Neuromuscular Junctions in Cerebral Palsy

Presence of Extrajunctional Acetylcholine Receptors

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Background: Cerebral palsy (CP) is the most prevalent neurologic disease in children. A primary deficit in CP is neuromuscular dysfunction; however, neuromuscular junctions in children with CP have not been studied. Evidence exists that up-regulation of acetylcholine receptors (AChRs) may be present in children with CP, and the current study was undertaken to examine this possibility.

Methods: Thirty-nine children with spastic CP and 25 neurologically normal children were enrolled in the study. Paraspinal muscles underwent biopsy during scheduled spinal fusion surgery. Two sets of assessments were performed on the biopsy specimens: (1) reverse-transcription polymerase chain reaction and Western blot analyses to evaluate the expression of the γ subunit of the AChR, and (2) histologic evaluation using a double-stain technique for AChR and acetylcholinesterase, wherein acetylcholinesterase staining defined the limits of the neuromuscular junction, and AChR staining that appeared outside of these limits indicated an abnormal distribution of AChRs.

Results: Reverse-transcription polymerase chain reaction and Western blot analyses showed that neither the CP nor non-CP samples had detectable γ-AChR subunit. Histologic analysis indicated that 11 of 39 children with CP and none of 20 children with idiopathic scoliosis scored positive for the presence of AChR outside of the neuromuscular junction (P = 0.0085).

Conclusion: A subset of children with CP have an abnormal distribution of AChR relative to the acetylcholinesterase found at the neuromuscular junction. The altered distribution of AChR in CP was not associated with a detectable presence of the γ-AChR subunit, suggesting that the nonjunctional AChRs in CP does not contain the γ subunit.

CEREBRAL palsy (CP) is a disorder of the central nervous system in which an insult sustained during fetal development or early postnatal life results in spasticity and motor dysfunction. The incidence rate for CP is approximately 0.7 per 1,000 live births. The prevalence of CP is increasing,† which is possibly related to the increasing number of infants who survive preterm delivery. Between 5 and 15% of roughly 40,000 preterm infants, weighing less than 1,500 g, develop major spastic motor deficits grouped under the rubric CP. Almost all patients with CP require surgical intervention and anesthesia from early life, so it is important to understand any aspects of CP that may affect the practice of anesthesia in these children.

An area for concern is the potential presence of abnormal neuromuscular junctions (NMJs) in patients with CP. Despite the high incidence of the disease and the involvement of the motor nervous system in the manifestation of the disease, the NMJs of children with CP have not been studied previously.‡ Based on indirect evidence obtained from studies of neuromuscular blocking agents, a possible up-regulation of nonjunctional acetylcholine receptors (AChRs) has been postulated in children with CP.§,‖

We conducted the current study to directly examine and compare NMJs in biopsy samples from children with and without CP. Our approach was to perform in vitro assessments on samples of erector spinae muscle taken during spinal fusion surgeries. Assessments included the determination of AChR subunit composition using molecular-biochemical assays and the detection of junctions by histologic staining. There were two null hypotheses in the study design: (1) there is no difference in the level of expression of γ-AChR subunits in muscles from children with CP when compared with muscles from children without CP; and (2) there is no difference between children with CP and normal children in the morphologic spread of AChRs in the NMJ.

Materials and Methods

Overall Design

The study design was prospective, nonrandomized, and partially blinded. Institutional review board approval was obtained for the study. Fetal tissue was used as control for reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting experiments. Per criteria established by the institutional review board, the fetal tissue was exempt from review as this tissue was from a preexisting sample without any patient identifiers.

Sixty-four children were enrolled in the study with parental consent. Thirty-nine children had stage 3 or 4 spastic quadriplegia,§ and 25 children were neurologically normal. The staging of children with CP was accomplished using clinical criteria, which are scored on a 5-point scale: 0 = normal with no neuromotor defect;
1 = minor impairment with evidence of neuromotor involvement, such as increased tendon reflexes, but no functional impairment; 2 = mild disability with only minor loss of function such as abnormal gait; 3 = moderate disability with some purposeful voluntary movement possible; and 4 = severe disability that prevents virtually all purposeful voluntary movement. All children were undergoing spinal fusion for scoliosis.

Biopsy material was obtained from the paraspinal muscles at the thoracolumbar junction on the concave side of the curve, as far away from the spine as possible. This site was chosen because the concave side is thought to be more affected and the thoracolumbar junction provides a consistent and easily identified area in all patients, despite their scoliosis. Muscle biopsy specimens (approximately 1 cm³) were placed in sterile specimen containers and immersed in wet ice immediately after surgical excision. The tissue was trimmed and mounted for transverse sectioning on small cork-chucks using 8% tragacanth gum at the base. Mounted samples were snap-frozen in N₂ chilled isopentane for 30 s and stored at −70°C until needed. Sections (8 μm thick) of the frozen material were taken for use in the histologic and biochemical assays described below.

It should be noted that the choice of the erector spinae muscle for this study was based on our need to obtain control muscles from otherwise healthy, age-matched children. Spinal fusion for the correction of scoliosis was the only surgical procedure conducted with sufficient frequency to allow the procurement of muscle samples from identical sites for both study and control groups of children.

**Exclusion Criteria**

Patient samples that lacked innervation sites, as judged by exhaustive sectioning and histologic staining, were excluded from the study. A total of five patients were excluded from the entire study by this criterion.

**Biochemical Determination of Acetylcholine Receptor Subunit Expression**

Two methods were used for the biochemical detection of AChR subunits. RT-PCR was conducted to determine the concentration of mRNA present for both the α and γ subunits. Western blotting was conducted to determine the concentration of γ-subunit protein present.

**Reverse-transcription Polymerase Chain Reaction.** Messenger RNA for the analysis of α and γ-AChR subunit expression was evaluated by RT-PCR. Samples of RNA were collected from sections of frozen material using kits from Qiagen (Valencia, CA) and analyzed using standard RT-PCR procedures from Promega (Madison, WI). Primers (Cruachem, Dulles, VA) specific for α-AChR (forward: dTCATCAAACACACACCGCTCAC; reverse: dCCATTGCAAGCTACTCCACTCTGC) and γ-AChR (forward: dAGCTGCTGAGATGACGTTC; reverse: dGCCCTTCTCTAGCTCTCCACG) resulted in the formation of a 531 base pair and a 199 base pair product, respectively, as verified by DNA sequence analysis. Single RT reactions were run, and the products were subjected to 30 cycles of PCR. The PCR products were visualized postelectrophoretically using ethidium bromide stain and an Eagle Eye Gel Documentation System (Stratagene, La Jolla, CA).

**Western Blotting.** Sections of frozen material were collected and solubilized in sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer containing Tris-HCl buffer, sodium dodecyl sulfate, glycercol, phenylmethylsulfonyl-fluoride, aminocaproic acid, benzamidine hydrochloride, and EDTA (all reagents from Sigma Chemical Co., St. Louis, MO). The samples were heated to 100°C for 2 min then centrifuged at 8,000g for 5 min to pellet any insoluble material. Samples were then partially purified using sequential ultrafiltration. Samples were diluted and subjected to ultrafiltration using a 30-kd cutoff Centricon-Plus-20° membrane (Fisher Scientific, Pittsburgh, PA). The retentate was recovered and passed through a 100-kd Microcon° (Fisher) filtration membrane, which was subsequently rinsed. The filtrate and rinse were combined then collected using a 30-kd cutoff Microcon° (Fisher) filtration membrane. In this way, proteins between 30 and 100 kd were partially purified. Samples were then resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer with β mercaptoethanol added and reheated to 100°C. The concentration of protein was determined using a Bicinchonininc-based assay (Pierce Chemical Co., Rockford, IL), and 50 μg of each sample was loaded onto polyacrylamide gels (BioRad Laboratories, Hercules, CA). The proteins were electrophoretically separated and transferred to polyvinylidene-difluoride (BioRad) membranes using Mini-Protean II and Mini-Blot equipment (BioRad). The membranes were blocked with 3% bovine serum albumin in phosphate-buffered saline, and bands were detected by enhanced chemiluminescence (BioRad) using an antibody specific for γ-AChR (Santa Cruz Biotechonology, Santa Cruz, CA).

**Histologic Evaluation of Neuromuscular Junctions**

To evaluate the presence of AChRs outside of NMJs, a double-stain method was developed to examine both AChR and acetylcholinesterase. In the past, researchers relied on measuring the spread of AChR staining patterns alone. In the current methodology, a second marker, namely, immunoreactive acetylcholinesterase, was used to indicate the limits of the NMJ. Acetylcholinesterase was chosen because of its well-characterized expression pattern and its localization to the functional NMJ. An antibody specific for acetylcholinesterase was used with a fluorescent secondary antibody to stain sections. In conjunction with the acetylcholinesterase detection,
AChRs were stained using α-bungarotoxin (α-BTX) conjugated with a second fluorophore. In this way, the limits of the NMJ, as defined by distinct acetylcholinesterase staining, and the distribution of AChR could be evaluated separately or simultaneously within a single section of biopsy material. Typically, the acetylcholinesterase was stained green using a fluorescein-conjugated secondary antibody, and the AChR was stained red using rhodamine-conjugated α-BTX. The addition of red and green fluorescence signals during simultaneous viewing of the fluorophores resulted in a yellow coloration where both molecules were present.

For microscopic localization of AChR, slides containing biopsy cryosections were fixed at room temperature for 25 min using neutral-buffered formalin. These were rinsed with phosphate-buffered saline and stained in a solution containing 2 μg/ml tetramethylrhodamine-conjugated α-BTX (Molecular Probes, Inc., Eugene, OR). After 1-h incubation at room temperature, the sections were rinsed with phosphate-buffered saline and restained using an antibody to acetylcholinesterase (AE-2; Biogenesis, Inc., Sansdown, NH) diluted 1:100 in phosphate-buffered saline and a fluorescein-conjugated secondary antibody (Jackson Labs, West Chester, PA). Samples were viewed and photographed on an Olympus BX-60 microscope (Olympus, Tokyo, Japan). The limits of the NMJ were defined as the extent of the acetylcholinesterase staining (which appeared green in the microscope), and samples were scored positive for abnormal presence of AChRs if α-BTX staining (which appeared red in the microscope) extended beyond this acetylcholinesterase-defined limit. When the acetylcholinesterase and AChR distributions were viewed simultaneously, areas of overlap appeared yellow. One investigator prepared photographs of each NMJ that was observed. Two blinded investigators evaluated the photographs for the presence or absence of abnormal AChRs. In this study, punctate signals were ignored as possible artifact or nonspecific staining. A patient was scored positive for the presence of abnormal AChRs when both scorers agreed that extrajunctional staining was present.

Statistical Analysis
Based on a pilot study of 10 patients, 40% of CP patients and 0% of non-CP patients were expected to have abnormal AChRs. Using a conservative estimate of 30% positives expected in the CP group, to achieve a power equal to 0.8, a sample size of 20 patients per group was determined. The presence or absence of abnormal AChRs, determined by histologic staining, was analyzed using chi-square analysis. The presence or absence of AChR subunits by RT-PCR or Western blotting was not analyzed because of the absence of detectable signal.
Results

Patient Demographics
The group undergoing spinal fusion surgery for idiopathic scoliosis (non-CP group) had a mean age of 14.2 yr (range, 7.8–15 yr) and a mean weight of 56.7 kg (range, 35–107 kg). The group undergoing spinal fusion surgery for scoliosis associated with CP (CP group) had a mean age of 11.5 yr (range, 7–15 yr) and a mean weight of 24.5 kg (range, 13–55 kg). All but eight patients in the CP group were receiving anticonvulsants. The most commonly used anticonvulsant was valproic acid, followed by phenobarbital, clonazepam, carbamazepine, primidone, and lamotrigine. None of the children with idiopathic scoliosis were receiving anticonvulsants.

Histologic Staining of Neuromuscular Junctions
Figure 1 shows representative staining patterns from NMJs of children with idiopathic scoliosis. These NMJs were all judged to be negative for abnormal presence of AChRs. Column A is the acetylcholinesterase distribution shown in green. Column B shows the AChR distribution in red fluorescence. Column C shows both red and green fluorescence signals visualized together; where the two distributions overlap, a yellowish color was seen. In all cases, the NMJs of children with idiopathic scoliosis appeared normal. In the samples from the 20 patients with idiopathic scoliosis, no AChRs were found outside the NMJ.

Figure 2 shows representative staining patterns from NMJs of children with CP. These NMJs were all judged to be positive for abnormal presence of AChRs. Similar to figure 1, column A shows the limits of the NMJ as indicated by acetylcholinesterase, column B shows the distribution of AChRs, and column C shows both signals together. After all three panels were viewed, the presence of abnormal AChRs was judged. The arrows in column C indicate the location of AChRs outside of the NMJ, based on red staining extending beyond the acetylcholinesterase-defined NMJ. In 11 of the 39 samples from patients with CP, abnormal spread of AChRs was found. Chi-square analysis indicated a significant difference between the CP group and the non-CP group (P = 0.0085).

Among the 11 patients with CP who scored positive for the presence of AChRs outside of the NMJ, six were receiving anticonvulsants. Conversely, of the 28 patients with CP who scored negative for the presence of AChRs outside of the NMJ, 25 were receiving anticonvulsants.

Expression of γ-Acetylcholine Receptor Subunits
We used two methods to look for expression of the γ subunit in these samples. RT-PCR using primers specific for the α and γ subunits was performed, and the results are summarized in figure 3A. The mRNA for α was present in each sample; however, the expression of the mRNA for γ was unexpectedly absent from all samples. These results suggested that AChRs containing the γ subunit were not present as a result of CP. Because little is known about the turnover of γ-AChR mRNA, Western

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Discussion

Denervation and immobilization are typical conditions
in which AChRs are found outside the NMJ. Neuro-
logic diseases, such as amyotrophic lateral sclerosis, pe-
ipheral neuropathy, and infantile spinal muscular atro-
phy, show the morphologic and histochemical changes
of denervation, consisting of scattered and grouped
small fibers with variable muscle fiber-type groupings. 
α-BTX-linked immunoperoxidase staining has indicated
that in many of the muscle fibers in these disorders,
AChRs are spread over the surface muscle fibers. These
diffusely spread AChRs have been linked to the hyper-
kalemia seen when these patients are exposed to succinylcholine.

The γ subunit of the AChR, which was expected to be
found in cases where nonjunctional AChRs were
present, was not detected by RT-PCR in the current
study. This was true even in patients who exhibited
extrajunctional AChRs by histologic assessment. Because
the turnover of AChR mRNA may be rapid, we attempted
to verify or refute the RT-PCR results by Western blotting
and obtained similar results. Based on our observations,
we concluded that some children with CP possess ex-
trajunctional AChRs but do not express the γ subunit;
however, it is also possible that the mRNA for γ-AChR is
significantly less stable than the mRNA for the α subunit
and that the amount of γ-AChR protein in our samples is
below the detection limit of the Western approach.

The protein subunit composition of skeletal muscle
AChRs changes both during the course of development
and in response to denervation of mature muscle. During
certain pathologic conditions, changes in the
distribution of AChRs have been reported. The most
common such pathologic state is denervation in which
there is an up-regulation of the γ-AChR subunit mediated
by transcriptional activation. Another classic case in
which AChR concentrations are increased is in burn
victims, who show a nontranscriptionally mediated up-
regulation. The latter study by Nosek and Martyn

Clinical experience supports the notion that children
with CP have extrajunctional AChRs. Children with CP
have a slightly increased sensitivity to the depolarizing
agent succinylcholine. The effective dose for 50% twitch
depression was lower in children with spastic quadriple-
gic CP than for age-matched normal children. Similarly,
the duration of action of vecuronium, a nondepolarizing
muscle relaxant, was significantly decreased in children
with CP when compared with children without CP. How-
ever, in this study, children with CP had also been
receiving anticonvulsant therapy. The known interaction
and up-regulation of AChRs when exposed to anti-
convulsants may have contributed to the vecuroni-
muscular resistance. Interestingly, in our study, only 6 of the
11 children who showed histologic evidence of AChRs
outside the functional NMJ were on anticonvulsants,
whereas 5 of 11 children who showed histologic evi-
dence of AChRs were not.

Other studies have demonstrated an increased require-
ment for nondepolarizing muscle relaxant with quanti-
tative increase in AChR. Chronic infusion of d-tubocurarine results in proliferation of AChRs and increased requirement for d-tubocurarine. This study points to the phenomenon of up-regulation of AChRs by long-term use of an agent that is an inhibitor of AChRs. There are clinical studies pointing toward up-regulation of AChRs in CP, although the cause of this up-regulation remains unclear.

In summary, our findings of an abnormal spread of AChRs in children with CP are consistent with clinical experience and previous studies. The γ subunit was not detected either by RT-PCR or by Western blotting experiments even in children who had histologic evidence of nonjunctional AChRs. This observation is consistent with observation by Nosek and Martyn, who detected a quantitative increase in AChR in a burn animal model without detecting γ subunit. These observations differ from results seen in other studies of changes after denervation in which expression of the γ subunit correlates with both an altered distribution and an up-regulation of AChRs.

In conclusion, children with spastic CP had histologic evidence of spread of AChRs beyond the confines of the functional NMJ when compared with children without CP. On further examination of the subunits by RT-PCR and Western blotting, no γ subunit was detected. These findings (spread of AChRs beyond the NMJs) are consistent with previous clinical findings in children with CP.

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