Chronic Recording of Regenerating VIIIth Nerve Axons With a Sieve Electrode

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Chronically recording regenerating VIIIth nerve axons with a sieve electrode. J. Neurophysiol. 83: 611–615. 2000. A micromachined silicon substrate sieve electrode was implanted within transected toadfish (Opsanus tau) otolith nerves. High fidelity, single unit neural activity was recorded from seven alert and unrestrained fish 30 to 60 days after implantation. Fibrous coatings of genetically engineered bioactive protein polymers and nerve guide tubes increased the number of axons regenerating through the electrode pores when compared with controls. Sieve electrodes have potential as permanent interfaces to the nervous system and to bridge missing connections between severed or damaged nerves and muscles. Recorded impulses might also be amplified and used to control prosthetic devices.

INTRODUCTION

One of the major goals of the decade of the brain has been the implementation of chronic interfaces to the nervous system. More than 25 years have elapsed since the concept of the sieve electrode as such an interface was first introduced (Llinas et al. 1973; Marks 1969); however, numerous technical difficulties have prevented its routine use. Hardware complications for a silicon-based wafer electrode (Llinas et al. 1973) have included difficulties in the consistent micromachining of quality electrodes, interfacing the micropores with manageable recording leads, and developing transdermal connectors for continuous data acquisition. Biologically, channeling of nerve fibers through the sieve pores has proven to be problematic, even in systems that exhibit vigorous regeneration. A further challenge has been the establishment of intimate contacts between nerve fibers and the recording substrate for the production of records with adequate signal-to-noise.

Limited success was achieved when Marks (1969) showed anatomic evidence of regeneration through a sieve implanted in bullfrog nerves. Further, Mannard et al. (1974), using silver wires embedded in epoxy, and Edell (1986), employing slotted gold microelectrode arrays, recorded from regenerated sciatic nerves in Xenopus and rabbit, respectively. Neural activity was demonstrated in rat peroneal nerve and frog auditory nerve using multiple-channel silicon arrays (Della Santini et al. 1997; Kovacs et al. 1994). Sieve electrodes were also used to record from regenerated rat glossopharyngeal (Akin et al. 1994; Bradley et al. 1997) and sciatic nerves (Navarro et al. 1998). However, these advances have been tempered by low success rates and the need to record from anesthetized or restrained animals.

This study is the first to report consistent success and repeated sampling of multiple single-regenerating axons via a transdermal connector in awake, unrestrained animals. Long-term chronic recording was achieved by implanting a sieve electrode in the path of regenerating VIIIth nerve fibers of the otolithic organs of a teleost fish, Opsanus tau. These nerves were previously demonstrated to regenerate ~60 days after transection and to display complete territorial overlap with their normal counterparts (Mensinger and Highstein 1999).

METHODS

Sieve electrodes possessing nine recording sites within a porous matrix were fabricated using micromachining techniques (Hetke et al. 1994) (Fig. 1, A and B). The electrodes consist of a recording head with a 20 μm-thick silicon support rim surrounding a 4 μm-thick internal diaphragm. The diaphragm was interspersed with iridium-coated recording sites (5–8 μm or 10–20 μm diameter) and additional noncoated pores (3–10 μm diameter). The recording sites were integrated via polysilicon leads into a 7 mm silicon ribbon cable that terminated in a rectangular bonding pad. The silicon leads were insulated from the external environment and internal diaphragm with a trilayer of dielectrics (silicon dioxide, silicon nitride, and silicon dioxide). For electrophysiological recording (bonded probes), the bonding pad was ultrasonically bonded to a flexible circuit board and connected via a flexible transdermal lead to a nine-pin connector (PI Medical). The entire bonded probe assembly, posterior to the electrode shaft, was coated with silastic (PI Medical) except for the nine-pin connector, which was sealed with a removable rubber plug. The impedance of each recording site (0.2–1.5 MΩ) was determined before implantation and regularly during recording sessions.

In later implants, based on the initial morphological results, several postproduction modifications were made to the electrode before implantation. The electrode head was electrostatically coated with a porous thin film of ProNectin L (Protein Polymer Technologies), a silk-like protein with laminin functionality (SLPL). The SLPL was electrosprun from a 150 mg/ml solution in 99% formic acid (Sigma).
across an air gap of 20 mm using DC voltage of 7 kV (Martin et al. 1997). The next modification was to attach a semipermeable nerve guide tube (NGT) to the electrode head (Fig. 1C). The NGT consisted of poly-(acrylonitrile-vinyl chloride) and was custom fabricated using a phase inversion through an annular spinneret. Small sections of tube were placed on a 27 gauge needle and heat shrunk into a tube several microns thick (450 μm diameter). The tubing was then cut into 400 μm sections, a slit was made in the middle of each section, and the electrode was lowered into the slit. A drop of Loctite 3341 medical adhesive that was immediately cured with an ELC-403 ultraviolet light (Electro-lite) was used to affix the guide tube to the outer electrode ring. Finally, the entubulated SLPL probes were dipped several times into a neural adhesive solution [0.4% solution of protamine sulfate (Sigma) and poly-d, l-lysine (Sigma)] just before implantation.

Toadfish (standard length 25 ± 2 cm) were obtained from the Marine Biological Lab in Woods Hole, MA and maintained at 15°C in flow-through or recirculating aquariums. All animal care and experimental procedures conformed to American Physiological Society and Washington University School of Medicine guidelines.

Fish were anesthetized in 0.001% MS-222 (Sigma) and injected with 0.1 ml of 2% pancuronium bromide (Sigma). The VIIIth nerve was transected with iris scissors at one of two locations: the anterior ramus of the VIIIth nerve, which included portions of semicircular canal and utricular nerves, or the anterior portion of the saccular nerve just lateral to its projection into the brain stem. Both unbonded (for morphological examination only) and wired probes were implanted. The sieve electrode was lowered into the transection site with a micromanipulator. The transected ends of the nerve were approximated to both sides of the electrode or teased into the NGTs. The bonding pad of the sieve electrode was mounted to the cranium with cyanoacrylate gel that was immediately hardened with a catalyst (Pacer), and the remainder of the cranium was sealed in a similar fashion. The incision was sutured in layers of fascia, muscle, and skin with the transdermal leads from the bonded probes protruding through the dermis.

For morphological examination, the toadfish were deeply anesthetized in 0.01% MS-222 and perfused through the heart with 0.9% NaCl, 0.1% heparin solution, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The implant was examined to determine if the electrode head was encapsulated with neural tissue. If so, the electrode and surrounding tissue were dissected from the brain and placed overnight in 20% sucrose solution in phosphate buffer. The tissue/electrode block was then placed in embedding medium and sectioned with a cryostat in 40 μm increments. The block was examined after each section to ascertain the presence of tissue contiguous with the electrode. If a gap in the tissue was detected before reaching 200 μm of the electrode face, the implant was considered to be unsuccessful. If sectioning revealed a continuous line of tissue, the tissue/electrode block was sectioned to within 100–200 μm of the electrode face. The block was then reversed and the process repeated. To label nerve fibers that might be extending through electrode pores, the block was incubated in goat serum (Sigma) for 30 min at room temperature, rinsed in phosphate buffer, and then incubated in Neuron Specific Enolase, rabbit anti-human (diluted 1:200) (Accurate Chemical Scientific) overnight at 4°C. The blocks were then incubated at room temperature for 1 h in tetramethylrhodamine goat anti-rabbit (diluted 1:200) (Molecular Probes). The block was then viewed with a Zeiss 510 laser scanning confocal microscope to

FIG. 1. A: scanning electron micrograph of a sieve microelectrode showing the nine active sites and the silicon leads (arrow). The iridium-lined active sites and unlined support pores for this probe design were 5–8 μm in diameter. B: scanning electron micrograph of the 8 μm iridium-lined active site. C: light micrograph of the sieve electrode with nerve guide tube. D: confocal micrograph of multiple regenerating axons growing through an active site of an entubulated silk-like protein with laminin functionality (SLPL) probe 60 days after implantation. Neural tissue has been reacted with a secondary tetramethylrhodamine antibody to neuron specific enolase. Arrows bracket the labeled tissue, which filled >50% of the pore. E: neural activity from the active site in D 45 days after transection. Scale bars (μm): A = 100; B = 8; C = 200; D = 20.
visualize labeled nerve processes. Images were imported into Abode Photoshop and printed with a Kodak XLS printer.

Postsurgical fish in which bonded probes were implanted were tested biweekly for neural activity. The unanesthetized fish were immersed in a shallow aquarium. The rubber cap on the nine-pin connector was removed and a long recording lead was inserted in the connector. The recording lead had minimal tension to prevent the connector from entering the water; however it did not impede fish movements, which were only restricted by the walls of the aquarium. The nine recording sites were tested sequentially, two channels at time, with an AM-systems AC differential amplifier model #1800, and the signal was filtered with a Tektronix 502 differential amplifier. After detection of neural activity, positive channels were recorded single-ended to determine the channel of signal origin. Neural activity was digitized with a Cambridge Electronic Design 1401 interface and stored on a PC using the Spike 2 software package (CED).

RESULTS

A total of 54 probes (both bonded and unbonded) were examined morphologically to assess the efficacy of neural regeneration through the electrode. Morphological examination of the uncoated implants without NGTs (n = 12) demonstrated that 28–35 days was the minimum time needed for the axons to regenerate into or through the implant site. By the end of this period, the electrode head was routinely encased in neural tissue. Histological sections through the tissue/electrode block revealed that, despite the vigorous regenerative response, most axons were not growing through the electrode but instead were traversing around it, with <6% of the pores containing neural tissue. The attachment of NGTs to the uncoated probes increased the success rate to ~20%. The addition of SLPL to the NGT probe (n = 18) proved more successful than the NGT alone as the SLPL/NGT probes had more than double the success rate of uncoated electrodes with NGT, as indicated by the percentage of axons growing through guide tubes. However, during implantation, some retraction of the transected nerves from the NGT was observed. Further success was attained with the addition of a nerve glue, which provided greater adhesion of the cut axons to the inside of the tube, and resulted in greater mechanical stability of the electrode/tissue block that is necessary for successful regeneration. Approximately 70% of these implants (n = 13) contained nerves in contact with both sides of the electrode, with 49% of the pores integrated with regenerating nerves. Figure 2 summarizes the effect of the different treatments.

Figure 3 shows a confocal micrograph taken from the distal face of an entubulated SLPL probe to demonstrate the widespread incursion of regenerating axons. Five of the nine recording sites and ~50% of the nonlined pores are filled with neural material. The arrow indicates the site from which the neural recordings in Fig. 1E were obtained. Figure 1D shows a
close-up of this 20 μm recording site, illustrating multiple axons regenerating through the pore.

To date, seven implanted electrodes have yielded electrophysiological data (Fig. 1E). Three uncoated electrodes \((n = 23)\) yielded spontaneous signals from single sites after implantation in the anterior ramus of the VIIIth nerve. Four SLPL electrodes with NGTs \((n = 18)\), one in the saccular nerve and three in the utricular portion of the anterior ramus, yielded both spontaneous and evoked action potentials in response to mechanical stimulation, with an average of two active channels per implant. The recordings were of high fidelity with background noise ranging from 5 to 20 μV and spike amplitudes averaging between 100 and 200 μV with peak amplitudes >500 μV. Multunit activity was also detected from several sites. Spontaneous discharge rate of the fibers ranged from 30 to 100 Hz. The earliest detection of neural activity occurred 29 days after nerve transection, with signals persisting up to 42 additional days in individual fish.

**DISCUSSION**

The results demonstrate that axons will regenerate through a sieve electrode and that chronic recordings are possible from these electrodes. Histological examination of the uncoated, nonentubulated electrodes showed that axons were growing around rather than through the probe. Therefore, to aid in channeling regenerating axons through the pores, three modifications were made to the electrode: 1) the electrode head was electrostatically coated with a porous thin film of ProNectin L, a silk-like protein with laminin functionality (SLPL); 2) a semipermeable nerve guide tube (NGT) was attached to the electrode head (Fig. 1C); 3) the entubulated SLPL probes were dipped into a neural adhesive solution just before implantation.

Because axons will grow on or through surfaces coated with extracellular matrix molecules such as laminin (Sanes 1989), the face of the electrode was coated with SLPL, a genetically engineered polymer. The polymer contains both GAGAGS crystalline silk-like sequences as well as several repeats of one of the identified laminin binding domains (IKVAV), making it a “super-sticky” laminin analogue (Martin et al. 1997). The morphology of the electrospun polymer coating consists of 1 μm-thick filaments (50–150 nm diameter). This provides a high surface area for cell attachment and maintains the ability to transfer electrical signals between the nerves and the device. The addition of SLPL combined with the NGT and neural glue greatly increased the number of axons regenerating through the pores.

One of the major functional advantages of the hardware designs implemented in this study was the ability to interface the probe with a transdermal connector, which allowed repeated, noninvasive sampling of the regenerating axons. We were able to detect neural activity within 29 days of nerve transection, with a median delay of 42 days posttransection. This was significantly faster than the 49–175 day intervals reported in previous studies, emphasizing the advantage of transdermal leads for repeated sampling. The regeneration kinetics were relatively consistent with our previous observations, which demonstrated that axons transverse the transection site within 14 to 21 days and begin to innervate central nuclei within 21 to 35 days (Mensinger and Highstein 1999). Histological examination of the implant sites indicated that the regenerating axons took approximately 28 to 35 days to either contact the electrode face or transverse the electrode. This slightly longer delay might be attributed to the greater distance the axons needed to transverse because of the presence of the probe. Furthermore, because degenerating axons provide a great attractant for regenerating nerves (Weis and Schroder 1989), the presence of the probe may have diluted the gradient created by the degenerating axons and delayed regeneration. Finally, the neural activity in regenerating axons may be delayed in onset or reduced in amplitude compared with control, thus preventing earlier detection.

Although morphological examination revealed that up to seven electrode sites contained neural tissue in a given electrode, the maximum number of sites that yielded neural activity was three in any one animal. It is assumed that electrical activity can only be recorded from sites in proximity to nodes of Ranvier, as the electrical insulation provided by the myelin sheath might prevent recordings at other sites along the axon. Although the internodal distance is significantly foreshortened in regenerating nerves [240 μm (controls) vs. <100 μm at 10 wk posttransection; Mensinger and Highstein 1999], increasing the chances of an electrode pore being near an active site or node, the minimum distance a node can be situated from an electrode pore and still produce recordable activity remains to be determined. This may explain why the number of recorded sites was always less than the number of electrode sites containing neural material.

Multiple unit activity was recorded from ~50% of the functioning electrode sites. Because initial testing was conducted differentially between two channels, the multiple unit activity could have been attributed to single units contributed by each channel. However, single-ended recordings from a number of these sites also showed multiple unit recording, indicating that multiple nerves were regenerating through the sites, consistent with the histological examination.

These experiments demonstrated that regenerating axons will grow through a sieve electrode and that neural recording is possible from unrestrained fish. Functional recovery with the nerves regenerating through the sieve roughly paralleled that seen in previous studies (Mensinger and Highstein 1999). The eventual integration of the sieve electrode with a telemetry device (Mensinger and Deffenbaugh 1998) promises to allow chronic recording from free swimming fish, which will aid in determining how the nervous systems functions in a natural environment. Information gained from such recordings might greatly enhance our knowledge of the neural correlates of behavior.

The sieve prosthesis also holds promise for clinical applications. The ability of mammalian peripheral nerves to at least partially regenerate after injury suggests that the device could serve as a conduit for signal transmission past injured nerves. In systems that have limited natural regeneration such as spinal cord, neurotrophins could be incorporated with the SLPL molecule to stimulate sufficient growth to have nerve sprouts enter the electrode and establish a neural interface for prosthesis control.

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