoneurons. J Neurosci *19*, 7925-39.

Varon, S., Raiborn, C., and Tyszka, E. (1973). In vitro studies of dissociated cells from newborn mouse dorsal root ganglia. Brain Res *54*, 51-63.

Vaux, D. L., and Korsmeyer, S. J. (1999). Cell death in development. Cell *96*, 245-54.

Vijayaraghavan, S., Huang, B., Blumenthal, E. M., and Berg, D. K. (1995). Arachidonic acid as a possible negative feedback inhibitor of nicotinic acetylcholine receptors on neurons. J Neurosci *15*, 3679-87.

Vogt, C. (1842). Untersuchungen uber die Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstertricians), J. u. Gassman, ed. (Solothurn, Switzerland.

Volpe, P., Biral, D., Pizzo, P., Salviati, G., and Margreth, A. (1993). Ontogenesis of chick iris intrinsic muscles: evidence for a smooth-to-striated muscle transition. Dev Biol *159*, 441-9.

Walicke, P. A., Feige, J. J., and Baird, A. (1989). Characterization of the neuronal receptor for basic fibroblast growth factor and comparison to receptors on mesenchymal cells. J Biol Chem *264*, 4120-6.

West, M. J., Slomianka, L., and Gundersen, H. J. G. (1991). Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the Optical Fractionator. Anat. Rec. *231*, 482-497.

Yang, X., Kuo, Y., Devay, P., Yu, C., and Role, L. (1998). A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis [published erratum appears in Neuron 1998 Apr;20(4):823]. Neuron *20*, 255-70.

Zhang, Z. W., Coggan, J. S., and Berg, D. K. (1996). Synaptic currents generated by neuronal acetylcholine receptors sensitive to alpha-bungarotoxin. Neuron *17*, 1231-40.