Injury to the peripheral nerves, and in particular to the brachial plexus, is a life-altering injury and the surgeon should strive to obtain optimal recovery when possible. In brachial plexus reconstructions it is imperative to determine if a nerve root is in continuity with the spinal cord (postganglionic injury) or not (preganglionic injury), as nerve grafting of a discontinuous root will be futile. Developing a test to identify the viability of a nerve (nerve in continuity with the spinal cord) is therefore necessary prior to using it as a source of motor axons. As such, various histomorphometric approaches have been proposed and continue to be used and advocated.2,16,18

A histomorphometric approach for differentiating between sensory and/or motor function and the presence of viable motor nerve fibers is Karnovsky staining. This type of staining visualizes acetylcholinesterase (AChE), an enzyme present in the neuromuscular junctions and motor neurons that is responsible for degradation of the neurotransmitter acetylcholine into choline and acetate. This staining technique was introduced clinically in 1975 by a Russian group, and first described in English by He and Zhong.10 Although evaluated by many for intraopera-

Key Words • brachial plexus • intraoperative testing • acetylcholinesterase • choline acetyltransferase • peripheral nerve
tive use,12,15,19 the staining procedure requires long incubation times, and thus is impractical for routine intraoperative use as a method to determine motor nerve viability.

Another technique described for intraoperative evaluation of motor nerve bundles is the radiochemical assay of choline acetyltransferase (ChAT), first introduced by Engel et al.3 Choline acetyltransferase is an enzyme that is synthesized within the body of a neuron during communication between the pre- and postsynaptic neurons. Choline acetyltransferase is transported to the nerve terminals where it acetylates choline, resulting in the formation of the neurotransmitter acetylcholine.23 Quantification of ChAT has been reported as a way of determining not only motor nerve bundles, but also possibly assisting in the differentiation between motor and sensory fibers as well as continuity of the nerve root.1,11 The potential of using ChAT quantification for clinical use has been reported in the last 2 decades.3,4 Hattori et al.7–9 reported that the level of ChAT activity could be used as a reliable indication of motor fascicles, based on a few clinical cases with intraoperative ChAT assays. However, several concerns have prevented the adoption of routine intraoperative ChAT usage. These concerns include high variability in measured levels, and the absence of studies comparing ChAT to other established assessments for motor neuron fiber activity. To address these concerns, a controlled animal study should be performed, which would be expected to demonstrate that the ChAT assay functions as good as, if not better than, other available techniques.

The purposes of this study were as follows: 1) determine the ChAT activity in the normal and injured sciatic/peroneal nerve in a rat model; 2) evaluate the correlation between ChAT and motor recovery (wet muscle weight); 3) find the relationship between ChAT activity and the isometric muscle force; and 4) elucidate the parallel between ChAT activity and Karnovsky acetylcholine staining.

**Methods**

**Experimental Animals**

Sixty Lewis rats were used, weighing 200 grams each, and cared for in accordance with the Guide for the Care and Use of Laboratory Animals Guidelines published by the NIH and the guidelines of our Institutional Animal Care and Use Committee. The study was approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

The animals were divided into 3 experimental groups. Group 1 animals underwent sciatic nerve transection without microsurgical repair; Group 2 nerves were transected and repaired; and Group 3 specimens sustained a sciatic nerve crush injury followed by nerve transection and repair. To minimize side-to-side variability during the initial procedure, odd-numbered rats received surgery on the left side and even-numbered rats on the right side. Twelve weeks after the surgery the maximal isometric tetanic force of the tibialis anterior muscle was measured.21 At the end of the procedure the sciatic and peroneal nerves as well as spinal roots were harvested to enable both Karnovsky staining and a radiochemical assay of ChAT.

Animals were single-housed in hooded cages at room temperature on a 12-hour light/dark schedule, and were given food and water ad libitum. Surgical procedures and electrophysiological evaluations were performed under intraperitoneal ketamine cocktail anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine). The surgical procedure was performed by 1 surgeon (T.V.) using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6; Carl Zeiss). Postoperatively, a single dose (0.05 mg) of the opioid analgesic buprenorphine (Reckitt Benckiser Pharmaceuticals) was administered subcutaneously and the rat was kept warm until fully awake and stable using a heating pad.

**Nerve Injury in Group 1**

In Group 1, the sciatic nerve of the experimental side was fully exposed by making a skin incision from the hip joint to the knee joint. Second, a gluteal muscle splitting allowed exposure of the sciatic nerve. The nerve was dissected up to the trifurcation of the peroneal, sural, and tibial branches. The sciatic nerve was then transected using microsurgical scissors. The gluteal muscle was approximated using 4-0 Vicryl sutures and the skin was closed using 4-0 nonabsorbable nylon sutures.

**Nerve Injury in Groups 2 and 3**

In Groups 2 and 3, the approach and resection of the sciatic nerve was identical to that in Group 1. The transected nerve in these groups was repaired using 6 10-0 nylon transverse epineural sutures. In Group 3, the nerve was crushed for 3 seconds both proximal and distal to the area of transection using a straight microsurgical clamp, then transected and repaired in the same manner as in Group 2. After repairing the nerve, the muscle and skin were closed according to protocol.

**Isometric Tetanic Force Measurement**

The isometric tetanic muscle force measurement was determined for the tibialis anterior muscle of both legs as detailed by Shin et al.20,21 Measurement of force in the normal hind limb allows the experimental limb force to be normalized, or expressed as a percentage of the uninjured side. To do so, each hind limb was secured to a testing board with Kirschner wires (DePuy Orthopedics). The proximal tibialis anterior tendon was released at its insertion, and the muscle was dissected from the surrounding tissue with preservation of its neurovascular pedicle. The distal tendon was then divided and attached to a force transducer (MDB-2.5, Transducer Techniques) using a custom clamp with the muscle positioned as closely as possible to its original orientation. The force transducer signal was processed by a computer using LabVIEW software (National Instruments). A bipolar stimulator (Grass SD9, Grass Instruments) was applied to the peroneal branch of the nerve using a miniature electrode (Harvard Apparatus). As described by Shin et al.,21 4 parameters require optimization to standardize the recording: muscle resting length or preload, stimulus intensity, pulse duration, and pulse frequency. After maximizing each value with a pulsed signal, a tetanic stimulus is used to measure the maximal force. After killing the rats with a pentobarbital overdose, the muscles were harvested for wet weight measurement.
Choline acetyltransferase assay for assessing nerve injury

**Tibialis Anterior Muscle Weight Index (TAMI)**

The tibialis anterior muscles of both sides were carefully dissected, harvested, and weighed. The result was normalized to minimize biological variability. As such, a percentage of the contralateral tibialis anterior wet muscle weight was used for statistical analysis.

**Histological Evaluation**

Next, the sciatic and peroneal nerves were harvested from both hind limbs. The segments of sciatic and peroneal nerve measured 4 mm in length. Peroneal segments were harvested in an area 5 mm distal to the anastomosis site and distal to their sciatic origin. In addition, several ventral and dorsal spinal rootlets were harvested as pure sensory and motor nerve controls, respectively. All nerve samples were immediately frozen at −80°C until ChAT assay and AChE staining could be performed in batches. The proximal half of each segment was prepared for Karnovsky staining (ACHE activity), and the distal segments sent to quantitate ChAT activity. As in the tetanic force study, the contralateral nonexperimental side served as a control.

**Acetylcholinesterase Activity Measurements (Karnovsky Staining)**

We used a modified Karnovsky staining method for staining the AChE activity as described by Karnovsky and Roots. The proximal segments of sciatic and peroneal nerves were cross-sectioned (10 μm) with a cryostat microtome. Serial sections were collected on aminopropylsilane-coated glass slides. The incubation medium was 0.1 mol/L NaOH-maleate buffer containing 0.1 mol/L Na citrate, 30 mmol/L CuSO\textsubscript{4}, 5 mmol/L K\textsubscript{3}[Fe(II)(CN)\textsubscript{6}], and 1.8 mmol/L acetylcholine iodide. Tetraisopropyl pyrophosphoramide (10\textsuperscript{−4} mol/L; Sigma) was used as an inhibitor of nonspecific cholinesterase activity. The sections were incubated for 24 hours at 37°C. The sections were rinsed in distilled water, dehydrated, and mounted. Sections were photomicrographed at a magnification of 250 and the number of AChE-stained fibers (motor fibers) was determined by direct counting.

**Choline Acetyltransferase Activity Measurements**

The measurement of ChAT activity was based on the radiochemical method of Engel et al. The sciatic and peroneal nerve sample was weighted. Each sample was placed into a plastic microtube containing 20 μl buffer A (20% weight to volume). Buffer A consisted of 50 mmol/L buffer A, 0.2 ml of 1.6 mmol/L sodium phosphate buffer, pH 7.4, 300 mmol/L sodium chloride, and 20 mmol/L ethylenediaminetetraacetic acid (EDTA). The tissue was sonicated and 10 μl was mixed with 20 μl of the incubation mixture (0.0476 μCi [1-\textsuperscript{14}C] acetyl-CoA, 18.8 μl buffer A, 0.2 μl of 1.6 μM/L choline chloride, and 1 μl of 5 μM/L physostigmine sulfate) was mixed and incubated for 30 minutes at 37°C. At the end of the incubation period, 100 μl of a solution of tetraphenylboron in diisobutyl ketone (15 mg/μl) was added. After centrifugation (2 minutes at 20,000 g), 40 μl of the organic phase was transferred to a counting vial and 10 μl of omnifluor in toluene was added. The ChAT activity was then measured using a liquid scintillation counter.

The preset time was 1 minute. The ChAT activity was expressed as count per minute (cpm). Using this technique, the background, measured without the needle sample, was subsequently subtracted from each data point. The ChAT activity was also normalized as activity per gram of nerve tissue (cpm/g). The rationale for normalizing tissue per gram was to eliminate variety in diameter when taking different nerve samples.

**Statistical Analysis**

The number of rats required in each group has been determined by the results of isometric tetanic muscle force tests obtained from a previous study. It was estimated that 17 rats in each group would provide an 80% power to detect a 10% difference in mean muscle force between the groups (α = 0.05, 2-sided). To guard against potential attrition, the sample size was increased to 20 in each group. Maximum isometric tetanic force was to be the primary outcome measurement.

All the results are presented as mean ± SD. Muscle force was analyzed as a percentage of normal force using the contralateral limbs as a measure of normal. The ChAT activity was normalized per gram of tissue (cpm/g). The correlation between the muscle force and ChAT activity was analyzed using a percentage based on the measurements retrieved from the contralateral side measurement. The correlation between muscle force and ChAT activity was calculated using Spearman’s rank correlation coefficient. The correlation among the 3 ChAT levels in the spinal nerve rootlets was determined using a Kruskal-Wallis test. Significance was set at p ≤ 0.05.

**Results**

All animals that entered the study survived the initial surgical procedure and no complications regarding gait or misbehavior occurred. We will discuss each assessment individually.

**Isometric Tetanic Force Measurement**

The maximal isometric tetanic force was 0 grams in Group 1, 420 ± 84 grams in Group 2, and 394 ± 81 grams in Group 3. The forces produced by the contralateral side for Groups 1, 2, and 3 were 700 ± 84, 630 ± 84, and 643 ± 95 grams, respectively. Normalized data had a mean force of 0%, 67.08% ± 0.17%, and 63.11% ± 0.15%, respectively. There was an obvious significant difference when comparing Group 1 to Groups 2 and 3 (p < 0.0001). After comparing Group 2 and Group 3, no significant difference was found (p > 0.05). Figure 2 depicts the TAMI for the 3 groups.

**Tibialis Anterior Muscle Weight Index**

Group 1 had a mean TAMI of 18.88% ± 6.23%; Groups 2 and 3 had muscle indices of 63.48% ± 8.66% and 64.41% ± 8.00%, respectively. Significant differences were found comparing Group 1 to Groups 2 and 3 (p < 0.0001) and no difference was found comparing Group 2 with Group 3 (p > 0.05). Figure 2 depicts the TAMI for the 3 groups.

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Figure 3 shows that the number of stained peroneal (motor) nerve fibers in Groups 1, 2, and 3 was 90, 650, and 671, respectively. The normal or contralateral side fiber counts in Groups 1, 2, and 3 were 700, 810, and 422, respectively. The number of fibers in the operated nerve proved to be significantly different compared with the amount of fibers in the contralateral side for all 3 groups (p < 0.001).

Choline Acetyltransferase Activity Measurements

Peroneal Nerve Tissue. Figure 4 is a detailed graphic illustrating the difference in ChAT activity. For peroneal nerve tissue, Group 1 had a mean ChAT level of 194331 ± 209738 cpm/g in the experimental side 12 weeks after surgery, compared with a mean contralateral level of 1952351 ± 1808458 cpm/g. The ChAT level in Group 2 was 850957 ± 735582 cpm/g in the operated side. The control side had 683817 ± 828641 in the operated nerve and a mean cpm/g of 1554967 ± 1889251 in the control side.

Sciatic Nerve Tissue. For sciatic nerve tissue, Group 1 had a mean ChAT level of 3686171 ± 1637909 cpm/g in the experimental side 12 weeks after surgery, compared with a mean contralateral level of 2845860 ± 1356948 cpm/g. The ChAT level in Group 2 was 2049127 ± 1311806 cpm/g in the operated side; the control side had 2192065 ± 1287224 cpm/g. Group 3 had a mean cpm/g of 2152752 ± 2017032 in the operated nerve and a mean cpm/g of 2516507 ± 2225408 in the control side.

Spinal Rootlets. Group 1 had a mean ChAT level of 1848 ± 1528 cpm/g in the ventral side 12 weeks after surgery, compared with a mean dorsal level of 54 ± 69 cpm/g. The ChAT level in Group 2 was 1457 ± 1477 cpm/g in the ventral rootlet, whereas the dorsal rootlet had 68 ± 89 cpm/g. Group 3 had a mean cpm/g of 1484 ± 2105 in the ventral rootlet and a mean cpm/g of 61 ± 76 in the dorsal rootlet.

Correlations

The correlation between the isometric tetanic force and the TAMI was 0.382. The correlation between the isometric tetanic force index and the normalized ChAT level (cpm/g) in Group 1 and 2 was 0.468. The correlation for the AChE staining and the isometric tetanic force measurement was 0.111. The correlation between the TAMI and normalized ChAT levels was 0.773. The correlation between the TAMI and the AChE-stained fibers was 0.640.

Correlating the AChE staining to the normalized ChAT analysis, we found a correlation of 0.712. Correlating the level of ChAT in the dorsal spinal nerve among Groups 1, 2, and 3, we found no significant difference (p = 0.202).
Discussion

This study was designed to determine the ChAT activity normalized to weight in the normal and injured sciatic/peroneal nerve in a rat model. Despite the performance of the assay in a clinical laboratory well experienced with the methodology, we found the radiochemical assay to have a very high level of variability in ChAT activity, even in normal specimens. The second and third goals were to evaluate the correlation between ChAT and motor recovery; this correlation proved to be weak, as both the TAMI and the isometric tetanic muscle force measurements did not correlate strongly with the ChAT levels. The fourth goal was to determine the correlation between AChE staining and ChAT levels, which also proved to be weak (r = 0.712).

Despite the description by Engel et al.3 that the number of motor nerve fibers was reflected by ChAT activity, the relationship between ChAT levels and tetanic isometric motor force has not been compared. Additionally, a comparison of ChAT activity and AChE staining has not been performed. However, the most important improvement in this study compared with previous studies is the correction for weight when analyzing the ChAT activity.

In the literature, studies primarily focused on the ChAT activity as counts per minute alone, rather than combining the complete arsenal of tests available to determine viability of a motor nerve root. Yajima et al. used ChAT levels as a single-measure instrument to distinguish between motor and sensory branches in the brachial plexus in a rat model and suggested it could be a valuable tool to differentiate pre- and postganglionic injury in the cervical roots. Their most important finding was that 90 days after a brachial plexus injury, the ChAT activity was reduced to 50% compared with normal ChAT levels.22

Hattori et al. extensively investigated the usefulness of ChAT activity in the identification of motor fibers in both a clinical and experimental setting.3-7 In one of the preliminary studies, the ChAT levels were evaluated in a beagle brachial plexus model. These investigators transferred and compared the spinal accessory and intercostal nerves to reinnervate the biceps and triceps muscles of the beagles. Similar to the previous studies investigating the ChAT enzyme, they failed to report the activity of ChAT calculated per gram of tissue. It is imperative to normalize the ChAT enzyme level per gram of tissue to exclude the variety in nerve diameter (which would be inevitable if obtaining a section). In addition to their finding that showed a low correlation between the ChAT assay and functional testing, they also failed to find a correlation between ChAT activity and the number of motor fibers.6 These results were confirmed by our results, as we were unable to demonstrate a high correlation between the tetanic isometric force measurement or TAMI with the ChAT assay. Nevertheless, the AChE staining proved to have an even worse correlation to both functional muscle assessments.

Kawanishi et al. reported that the ChAT activity of the intercostal motor nerves had an average cpm of 2236.14 In this study, ChAT activity was also not depicted as activity per gram of tissue, but as activity per nerve section, and thus did not take diameter differences into account. This finding and the clinical results found by Hattori et al. lead to the hypothesis that nerve fibers with a ChAT level greater than 2000 cpm had an adequate quality and quantity of intact motor fascicles to ultimately gain function after a nerve reconstructive procedure.6 This hypothesis was also based on earlier findings from the same group in which the ChAT assay in a pure sensory nerve had levels of 345 ± 106 cpm.6 However, these optimistic conclusions on the usefulness of the ChAT assay were made on the basis of an animal-based experiment by Hattori et al. in which no direct correlation was found between the amount of ChAT activity and number of motor fibers, and on 3 small clinical studies (1 case report with 3 cases 9 and 2 intraoperatively measured ChAT activity studies with a total of 16 patients10). Additionally, these studies did not normalize the ChAT activity for weight. The minimum ChAT assay of 2000 cpm, which has been arbitrarily defined by Hattori, has thus been recommended without evidence proved in basic experimental models. The lack of experimental studies validating this definition and current reports do not adequately define the correlation between the ChAT activity and ultimate motor outcomes.

In this study we chose to use the rat sciatic nerve, because it is responsible for both sensory and motor innervation. Also, we included the peroneal nerve as a pure motor
nerve control. The tremendous variability in all groups, as well as the weak correlations with the functional muscle assessments and the AChE staining, do not support the use of the ChAT assay to determine motor nerve viability. The ventral and dorsal rootlets were harvested as controls and their results correlated well, regardless of the nerve injury, with the expected nature of the rootlets (that is, ventral being purely motor and dorsal being purely sensory). We are unable to determine the cause of the huge variability in the amount of ChAT activity within the groups (intraclass correlation coefficient), but previous reports also demonstrated significant variability in ChAT assay levels.

Conclusions

We can conclude from these data and the dissatisfactory correlation between functional testing and the ChAT assay that intraoperative measuring of ChAT activity is not a reproducible and accurate measure of motor nerve viability. Therefore, the search for a method to determine motor nerve viability intraoperatively should continue. Whatever this technique may be, it should not only be time and cost efficient, but also demonstrate low variability, high specificity, and a side-to-side consistency.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Shin, Vathana, Bishop. Acquisition of data: Shin, Vathana, Friedrich. Analysis and interpretation of data: Shin, Vathana, Nijhuis, Friedrich. Drafting the article: Shin, Vathana, Nijhuis, Bishop. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Shin. Administrative/technical/material support: Fried- rich. Study supervision: Shin, Bishop.

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