An Electrophysiologic Model for Functional Assessment of Effects of Neurotrophic Factors on Facial Nerve Reinnervation

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Objectives: To establish a sound objective model for assessing the effects of neurotrophic factors on facial nerve function after injury and to compare the effects of brain-derived neurotrophic factor (BDNF) with its neutralizing antibody on facial nerve function after injury.

Design: Prospective electrophysiologic analysis of recovery of function 4 weeks after axotomy involving facial nerve transection and primary end-to-end reanastomosis in adult rats and blind comparison with randomized intramuscular injection of either BDNF, monoclonal antibody to BDNF in neutralizing concentration, or control solution.

Results: There were no statistically significant differences between groups in latencies, duration, amplitude, area, or conduction velocity before axotomy, and recorded conduction velocities were consistent with previously reported values, which suggests that the recordings were reliable and reproducible. After transection, there was a mean increase in latency 1 and decreases in latency 2, integrated average area, muscle action potential duration, amplitude, and conduction velocity for all 3 groups. When the groups were compared after transection, the anti-BDNF group showed a significant decrease in conduction velocity and muscle action potential duration (Kruskal-Wallis P = .01 and P = .008, respectively) compared with the other groups. There were no statistically significant differences in latencies, amplitude, or area among the groups.

Conclusions: We have established an electrophysiologic model for objective assessment of facial nerve function in the rat. Future studies should combine functional electrophysiologic assessment and histologic examination to provide a more robust model for studying the effects of neurotrophic factors on facial nerve reinnervation and synkinesis.

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NEUROTROPHINS (NTS) ARE a family of proteins comprising nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, NT-4/5, and NT-6. These endogenous soluble proteins have been shown to inhibit neuronal apoptosis and to promote neuronal survival, development, regeneration, plasticity, and neuron-related enzyme synthesis.1-4 Brain-derived neurotrophic factor is a potent and effective trophic factor for motor neurons and other neurons of the peripheral and central nervous systems.1-4 Furthermore, retrogradely transported BDNF has been demonstrated to prevent injury-induced cell death of facial motor neurons in neonatal rats,5-10 and BDNF has been shown to improve the histologic outcome of facial nerve regeneration.1 Brain-derived neurotrophic factor positively influences the branching of axonal arbors6 and promotes axon outgrowth.7 After axotomy of the facial nerve, a series of intrinsic and extrinsic neuronal events occurs.11 Intrinsically, these events include the following: (1) anterograde “Wallerian degeneration” at 1 to 14 days in which distal myelin swells, retracts, and fragments; (2) retrograde signaling of injury in the form of calcium and sodium leakage from the site of axotomy and generation of an “injury current,” increases in cytokine production, and loss of trophic signals from target-derived sources; and (3) retrograde degeneration marked by neuronal cell body swelling and nuclear eccentricity, sealing of the seeping cut proximal end, and formation of an axonal bulb from which the proximal axon will regrow. Extrinsically, Schwann cells are activated by cytokines, trophic factors, and macrophage factors. They have phagocytic activity for myelin and cell debris; produce cytokines and growth factors, including BDNF12, and align themselves along the chains of Bungner13 to form a guiding scaffold for regenerating axons to

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their target(s). While this environment is supportive for axonal regrowth and facilitates regenerating axons entering the distal stump, the problem of aberrant or misguided regeneration, known as synkinesis, makes complete functional recovery difficult to achieve. This is due to either the misrouting of an axon to the wrong fascicle (resulting in an axon innervating the wrong muscle) or the presence of extra branches from one or many motoneurons "hyperinnervating" a muscle. In addition, central changes, including synaptic stripping, may contribute to the development of synkinesis.

Streppel and colleagues recently hypothesized that aberrant axonal branching could be due to the increased expression of trophic molecules at the lesion site and that inhibition or blockade of these factors could reduce branching and improve the accuracy of reinnervation. They showed that local application of neutralizing concentrations of antibodies to BDNF and other neurotrophic factors diminished the number of axonal branches after facial nerve transection without any notable harmful effect on neuronal survival. In particular, anti-BDNF showed the greatest effect, reducing the portion of branched neurons to 14%.

These findings raise a central question as to the therapeutic role of BDNF and other neurotrophic factors in peripheral nerve regeneration: Which is more important to the functional outcome of nerve regeneration, the promotion of survival of the regenerating motoneurons or the accuracy of reinnervation? While the techniques of double and triple retrograde labeling (to identify neurons that project axons down multiple branches of the facial nerve) and immunocytochemical analysis and myelin staining together provide a sound histologic model for the representation of synkinesis, objective assessment of functional outcomes is difficult. The ultimate therapeutic potential of strategies aimed at reducing axonal branching remains largely unknown. Efforts using video-motion analysis to measure the protraction, amplitude, angular velocity, and acceleration of vibrissal movements have provided a useful method of grading functional outcomes, although these techniques often rely on a subjective measurement of the degree of movement.

In the present study we attempted to establish, through the capture and analysis of a series of electrophysiologic recordings made before and after nerve transection, a sound objective model for assessing the effects of neurotrophic factors on facial nerve function after axotomy.

**METHODS**

**ANIMALS**

Experiments were carried out on 30 male Sprague-Dawley rats weighing 300 to 350 g. In all rats, the left facial nerve underwent transection followed by "direct reanastomosis." All surgical procedures were performed under aseptic conditions. Rats were anesthetized with xylazine hydrochloride (3 mg/kg) and ketamine hydrochloride (100 mg/kg) by intramuscular injection.

**SURGICAL PROCEDURES**

The main trunk of the facial nerve was exposed at the point where it exits from the stylomastoid foramen and transected just beyond the origin of the digastric nerve. Under a Zeiss operating microscope (Carl Zeiss Inc, Thornwood, NY), the transected nerve was immediately repaired by end-to-end anastomosis using 10-0 perineural microsutures (Ethicon Inc, Somerville, NJ). The muscle layers and skin were closed with 6-0 silk sutures. During the postoperative period, none of the rats displayed complications such as self-mutilation, ulcers, infections, overt signs of discomfort, or an inability to eat.

Current knowledge indicates that BDNF is target derived, taken up at the synaptic terminals, and transported retrogradely to the facial nucleus. Therefore, we chose to administer BDNF by intramuscular injection at the target muscle, the vibrissal pad of the rat. At the time of surgery, each rat was randomly assigned to receive a solution of either recombinant BDNF, monoclonal antibody to BDNF, or a control vehicle solution (phosphate-buffered saline) injected intramuscularly into the vibrissal pad. Methylated BDNF was supplied by Amgen Inc, Thousand Oaks, Calif, and anti-BDNF was supplied by R&D Systems Inc, Minneapolis, Minn. For anti-BDNF, a neutralizing concentration of 7.5 µg/mL was used. For the BDNF solution, it has been shown that administration of 5 mg/kg per day over a period of at least 7 days optimally attenuates the axotomy-induced reduction in choline acetyltransferase immunoreactivity in motor neurons in the rat facial nucleus. Thus, a dosage of 1.5 mg was used, and intramuscular injections of all solutions were made every other day for 4 weeks with the rats under halothane gas anesthesia. One rat in the BDNF group died during the 4 weeks.

**ELECTROPHYSIOLOGIC TESTING**

Experiments were carried out on 30 male Sprague-Dawley rats weighing 300 to 350 g. In all rats, the left facial nerve underwent transection followed by "direct reanastomosis." All surgical procedures were performed under aseptic conditions. Rats were anesthetized, and the compound muscle action potentials (MAPs) of the right and left vibrissal muscles were recorded by 2 monopolar needle electrodes inserted subcutaneously at symmetric points in the vibrissal array of the 2 sides (Figure 1). The main trunks of the right and left facial nerves were exposed and stimulated with a single electrode with a rectangular current pulse of 0.05-millisecond duration delivered from a VikingQuest Portable 2-channel electromyograph (Nicolet Biomedical, Madison, Wis); the position of the stimulating electrodes was optimized to obtain a MAP threshold of less than 1.5 mA. All MAPs were obtained by supramaximal nerve stimulation and recorded with VikingQuest data-acquisition software. The program calculated the latencies of evoked MAP re-
RESULTS

Initial recordings were obtained on both the right and left sides of each rat prior to transection and reanastomosis on the left side. The differences in the recorded values for latencies, duration, amplitude, area, and conduction velocity between the right and left nerves were calculated for each rat, as were the median difference and range for each group (Table 1). The 3 groups were then compared using the Kruskal-Wallis test. There was no statistically significant difference (P > .05) between groups in the median values obtained for the difference between the right and left nerve recordings for any of the recording parameters (latency 1 or 2, duration, amplitude, area, or conduction velocity; Table 1). The mean preoperative values for conduction velocity were 35.4, 35.75, and 34.0 m/s in the BDNF, anti-BDNF, and control groups, respectively.

Four weeks after the axotomy, recordings were again made on the left side, and these values were compared with the right (nontransected) side recordings made previously. The signed-rank test was used to compare the left (transected) and right (nontransected) sides for each recording parameter. With the exception of latency 2 in the anti-BDNF and control groups, all other recordings showed a significant change in function on the transected nerve compared with the nontransected side (P < .05) (Table 2).

The preaxotomy and postaxotomy values for latencies, duration, amplitude, area, and conduction velocity for the left (transected) nerve were then compared using the Kruskal-Wallis test (Table 3). The anti-BDNF group showed a significant decrease in median duration of the MAP and conduction velocity compared with the BDNF and control groups (P < .01 and P < .05, respectively) (Figure 2). There were no significant differences in latencies, amplitude, or area between groups (Table 3).

Neurotrophic factors have been shown to inhibit neuronal apoptosis and to promote neuronal survival, development, regeneration, plasticity, and neuron-related enzyme synthesis, which makes them promising candidates for novel therapeutic strategies aimed at improving facial nerve function after injury. However, their promise has been largely unrealized because the exact mechanisms of their actions on regenerating neurons are far from understood. The recent findings by Streppel and colleagues add further complexity: their results suggest that the focal application of antibodies to neurotrophic factors reduces overall axonal branching with outward adverse effect on overall neuronal survival. This finding points toward a possible therapeutic strategy for reducing synkinesis, a debilitating and often inevitable consequence of facial nerve reinnervation after injury. Guntinas-Lichius and colleagues found that minimizing axonal branching using transplanted olfactory mucosa promotes the recovery of vibrissal motor performance after facial nerve repair.

### Table 1. Values of Preaxotomy Left Nerve Recordings Minus Right Nerve Recordings*

<table>
<thead>
<tr>
<th>Recording</th>
<th>BDNF</th>
<th>Anti-BDNF</th>
<th>Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency 1, ms</td>
<td>0.20 (-0.10 to 0.50)</td>
<td>0.00 (-0.10 to 0.20)</td>
<td>0.05 (-0.90 to 0.80)</td>
<td>.32</td>
</tr>
<tr>
<td>Latency 2, ms</td>
<td>0.30 (-0.10 to 0.80)</td>
<td>0.10 (-1.10 to 3.80)</td>
<td>0.40 (-1.90 to 2.00)</td>
<td>.49</td>
</tr>
<tr>
<td>Duration, ms</td>
<td>0.00 (-0.20 to 0.60)</td>
<td>0.10 (-1.20 to 3.70)</td>
<td>0.05 (-2.20 to 1.20)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Amplitude, mA</td>
<td>0.70 (-2.80 to 13.40)</td>
<td>-0.15 (-4.10 to 3.40)</td>
<td>2.65 (-5.20 to 32.60)</td>
<td>.58</td>
</tr>
<tr>
<td>Area, mm²</td>
<td>0.60 (-2.30 to 8.20)</td>
<td>0.50 (-2.30 to 3.30)</td>
<td>2.50 (-5.20 to 18.90)</td>
<td>.44</td>
</tr>
<tr>
<td>Conduction velocity, m/s</td>
<td>-5.00 (-16.00 to 5.00)</td>
<td>-1.00 (-8.00 to 5.00)</td>
<td>0.00 (-41.00 to 91.00)</td>
<td>.14</td>
</tr>
</tbody>
</table>

Abbreviation: BDNF, brain-derived neurotrophic factor.
*Unless otherwise indicated, data are reported as mean (range) values.

### Table 2. Postaxotomy Comparisons of Left (Transected) Minus Right (Nontransected) Nerve Recordings*

<table>
<thead>
<tr>
<th>Recording</th>
<th>BDNF</th>
<th>Anti-BDNF</th>
<th>Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency 1, ms</td>
<td>0.80 (0.60 to 1.10)</td>
<td>0.75 (0.40 to 1.30)</td>
<td>0.40 (0.00 to 1.40)</td>
<td>.004</td>
</tr>
<tr>
<td>Latency 2, ms</td>
<td>-1.00 (-1.60 to 1.20)</td>
<td>-0.45 (-2.20 to 0.80)</td>
<td>-1.10 (-1.80 to 1.30)</td>
<td>.07</td>
</tr>
<tr>
<td>Duration, ms</td>
<td>-1.80 (-2.60 to 0.50)</td>
<td>-1.05 (-3.50 to 0.40)</td>
<td>-1.60 (-2.60 to 1.30)</td>
<td>.01</td>
</tr>
<tr>
<td>Amplitude, mA</td>
<td>-4.20 (-5.90 to -0.80)</td>
<td>-3.10 (-6.80 to -0.40)</td>
<td>-2.65 (-6.40 to 2.70)</td>
<td>.02</td>
</tr>
<tr>
<td>Area, mm²</td>
<td>-5.40 (-7.30 to -2.00)</td>
<td>-4.55 (-6.30 to -0.30)</td>
<td>-3.85 (-8.40 to 0.90)</td>
<td>.004</td>
</tr>
<tr>
<td>Conduction velocity, m/s</td>
<td>-14.00 (-26.00 to 0.00)</td>
<td>-12.50 (-21.00 to -8.00)</td>
<td>-10.00 (-21.00 to -1.00)</td>
<td>.002</td>
</tr>
</tbody>
</table>

Abbreviation: BDNF, brain-derived neurotrophic factor.
*Unless otherwise indicated, data are reported as mean (range) values.
While Streppel and colleagues\textsuperscript{21} relied entirely on histologic analysis for their experiments, Guntinas-Lichius et al\textsuperscript{22} used a video-motion analysis system to assess vibrissa motor performance. Both models could be improved and complemented by an objective electrophysiologic system for the analysis of facial nerve function in the rat. The electrophysiologic measurement of MAPs described herein allows for accurate recording of several parameters of the waveform, including the initial and second latencies, duration of the MAP, conduction velocity, peak-to-peak amplitude, and the area under the curve of the rectified MAP.

The maximum peak-to-peak amplitude of the MAP is a function of 3 factors: (1) the population of motor nerve fibers responding to the stimulus, (2) the synchronization of their responses, and (3) the size of the motor unit innervated by the axons. As axon regeneration and remyelination proceed, more muscle fibers are recruited, and their responses become increasingly more synchronized, thereby increasing the amplitude of the MAP\textsuperscript{29}.

There were no statistically significant differences between groups in latencies, duration, amplitude, area, or conduction velocity before axotomy (Kruskall-Wallis comparison of medians; \( P > .05 \)). This finding suggests that the recordings made prior to nerve transaction were reproducible and provided a reliable evaluation of normal facial nerve function. The mean conduction velocity of the facial nerve in the BDNF and anti-BDNF groups was consistent with values previously reported (range, 35.0-64.6 m/s).\textsuperscript{30} In the control group it was 34 m/s.

After transection, when the transected nerve was compared with the nontransected side, there was a mean increase in latency 1 and decreases in latency 2, integrated average area, MAP duration, amplitude, and conduction velocity in all 3 groups. With the exception of latency 1 in the anti-BDNF group, all differences were consistent with a signed-rank \( P \) value less than .05, accurately reflecting the functional impairment of the transected nerve compared with the normal nontransected nerve. When recordings for the transected nerve were compared before and after axotomy, latency 1, amplitude, area, and conduction velocity all achieved signed-rank \( P \) values less than .05 for all 3 groups, again reflecting changes in the nerve after axotomy compared with its normal function preoperatively.

Interestingly, when groups were compared after transection, the anti-BDNF group showed a significant decrease in conduction velocity and MAP duration (\( P = .01 \) and \( P = .008 \), respectively; Kruskal-Wallis test) compared with the other groups. However, there were no statistically significant differences in latencies, amplitude, or area among the groups. This would seem to suggest that reducing axonal branching impaired conduction of the impulse while not affecting the overall size or strength of the impulse.

We have established an electrophysiologic model for objective assessment of facial nerve function in the rat and a method for studying the effects of neurotrophic factors on facial nerve reinnervation. Future studies combining functional electrophysiologic assessment and histologic examination should yield a more robust and comprehensive evaluation of strategies aimed at reducing synkinesis.

### Table 3. Comparison of Preaxotomy and Postaxotomy Left (Transected) Nerve Values*

<table>
<thead>
<tr>
<th>Recording</th>
<th>BDNF</th>
<th>Anti-BDNF</th>
<th>Control</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency 1, ms</td>
<td>0.50 (0.20 to 1.00)</td>
<td>0.80 (0.30 to 1.20)</td>
<td>0.25 (0.00 to 1.00)</td>
<td>.07</td>
</tr>
<tr>
<td>Latency 2, ms</td>
<td>0.65 (-0.80 to 2.40)</td>
<td>-0.05 (-3.90 to 0.90)</td>
<td>0.10 (-0.90 to 1.30)</td>
<td>.22</td>
</tr>
<tr>
<td>Duration, ms</td>
<td>0.05 (-1.80 to 1.90)</td>
<td>-0.85 (-4.30 to 0.40)</td>
<td>-0.05 (-1.00 to 2.40)</td>
<td>.008</td>
</tr>
<tr>
<td>Amplitude, mA</td>
<td>-10.55 (-21.50 to -4.00)</td>
<td>-4.70 (-6.30 to -1.60)</td>
<td>-8.50 (-33.40 to 0.00)</td>
<td>.07</td>
</tr>
<tr>
<td>Area, mm(^2)</td>
<td>-4.95 (-9.90 to -2.90)</td>
<td>-4.80 (-6.10 to -2.90)</td>
<td>-5.60 (-18.70 to 0.00)</td>
<td>.35</td>
</tr>
<tr>
<td>Conduction velocity, m/s</td>
<td>-2.50 (-12.00 to 3.00)</td>
<td>-13.50 (-18.00 to -5.00)</td>
<td>-4.00 (-90.00 to 1.00)</td>
<td>.01</td>
</tr>
</tbody>
</table>

Abbreviation: BDNF, brain-derived neurotrophic factor.

*Unless otherwise indicated, data are reported as mean (range) values.

### Figure 2. Median change in the duration of muscle action potential (MAP) (A) and conduction velocity (B) of the regenerated nerve compared with its normal preoperative function. BDNF indicates brain-derived neurotrophic factor.
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