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In vitro model of glial scarring around neuroelectrodes chronically implanted in the CNS

Vadim S. Polikov[a], Michelle L. Block[b], Jean-Marc Fellous[a], Jau-Shyong Hong[b], W. Monty Reichert[a,*]

[a]*Department of Biomedical Engineering, Duke University, Durham NC 27708-0281, USA*
[b]*Neuropharmacology Section, National Institute of Environmental Health Sciences, 111 Alexander Drive, Research Triangle Park, NC 27709, USA*

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Abstract

A novel in vitro model of glial scarring was developed by adapting a primary cell-based system previously used for studying neuroinflammatory processes in neurodegenerative disease. Midbrains from embryonic day 14 Fischer 344 rats were mechanically dissociated and grown on poly-D-lysine coated 24 well plates to a confluent layer of neurons, astrocytes, and microglia. The culture was injured with either a mechanical scrape or foreign-body placement (segments of 50 μm diameter stainless steel microwire), fixed at time points from 6 h to 10 days, and assessed by immunocytochemistry. Microglia invaded the scraped wound area at early time points and hypertrophied activated astrocytes repopulated the wound after 7 days. The chronic presence of microwire resulted in a glial scar forming at 10 days, with microglia forming an inner layer of cells coating the microwire, while astrocytes surrounded the microglial core with a network of cellular processes containing upregulated GFAP. Vimentin expressing cells and processes were present in the scrape at early times and within the astrocyte processes forming the glial scar. Neurons within the culture did not repopulate the scrape wound and did not respond to the microwire, although they were determined to be electrically active through patch clamp recording. The time course and relative positions of the glia in response to the different injury paradigms correlated well with stereotypical in vivo responses and warrant further work in the development of a functional in vitro test bed.

Keywords: Neural prosthesis; Foreign-body response; In vitro test; Cell culture; Brain; Biocompatibility

1. Introduction

While the promise of using thought controlled robotics to treat paralysis is very exciting, the electrodes implanted in the brain to record neuronal signals have failed to create a stable, long-term interface for obtaining the necessary control signals [1–3]. It is generally believed that a well defined, although poorly understood tissue reaction against the recording electrodes is the primary cause of the signal degradation experienced over time [4]. The in vivo response to the implantation of a chronic electrode can be divided into two stages: the initial acute response to the mechanical injury of insertion, and the chronic response resulting in a glial scar [4,5]. As the electrode is inserted into the cortex, its path severs capillaries, extracellular matrix, and glial and neuronal cell processes. Activated, proliferating microglia, the immune cells of the CNS, appear around the implant site as early as 1-day postimplantation as they migrate towards the injury site to initiate wound healing and debris clearance [5–7]. Astrocytes are also activated within the first few days and upregulate glial fibrillary acidic protein (GFAP) as far as 500 μm away from the injury site [5]. This initial reaction is transitory however, as electrode tracks are not found in animals after several months when the electrode is inserted and quickly removed [8–11].

If an implant is chronically present, a foreign-body reaction is observed, although it is unclear whether such a reaction occurs due to unresolved acute inflammation, the toxic nature of the implant material, chronic micromotion between the implant and the neural tissue, or through some

*Corresponding author. Tel.: +1 919 660 5151; fax: +1 919 660 5362.
E-mail address: reichert@duke.edu (W.M. Reichert).

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other mechanism [5,9,12–14]. This reaction is characterized by the presence of both reactive astrocytes and activated microglia [5,8,15,16]. Microglia will cluster around the implant in a reactive tissue sheath and will persist for the life of the implant [5,14,16,17]. The astrocytes surround this inner core of microglia in an encapsulation layer referred to as the "glial scar" [5,10,15,17–22]. Imaging studies in the first 2 weeks after insertion have revealed a reactive astrocyte region surrounding the implants extending out 500–600 μm [5]. This region decreased over time, but the layer of cells immediately adjacent to the implant become denser and more organized. The mesh of astrocytic processes becomes stronger and more compact until a complete glial scar is formed at around 6 week post-implantation.

The biological testing of neuroelectrodes has been almost exclusively performed in vivo, where microelectrode designs aimed at overcoming the tissue response are implanted into an animal model (typically rat cortex), followed by sacrifice of the animal at various time periods, and assessment of the extent of the tissue reaction to the implant (see recent review [4] and references therein). Yet, after more than two decades of in vivo testing, the mechanisms behind the signal degradation of chronically implanted electrodes remain unclear. The neural implant field would benefit from an in vitro system capable of dissecting the complicated mechanisms behind implant failure and that allows high throughput testing of new neuroelectrode designs.

This paper presents an in vitro cell culture system that has been adapted from a culture system used to study neuroinflammatory processes for the past 15 years. This culture contains all of the brain cell types known to play a major role in the tissue reaction, and that successfully recreates many of the hallmarks of glial scar formation. The cellular responses of neurons, astrocytes, and microglia to injury were characterized using immunocytochemistry. Mechanical injury, in the form of a scrape to the confluent cellular layers, and chronically placed stainless steel microwire, mimicking the presence of a foreign body, resulted in cellular responses that were similar to those documented in vivo. This system will be employed as a useful tool for the neuroelectrode biocompatibility field in understanding the causes of implant failure.

2. Materials and methods

2.1. Reagents

Cell culture ingredients were obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies against the CR3 complement receptor (OX-42) and against MAP-2 were obtained from Chemicon (Temecula, CA, USA). Polyclonal antibody against IBA-1 was obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA). Polyclonal antibody against GFAP was bought from DAKO Corporation (Carpinteria, CA, USA). Monoclonal antibody against vimentin was bought from Sigma–Aldrich (St. Louis, MO, USA). The Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). 50 μM diameter stainless steel microwire was bought from A-M Systems (Carlsborg, WA, USA). Secondary antibodies with fluorescent tags Alexa 594 and Alexa 488 were bought from Molecular Probes (Invitrogen Corporation, Carlsbad, CA, USA).

2.2. Animals

Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). Housing and breeding of the animals were performed in strict accordance with the National Institutes of Health guidelines at the National Institutes of Environmental Health Sciences (Research Triangle Park, NC, USA).

2.3. Primary mesencephalic neuron-glia cultures

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 13–14 rats, as described previously [23]. Briefly, dissociated cells were seeded at 5×10^5/well into poly-D-lysine-coated 24-well plates. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 10% horse serum (HS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin. Seven-day-old cultures were used for treatment after a media change to MEM containing 2% FBS, 2% HS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin. Data shown are representative of at least 3 different culture preparations.

2.4. Scrape (mechanical injury) model

At treatment time, a rectangular area in the middle of the culture well, approximately 2 mm on each side, was cleared of cells with a 2 mm long scrape of the tip of a cell scraper (#3010, Corning Inc., Corning, NY, USA). The injury was inflicted after the media change so that soluble factors released during the injury were present.

2.5. Wire (foreign body) model

Wire was cut into 3–5 mm pieces and soaked in 70% ethanol for at least 30 min, after which it was allowed to dry in a laminar flow hood. At treatment time, 3–4 pieces of wire were placed into each treatment well at random locations using sterile forceps, so that the pieces would sink and rest atop the cultured cell layer.

2.6. Immunostaining

Microglia were detected with the OX-42, which recognizes the CR3 receptor as described [24], or anti-IBA-1 antibody, which recognized a calcium binding protein specific to microglia, astrocytes were detected with an antibody against GFAP, and neurons were imaged by staining with MAP-2 as described previously [25,26]. Immature glia were detected with an antibody against vimentin. Briefly, formaldehyde (3.7%)—fixed cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution (20 min), primary antibody (overnight, 4 °C), biotinylated secondary antibody (1 h), and ABC reagents (1 h). Color was developed with 3,3′-diaminobenzidine. Images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN, USA) operated with the MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). Fluorescently labeled cultures were stained in the same way, except a fluorescently labeled secondary antibody was used in place of ABC reagents. Fluorescent images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (Sensicam QE, Cooke

Corporation, Romulus, MI, USA) operated with the IPLab software (Scanalytics, Rockville, MD, USA).

2.7. Patch clamp recordings

Patch-clamp was performed under IR-DIC visual control at room temperature, in the treatment medium. Whole-cell patch-clamp recordings were achieved using glass electrodes (4–10 M) containing the following (in mM): 140 KmeSO4, 10 HEPES, 4 NaCl, 0.1 EGTA, 4 Mg-ATP, 0.3 Mg-GTP, and 14 phosphocreatine. Recordings were performed in the culture medium (MEM). Data were acquired in current-clamp mode using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA). Data were digitized using a custom software written using LabView 6.1 (National Instrument, Austin, TX), and data were acquired with a PCI16-E1 data acquisition board (National Instrument). The data acquisition rate was 10 kHz. Stimuli were designed on-line, or off-line as text files. All experiments were performed in accordance with animal protocols approved by the National Institutes of Health. Data shown are representative of 5 cells recorded from two different culture preparations. Data were analyzed off-line using MATLAB (MathWorks, Natick, MA) and Excel (Microsoft Corp, Redmond, WA).

3. Results

3.1. Scrape model

To characterize the response of the neuron-glia culture to a mechanical injury of the sort experienced by neural tissue upon implant insertion, cultures were scraped with a cell scraper to create an area empty of cells and filled with cell debris. Using immunocytochemistry to stain for different cellular markers, the time course of the cellular response for astrocytes and microglia is shown in Fig. 1A. GFAP positive astrocytes (arrows) begin to extend short processes into the wound area at 6 h post injury, extend their processes considerably through 24 and 48 h, and by 7 days have completely repopulated the wound area, expressing an activated, enlarged phenotype. Microglia enter the wound site much quicker (in as early as 1 h) and by 6 h have already spread out within the wound. The microglial cell numbers increase throughout the 7-day time course as they are attracted by the open space, the chemoattractants released by other microglia, or to the cellular remains within the scrape. By 48 h, there are more microglia inside of the scrape wound than in the surrounding tissue, as microglia migrate into the wound and line up along the striations of cell remains created by the cell scraper. Neurons, however, do not grow back into the wound area, even after it is repopulated by astrocytes, although some processes are extended beyond the initial boundary of the scrape injury (data not shown). GFAP−/vimentin+ astrocyte precursor cells (arrowheads, Fig. 1A) can be faintly seen (due to background staining) as spindle-shaped cells migrating ahead of the astrocyte processes at 6 h, spreading inside the wound at 24 and 48 h, and, after maturing and upregulating GFAP, eventually resulting in a carpet of activated GFAP+/Vim+ astrocytes at 7 days.

3.2. Wire model

To characterize the response of the neuron-glia culture to a chronic foreign-body placement, 3–4 pieces of 3–5 mm long, 50 μm diameter stainless steel microwire were placed into each treatment well with forceps and allowed to sink onto the cell layer. The wire is of the size and type commonly used by neurophysiologists to make in vivo recording arrays. Immunocytochemistry was again used to identify the cellular response to these model electrodes (Fig. 1B). Microglia migrated to the microwire and sat atop the wire as early as 6 h after wire placement. The number of microglia on the wire increased over time until the entire wire was coated with a layer of cells. This microglial layer remains for as long as the cultures have been maintained (14-day posttreatment), although at later times the cells express a larger, rounded phenotype indicative of multinucleated giant cells. Astrocytes do not seem to respond to the microwire until around 7–10-day post treatment, at which time they begin to form a cellular sheath reminiscent of the glial scar around the microglia coated wire. A more detailed look at the scar formation (Fig. 2) shows that while this astrocytic scarring is commonly seen around every piece of microwire in the culture, there is a wide variation in the intensity of scar formation around different wires, and in fact around different parts of the same wire. Furthermore, the in vivo layering of astrocytes surrounding a microglial core sitting atop an implant is maintained in this culture system as seen in Fig. 3A and B. Previous in vivo studies have also shown upregulation of vimentin, a structural protein expressed in immature cells, around glial scars. Staining for vimentin revealed it to be a useful marker for glial activation around the microwire, as vimentin was highly upregulated in the astrocyte processes forming the glial scar (Fig. 3C).

Staining for MAP-2 revealed that neurons are not affected by their proximity to the wire, as both neuronal soma and processes were maintained in the same density next to the wire as in areas of the culture away from the foreign body (Fig. 4A). To verify whether the neurons in culture were healthy and electrically active, patch clamp recordings on individual neurons were performed. Neurons were identified by morphological markers and patch clamping was performed on neuron-glia culture preparations, at different post treatment time points, while the cells were in their treatment medium. Action potentials were clearly distinguishable (Fig. 4C) and had a physiologically accurate shape profile (Fig. 4D). Finally, it was confirmed that the neurons would respond to stimuli such as a depolarizing current by electrically activating the cells into producing action potentials (Fig. 4E). Note that because recordings were made in the culture medium, the giga-ohm seal was typically of poor quality, and voltage deflections were smaller than would be expected in an Artificial Cerebrospinal Fluid extracellular environment. Nevertheless, action potentials were clearly visible, and had typical shapes.

4. Discussion

The development of implantable neuroelectrodes is following the well-worn path of all implantable biomedical

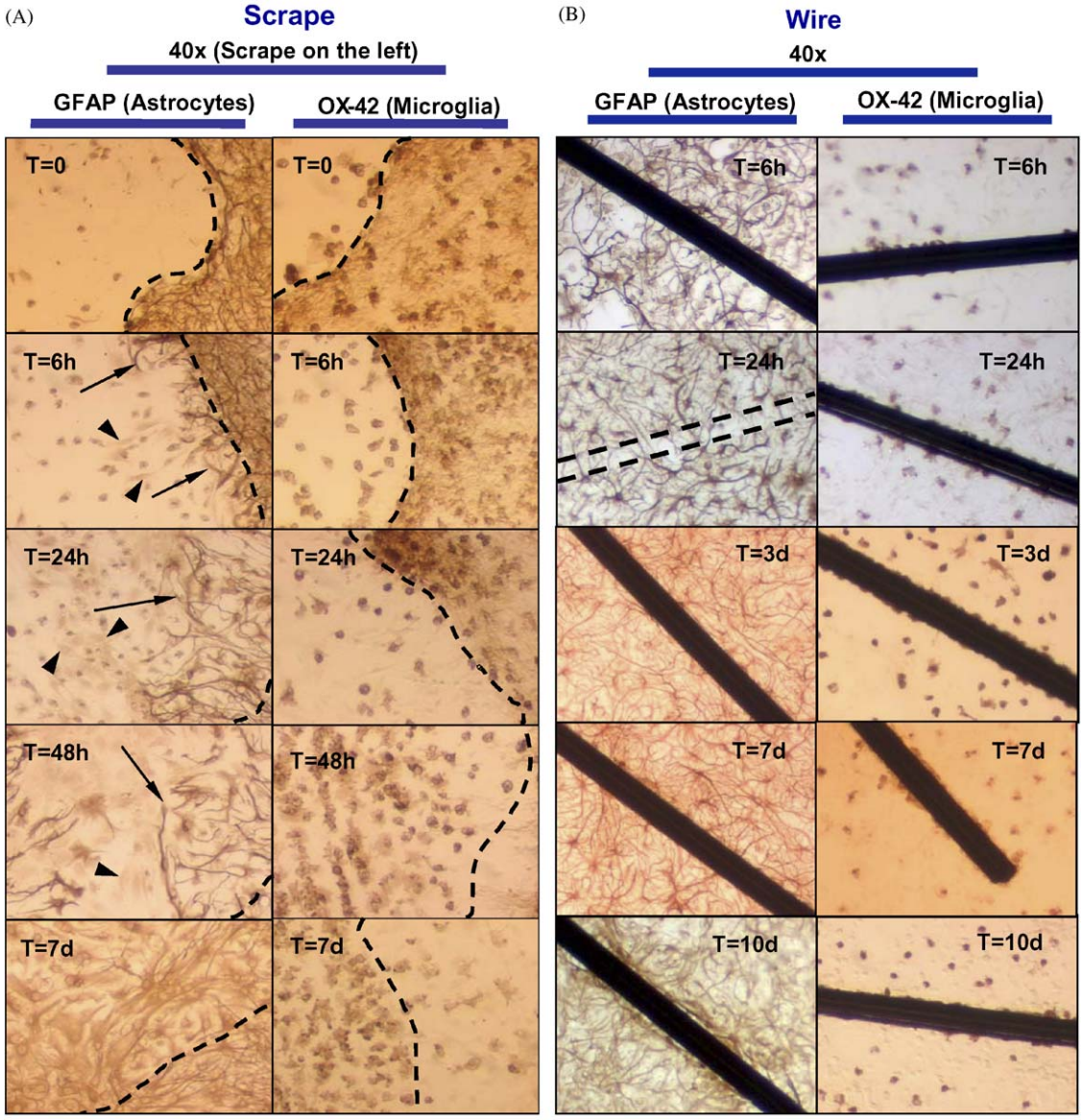

Fig. 1. (A) Time course of cellular events in response to the Scrape wound. The area scraped free of cell is on the left of the dotted line. The left panel shows the time course of astrocytes as stained for GFAP and the right panel shows the microglial response over time as stained for MAC-1 (OX-24 antibody). Astrocytes are seen to send processes (arrows) into the wound beginning at 6 h and continuing through 48 h, and completely re-colonize the wound by 7 days. GFAP negative spindle-shaped precursor cells (arrowheads) that do not stain for microglial markers but stain for vimentin (not shown) migrate into and colonize the wound ahead of the GFAP positive processes. Microglia migrate to and spread out within the wound by 24 h and their numbers increase over time, until by 7 days there are more microglia inside the wound than in the surrounding culture. (B) Time course of cellular events in response to the Wire placement. Microglia attach to the wire as early as 6 h and increase in numbers until a layer of microglia 1–2 cells thick is formed covering the length of the wire. This layer remains through 10 days in culture. Astrocytes show now response to the microwire until 7 days after treatment, when the beginnings of a response may be seen. By 10 days after treatment, a layer of activated astrocytes with upregulated GFAP forms around the microwire, mimicking the glial scarring seen in vivo.

sensors and electrodes; i.e. employing in vivo assessment for determining device suitability for chronic implantation. In vivo implantation studies are time consuming, expensive, and nearly impossible to control down to a level where the molecular mechanisms of implant failure can be understood [27]. The result is that innovations addressing implant-tissue compatibility invariably lag behind innovations in electrode design. This in vivo experimental paradigm has produced only incremental improvements in neuroelectrode tissue compatibility because it is used for what it does poorly, the primary screening of implant modifications, rather than for what it does well, the validation of implants that were pre-screened in vitro.

This paper presents a novel in vitro model of the tissue response to chronically implanted neuroelectrodes. The model is an adaptation of the neuron-glia culture system developed over the past 15 years in the laboratory of Dr. Hong for examining the neuroinflammatory

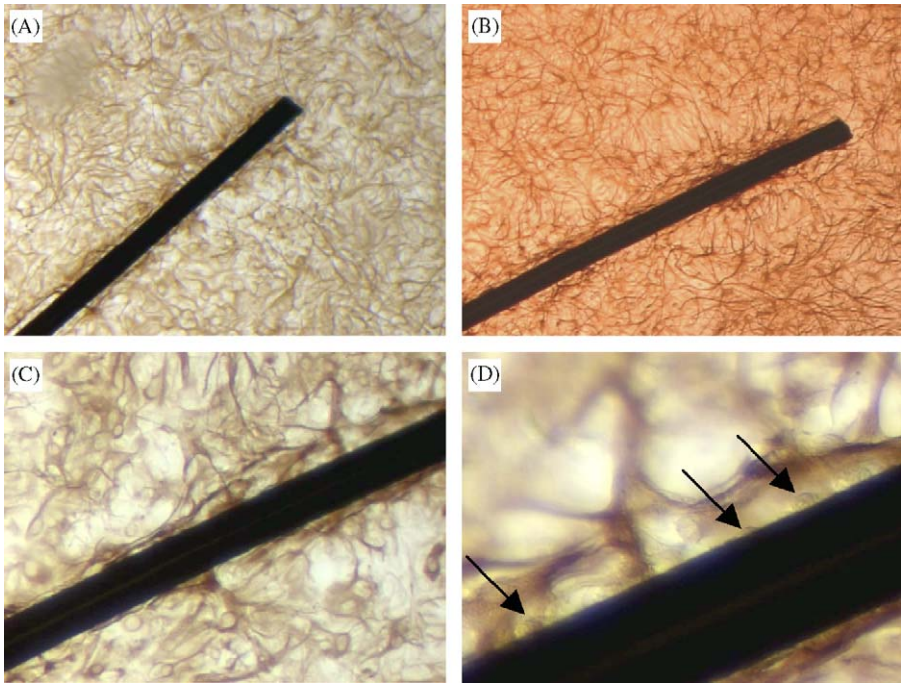

Fig. 2. Detail of the astrocyte glial scarring response to microwires placed in culture. All images are of cultures fixed 10 days after wire placement and stained for GFAP. For size reference, all wires are 50 um in diameter. (A–B) Astrocyte processes with upregulated GFAP surround a microwire along its entire length in a pattern similar to that seen in vivo around recording electrodes. (C–D) Higher magnification images of glial scarring in (A). Unstained cells (microglia) can be seen sitting directly on the wire inside of the GFAP positive processes (arrows).

mechanisms behind neurodegenerative diseases. The strength of the system is the physiologically relevant mix of neurons, astrocytes, and microglia that cannot be replicated by traditional cell-line based in vitro systems. Furthermore, this culture system has been verified by numerous in vivo studies, lending credibility to the in vitro data [24,28–30]. As a result, the culture system is able to reproduce many of the hallmarks of the glial response to implanted neuroelectrodes.

The relative locations of the astrocytes and microglia and the time course of their responses in both the scrape and the microwire models correlate well with previously published in vivo data. Microglia are know to be the first responders to any injury in vivo as they constantly sample their local environment and migrate through neural tissue [7,31–33]. Therefore, it is not surprising that microglia were seen inside the scrape wound and atop the microwire in the first time points assayed, 1 and 6 h, respectively. The commonly observed microglial accumulation at the site of injury was also seen in this culture system as microglia increased both within the mechanically damaged area and near the wire to levels that were visibly higher than in the surrounding culture.

As observed in vivo, the response of the astrocytes followed that of the microglia. Astrocytes present at the borders of the scrape extended their processes into the empty area and continue to elongate those processes over several days. However, the majority of astrocytes populating the interior of the scrape after 7 days are morphologically distinct from the astrocytes in the rest of the culture. Typically, the astrocytes are thickened, have fewer processes, and arise from the Vim+/GFAP− precursor cells that migrate into the wound. Vim+/GFAP− cells have been shown to form a thin sheath around implanted electrodes in the region that is interior to the majority of the astrocytes forming the glial scar [14]. The distribution of cells responding to the scrape correlates well to the concentric profiles of cells around the probe: an inner layer of microglia, an intermediate layer of Vim+/GFAP− cells, and an outer layer of astrocytes. The concentric profiles are also seen around the microwire, where microglia form the inner core of the glial scar and the astrocytes form the outer region. Although increased vimentin staining was seen around the microwire, a tight concentric ring was not observed.

Every stainless steel microwire placed in culture thus far has developed an adherent layer of microglia, yet there is visible variation in the astrocyte response to the microglia coated microwire. Some microwires develop a thick scar while others have only a few highly GFAP+ astrocyte processes around the foreign-body. There is even variation on each microwire, with some sections of the microwire having thicker scarring than other sections. This variability may be a cause of concern in developing a reproducible in vitro model, however the in vivo response has been shown to be just as variable, if not more variable [16,17,34]. The variability of the in vivo tissue response has also been linked to the unreliable recording capabilities of implanted electrode arrays [13].

There is a clear difference between embryonic and adult mammalian CNS responses to injury [35–37]. Embryonic

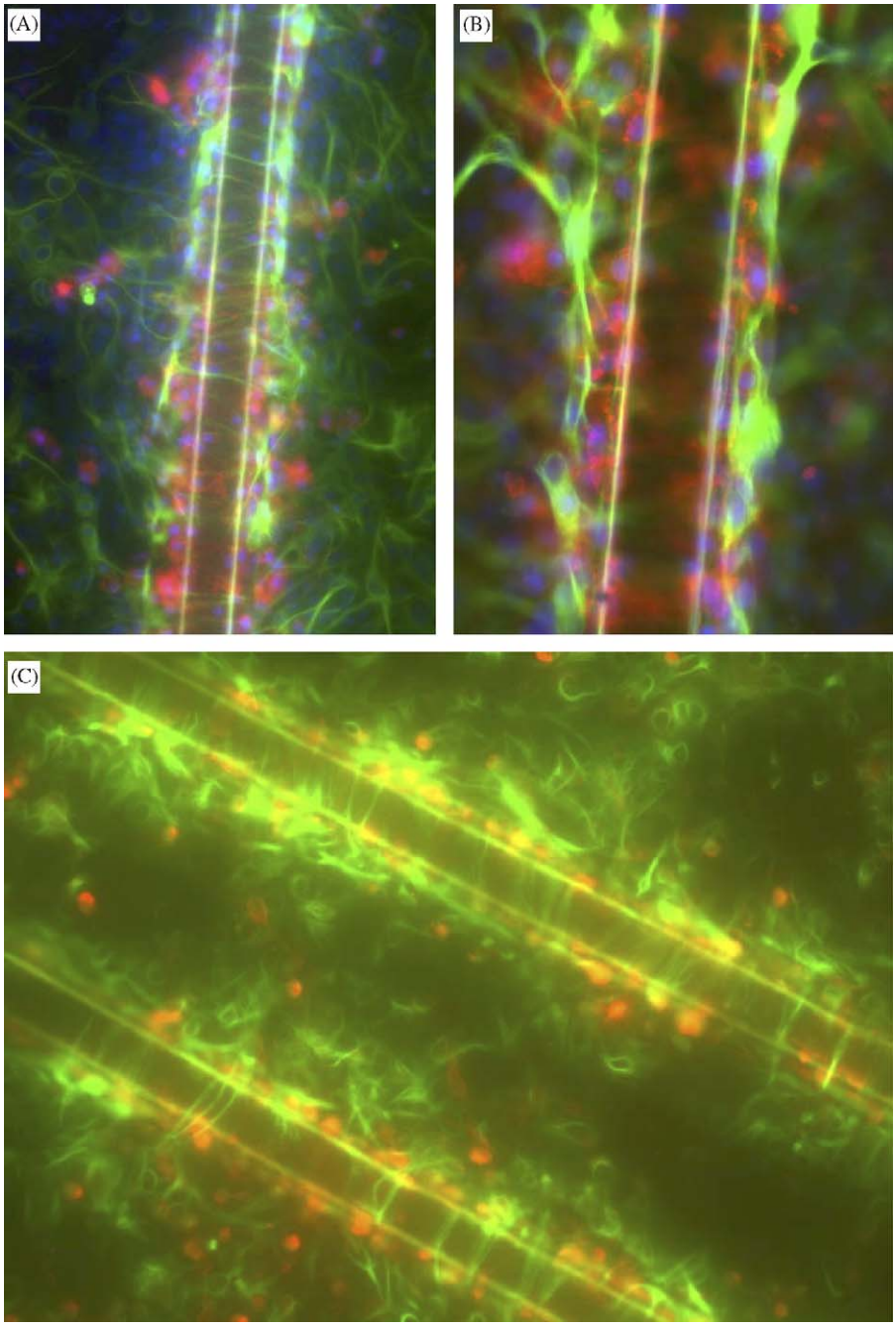

Fig. 3. (A–B) Triple fluorescent labeling with DAPI staining nuclei blue, GFAP staining green, and OX-42 staining microglia red shows the relative positions of different cells near the wire after 10 days in culture. Just as observed in vivo, there is a layer of microglia (Red) adjacent to the microwire and astrocytes (Green) outside of the microglial layer showing upregulated GFAP. The image in (B) shows the glial scarring at a higher magnification, clearly visualizing the prevalence of microglia around the microwire. For reference, the wire diameter is 50 um in all images. (C) Dual fluorescent labeling with Vimentin staining immature/activated astrocyte processes green and OX-42 staining microglia red. The layer of microglia sitting on the wire is surrounded by a bright halo of vimentin positive astrocyte processes forming the glial scar 10 days after wire placement.

cells regenerate readily from mechanical insults and do not produce a typical glial scarring response [38,39]. One line of experiments has pointed to a critical period of development around postnatal week two or three after which a mature glial scar is capable of forming [38,40]. The embryonic day 14 cultures described in this paper are prepared before this critical period and may not fully represent the adult scarring environment. However, culture preparations after the critical period are severely limited in their utility and relevance as the minimal regenerative potential of adult cells leaves only the most hardy, highly activated glia while all of the neurons and most of the glia die off. This culture system also does not contain vasculature, which has been shown to play an important role in the glial scarring process [41], although the presence of serum in both the growth medium and the treatment medium may help to

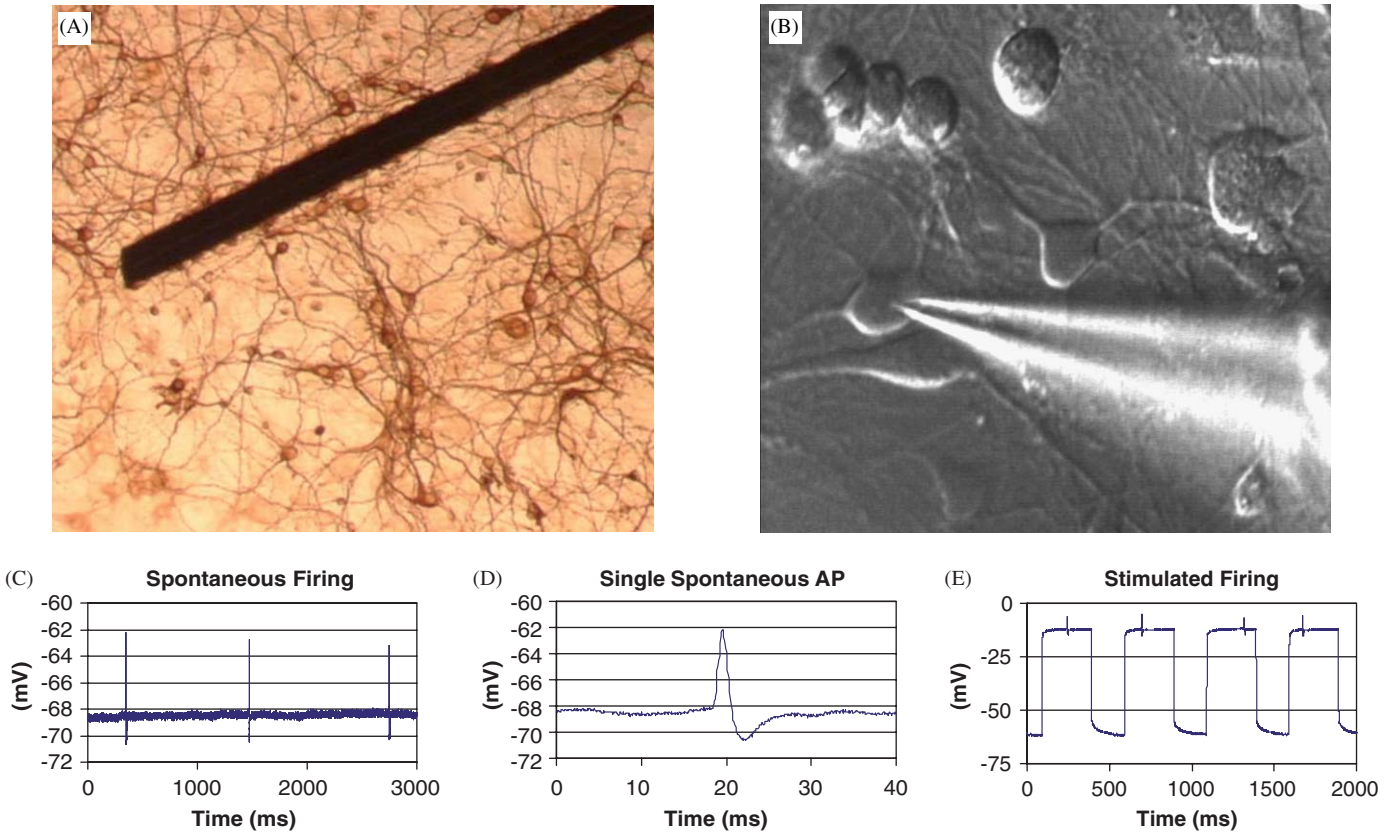

Fig. 4. Neuronal responses in the culture: (A) MAP-2 staining shows a network of neuronal processes and soma that are not affected by the microwire 14 days after it was placed in culture. (B) Light microscope image of the microelectrode in a patch clamp on a neuron within the neuron-glia culture. (C) Spontaneous firing of a neuron in a current clamp mode trace in which three action potentials are clearly seen. (D) A higher resolution picture of the first action potential in C, showing the physiologically accurate shape of the action potential. (E) The clamped neurons could be activated with depolarizing current. Four action potentials can be seen in response to the four depolarizing steps.

alleviate this deficiency. Although there will always be differences between an in vitro culture and the in vivo environment, the similarities between the glial response in this culture to an in vivo mechanical injury or to a foreign-body implantation suggest that these embryonic cultures are capable of providing useful insights into the scarring process.

The response of this in vitro system that contrasts most with some observed in vivo scarring processes is the neuronal behavior. The neurons in this culture seem to be unaffected by the proximity of activated glial cells responding to both the scrape and the microwire. In vivo, neurons have been found to be depleted by an unknown mechanism around a recording electrode, presumably leading to signal degradation over time and eventual implant failure [8,17]. Such depletion was not observed around the microwire or on the boundaries of the scrape. Neurons extended processes around and under the microwire and there was no observable difference in neuronal cell body density around the microglia and astrocytes surrounding the microwire. Although neurons did not re-colonize the scrape area, bordering neurons sent a few processes into the scrape atop of growing astrocyte processes. The lack of a clear neuronal response could be due to the same enhanced regeneration potential that allows neurons to survive the culture preparation. Alternatively, since the neurons do not survive in culture after 10-day posttreatment (17-day postisolation), it may take longer than 10 days to see the neuronal depletion observed in vivo after 4–6 weeks. If the depletion of neurons in vivo around the electrode occurs because the neurons are pushed away by glial cells forming the glial scar, this may not be reproduced in a two-dimensional in vitro culture where cells can grow atop one another and are not limited by three-dimensional space restrictions. Finally, microwire sitting in cell culture does not reproduce the micromotion and resultant injury that may occur at the interface between a tethered, rigid electrode and the soft neural tissue within a free-floating brain. It may be beneficial to examine the role of micromotion by physically moving the microwire inside the culture at various stages of glial scar formation or mimicking micromotion with culture plate vibration or media stirring.

To test whether the neurons in this culture system display functional characteristics similar to cells in vivo, patch clamp recordings were performed to assess the activity of the neurons. The neurons were confirmed to be

spontaneously active, maintained a physiologically accurate action potential shape, and were responsive to depolarizing currents. This ability to record electrical signals from the culture bodes well for future work in creating a functional in vitro test bed capable of correlating the tissue reaction to neuroelectrode recording performance.

5. Conclusions

Owing to the cost of in vivo experimentation in terms of resources, time, and animals, an in vitro cell culture system which recreates many of the characteristics of the in vivo reaction to chronically implanted neuroelectrodes would be of significant benefit to the brain implant field as a test bed for novel neuroelectrode innovations and as a way to dissect the complicated mechanisms behind implant failure. Such an in vitro model was developed by adapting a culture system previously used for neuroinflammatory disease research. This culture system contains a physiologically relevant mix of astrocytes, microglia and neurons, resulting in cellular responses that closely mimic the tissue response seen in vivo. The response of the different cell types to a mechanical injury and to a foreign body contacting the cells was characterized in an effort to recreate the acute and chronic injury response seen in vivo. Microglia were observed responding to both types of injuries within a few hours, eventually coating a microwire placed in the culture. Astrocytes filled up the space left vacant by the mechanical injury and upregulated GFAP in a glial scar around a microwire in the culture. Both the time courses and relative positions of the glia in response to the different injury paradigms were similar to the response seen in vivo and the neurons were spontaneously active in culture, suggesting further work in the development of a functional in vitro test bed.

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