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# Acute and long-term effects of botulinum neurotoxin on the function and structure of developing extraocular muscles

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**Strabismus is a misalignment of the visual axes, due to an imbalance in extraocular muscle (EOM) function. Botulinum neurotoxin (BoNT) treatment can correct the misalignment with permanent therapeutic effects in infants, possibly because the toxin causes structural alterations in developing EOM. To determine whether BoNT indeed permanently weakens developing EOMs, we examined the chicken oculomotor system. Following injections of BoNT in hatchling chicks, we quantified physiological parameters (contractile force measurements) and morphological parameters (myofiber morphometry, innervation, quantitative transmission electron microscopy of mitochondria/fiber types). Treatment of developing EOM with BoNT caused acute reductions of muscle strength and mitochondrial densities, but minimal changes in muscle fiber diameter and neuromuscular junction structures. Contrary to expectations, contractile force was fully recovered by 3–4 months after treatment. Thus, permanent therapeutic effects of BoNT most likely do not cause permanent changes at the level of the peripheral effector organ, but rather involve central (CNS) adaptive responses.**


*Keywords:* Oculomotor; Motor endplate; Neuromuscular junction; Eye muscle; Contractile force; Mitochondria; Botulinum toxin; Strabismus

## Introduction

Strabismus is a misalignment of the visual axes, due to an imbalance in extraocular muscle (EOM) function. Precise muscle force regulation is required to align the eyes for coordination of functional and binocular vision. Failure to maintain proper visual alignment results in a condition known as strabismus. Strabismus is one of the most prevalent ophthalmic disorders, affecting nearly 5% of the human population (Magramm, 1992; Stidwill, 1997; Tychsen, 2005). Infantile (pediatric or congenital) strabismus begins during the first year of life (Tychsen, 2005). Early treatment of infantile strabismus is particularly important because visual alignment is required for proper binocular vision development and stereopsis (Hubel and Wiesel, 1965; Ing, 1981; Fenstemaker et al., 2001; Kind et al., 2002). Eye misalignment during this critical period can disrupt binocular fusion and may lead to amblyopia (permanent loss of vision in one eye) through the neural mechanism of suppression (Birch et al., 1990).

In the early 1970s, Scott and colleagues pioneered the use of botulinum neurotoxin (BoNT) as an effective alternative to conventional strabismus surgery (Scott et al., 1973). Since this time, BoNT has been effectively used for eye realignment in children and adults, but its use has been limited due to its relatively short duration of action, which often necessitates reinjection or transition to incisional surgery (Scott, 1980; Lee et al., 1988; Biglan et al., 1989; Ing, 1993; Tejedor and Rodriguez, 2001). In contrast, the application of BoNT in the treatment of pediatric strabismus, particularly infantile esotropia, has demonstrated permanent therapeutic results, but the effectiveness is related to the age at which the treatment is performed. Administration before the critical age of 7 months resulted in the lowest recurrence of esodeviation, whereas treatment after the age of 8 months did not provide the expected "stable" outcome and required additional applications (Magoon, 1989; Campos et al., 2000; McNeer et al., 2000, 2003).

These results suggest that the toxin may have permanent muscle-weakening effects in developing EOM (Campos et al., 2000). However, it is also possible that the toxin-induced changes are transient and the lasting therapeutic effects may involve central adaptive responses that "self-adjust" to maintain the stable binocular state (Campos et al., 2000; Tychsen, 2005). Although about 300 million people worldwide would benefit from BoNT treatment for eye alignment during their first year of life, the principal mechanism of action of this therapy in the oculomotor system is unknown, and the physiological and structural effects of the toxin during the relevant early phase of EOM development have not been examined. While a primate model system would theoretically be optimal for this task, it is prohibitively expensive to use adequate numbers of monkeys necessary for conclusive statistical analyses (e.g., Spencer and McNeer, 1987; Spencer et al., 1992). Mice and other rodents, while less costly, have relatively small eyes and make it technically difficult to manipulate EOMs for contractile force measurements. To conclusively determine BoNT's effects during development, an additional model system is needed that is both cost-effective and

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provides an easily accessible EOM system that is similar to humans. Avian models are particularly well suited for this task because of their relatively large eyes, well developed eye muscles, accessibility during development, and an evolutionarily conserved extraocular motor system (Maier et al., 1972; Heaton and Wayne, 1983; Porter et al., 1995). Experiments utilizing chickens in developmental studies are faster and more cost-effective than comparable ones in mammals, allowing for sufficiently large "*n*" values. Although chickens are lateral-eyed animals, they are a valuable experimental model to obtain precise information on EOM force, in particular during critical periods of visual and oculomotor system development. Here we determined in a chicken animal model whether BoNT permanently alters structural and/or physiological parameters of developing EOMs. Our study, the first comprehensive functional and structural analysis of BoNT's long-term effects in the oculomotor system, indicates that the success of BoNT treatment does not involve long-term peripheral alterations at the level of the EOM.

## Materials and methods

### *Materials*

Fertilized White Leghorn chicken eggs were obtained from a local supplier (California Golden Eggs, Inc.) and incubated in a humidified force-draft incubator at 37–38°C. Date of hatching was designated post-hatch day 0 (P0). A total of about 260 chickens were used for this study. Animals were housed in a brooder with controlled temperature (23–25°C) on a 12 h light/dark cycle and were provided chicken feed and water ad libitum. Experimental procedures described in this study were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno. Botulinum neurotoxin type A (BoNT; 150 kDa) was supplied by List Biological Laboratories Inc. (Campbell, CA; Product 130A). Primary antibodies were monoclonal anti-neurofilament 200 and anti-neurofilament 68 from Sigma (St. Louis, MO), and synaptic vesicle antibody SV2 (supernatant) from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA). Biotinylated secondary antibody (horse anti-mouse) was from Vector Laboratories (Burlingame, CA). Tetramethylrhodamine-conjugated α-bungarotoxin (Rh-αBTX) and Alexa 488 were from Molecular Probes (Eugene, OR).

### *Botulinum neurotoxin preparation*

Botulinum neurotoxin type A (BoNT) was diluted to a concentration of 2.0 ng/μl in sterile phosphate-buffered saline (PBS) containing bovine serum albumin (BSA, 1.0 mg/ml). The BSA was added to insure maximum recovery and to minimize nonspecific loss of toxin potency during handling. Aliquots were stored at −80°C until use. Reconstituted BoNT has previously been shown to maintain potency if refrozen or refrigerated for 2 weeks before use (Sloop et al., 1997). Similarly, we found no evidence of decreased potency of toxin preparations frozen for up to 4 months before use. Prior to administration, BoNT was thawed and diluted in sterile PBS to give a final injection dose of 0.25 ng or 0.50 ng in a volume of 10 μl.

### *Determination of botulinum neurotoxin dose*

The primary method of measurement for BoNT potency is based on units. A unit is defined as the amount of toxin that is lethal in 50% ($LD_{50}$) of female Swiss Webster mice (body mass ~20 g) following intraperitoneal (IP) injection (Schantz and Johnson, 1992; Aoki, 2001; Kedlaya, 2004). Various commercial preparations of BoNT differ in their unit value: BOTOX®, 0.05 ng/U (Allergan, Inc.); DYSPORT®, 0.025 ng/U (Ipsen, Inc.); Oculinum®, 0.25 ng/U. Disparity in potency and the lack of correlation between the recommended doses have led to considerable confusion (Brin and Blitzer, 1993). List Biological Laboratories Inc., supplier of BoNT used in this study, does not specify a unit value or $LD_{50}$ for their product. To find the optimal dosage of BoNT for the present study, we used a regional chemodenervation assay modified from Pearce et al. (1995). At day of hatching (P0), increasing doses of toxin (0.125–3.0 ng) were injected into and above the superior oblique muscle (see Injection procedure, below). Four to five chicks were injected for each dose. Muscle paralysis was assessed, based on isometric twitch tension measurements (see Stimulation parameters, below), and the remaining force at 2 and 7 days post-injection (Fig. 1). Time duration was based on a previous study showing that both DYSPORT® and BOTOX® reached their maximal effect of muscle paralysis within 2 to 3 days, respectively (Pearce et al., 1995). Toxin activity for a given preparation was assessed by evaluating the percent decrease in maximum isometric twitch tension for the superior oblique muscle.

### *Injection procedure*

At day of hatching (P0) chicks were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, intramuscular) as assessed by the absence of withdrawal to digit pinch. The head was fixed in a stereotaxic frame and secured with a beak clamp. Using aseptic techniques, a dose of either 0.25 ng or 0.50 ng of BoNT (total volume: 10 μl) was injected into and above the belly of the superior oblique muscle. The thinness of the superior oblique muscle at this early stage of development necessitated partial delivery of the toxin to the muscle's exterior. BoNT has previously been shown to penetrate through muscle fascia, but spread is reduced by approximately 20–25% (Shaari et al., 1991). The toxin was delivered slowly (over 10 s) to allow diffusion of the toxin into the muscle and minimize leakage into the orbit (Christiansen et al., 2003). Control injections were identical to experimental injections, using an equivalent volume of sterile PBS. Preparations of BoNT and PBS

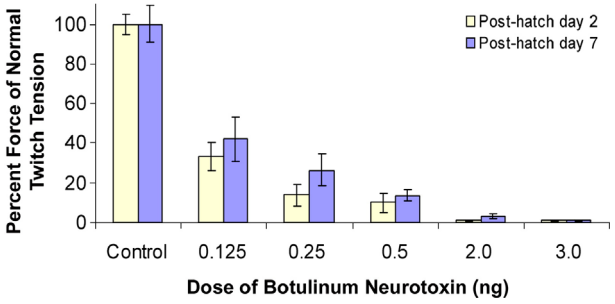


Fig. 1. Extent (severity) of extraocular muscle paralysis produced by increasing doses of botulinum neurotoxin (BoNT). On the day of hatching (P0), chicks were injected with a single dose of BoNT into and above the belly of the superior oblique muscle. The effect is shown as a function of dose and the percent of twitch tension is compared to controls (saline injected) for two time points: 2 days and 7 days after injection. For absolute values of force tension, see Fig. 2. Three to five chicks were used for each condition. Error bars = SEM.