Biosensors and Bioelectronics 24 (2009) 2365–2370

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Passaged neural stem cell-derived neuronal networks for a portable biosensor

Thomas J. O'Shaughnessy*, Jinny L. Liu, Wu Ma[1]

Center for Bio/Molecular Science and Engineering, Code 6900, Naval Research Laboratory, Washington, DC 20375, USA

ARTICLE INFO

Article history:
Received 11 September 2008
Received in revised form
14 November 2008
Accepted 3 December 2008
Available online 9 December 2008

Keywords:
Neural stem cells
Differentiation
bFGF
BDNF
Microelectrode array
Biosensor

ABSTRACT

We have previously demonstrated a portable biosensor that utilizes networks of mammalian neurons on microelectrode arrays (MEAs) as the sensing element. These neuronal cultures on MEAs are derived from primary neuronal tissues and are short-lived. In order to extend the shelf life of neuronal networks for use in a fieldable sensor technology, a renewable source of networks is needed. Neural stem and progenitor cells are capable of self-renewal and differentiation into functional neuronal networks. The purpose of this study was to develop a strategy for growing passaged neural stem and progenitor cells on MEAs under controlled conditions to produce differentiated neurons and glia comprising functional neuronal networks. Primary and passaged neuroepithelial stem and progenitor cells dissociated from embryonic day 13 rat cortex were seeded on MEAs and maintained with serum-free medium containing basic fibroblast growth factor (bFGF) combined with brain-derived neurotrophic factor (BDNF). These culture conditions lead to abundant neurons, with astrocytes as supportive cells, forming synaptically linked networks of neurons. Spontaneous action potentials were best recorded from networks derived from primary or passaged progenitor cells 4–5 weeks after initial culture. The passaged progenitor cell-derived networks on MEAs responded to the $GABA_A$ antagonist bicuculline, the NMDA glutamate inhibitor APV, and the non-NMDA glutamate antagonist CNQX indicating active synapses were present. Passaged neural stem and progenitor cell-derived networks on MEAs have properties similar to networks derived from primary neuronal cultures and can serve as a renewable supply of sensor elements for detection of environmental threats.

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1. Introduction

Development of environmental biosensors based on living cells is occurring at a rapid pace. These biosensors use functional biology to detect both unknown toxicants as well as hazardous combinations of otherwise non-threatening compounds. This fills a void in the detection capabilities of specific detector systems that can rapidly identify and quantify the presence of the compounds they are designed to detect. These types of systems cannot, however, detect compounds for which they have not specifically been designed, nor can they determine if a mixture of harmless compounds is, in fact, dangerous to humans. As such, the cell-based biosensors can play a synergistic role with the more traditional, specific detector systems. However, there are logistical hurdles that are limiting the acceptance of cell-based sensors for field use (O'Shaughnessy and Pancrazio, 2007). In particular, the source and delivery of the cells needed to function as the biosensor elements.

Our laboratory has previously reported the development of a biosensor system that combines microelectrode array technology with neuronal cell culture techniques to yield physiologically relevant information about a broad set of known and unanticipated threats (Gray et al., 2001; Pancrazio et al., 2003). Extracellular monitoring of the bioelectrical activity from the cultured neuronal networks grown on microelectrode arrays provides a non-invasive method for monitoring network activity. Changes in the rates and firing patterns of action potentials in the network indicate the presence of neuroactive compounds. This system has been successfully demonstrated in recent exercises in which detection of toxins in both potable water samples (O'Shaughnessy et al., 2004) and in sea water were performed (Kulagina et al., 2004, 2006). A strong correlation between neuronal network sensitivity and lethal dosages of a range of environmental threats has also been presented (O'Shaughnessy and Pancrazio, 2007). However, the neuronal networks used in the biosensor are primary cultures obtained from mice. This reliance on obtaining cells directly from an animal source presents a distinct disadvantage to the neuronal network biosensor system. While under laboratory conditions, the neuronal networks have been shown to survive as long as 9 months (Gross and Rhoades, 1995), their life expectancy for field use

* Corresponding author at: Center for Bio/Molecular Science and Engineering, Code 6900, Naval Research Laboratory, 4555 Overlook Avenue, Washington, DC 20375, USA. Tel.: +1 202 404 6120; fax: +1 202 767 9594.
E-mail address: thomas.oshaughnessy@nrl.navy.mil (T.J. O'Shaughnessy).
[1] Present address: Stem Cell Center, Developmental Biology, American Type Culture Collection (ATCC), Manassas, VA 20110, USA.

0956-5663/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bios.2008.12.007

is limited to perhaps 2 weeks, thus requiring constant replacement.

A potential solution to this problem is to use neural stem and progenitor cells as a renewable source of neuronal networks. Neural stem cells can be made to proliferate and under controlled conditions they are able to differentiate into functional neuronal networks (Mistry et al., 2002). Mistry et al. (2002) found that mitogenic growth factors can synergize with N-CAM or neurotrophins to generate spontaneously active neuronal networks from primary neural progenitors obtained from the hippocampus. The neurons in these networks were found to express traits similar to mature hippocampal neurons.

The present work was aimed at determining a set of suitable culture conditions to allow for the generation of functional neuronal networks from neural stem and precursor cells derived from the early cortex. It was of particular interest to demonstrate that functional networks could be generated not only from primary neural stem and progenitor cells, but also cells that had been grown and passaged in culture. A large number of neural stem and progenitor cells can be produced through passaging. The ability to generate functional networks from a renewable supply of stem and progenitor cells represents a distinct benefit for a neuron-based biosensor as it removes the reliance on primary dissection to obtain the sensor element, i.e. the neuronal networks.

2. Materials and methods

2.1. Cortical stem and progenitor cell cultures on microelectrode arrays

The dissociation and expansion of cortical precursor cells have been previously described (Ma et al., 1998). Briefly, embryos were removed from the dams at embryonic (E) day 13 and placed into Earle's balanced salt solution (EBSS; Invitrogen, Carlsbad, CA). Embryonic day 1 was defined as the day of conception established by the presence of a vaginal plug. The crown-rump length was measured to confirm the embryonic age. The cortical neuroepithelium was dissected from E13 rats. Dissected areas corresponded to the formative dorsal telencephalon according to the atlas of the prenatal rat brain (Altman and Bayer, 1995). Tissue was dissociated by mechanical trituration in EBSS with a sterile, fire-polished glass Pasteur pipette. The cells were collected by centrifugation and resuspended in serum-free medium consisting of Neurobasal medium (NB; Invitrogen) supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine (Invitrogen), and containing 60 ng/ml of recombinant human basic fibroblast growth factor (bFGF; Intergen, Purchase, NY). NB/B27 has been demonstrated to support the long-term survival of embryonic central neurons and tumor cell lines of neuronal origin (Brewer et al., 1993). Cells (30×10^3) in NB/B27 medium containing bFGF were plated onto microelectrode arrays (MEAs) that were pre-treated with poly-D-lysine (PDL; BD, Franklin Lakes, NJ) and fibronectin (Invitrogen). Surface treatment consisted of a 20 μg/ml solution of PDL in water being placed on the surface of the MEAs and left overnight at room temperature. The arrays were then rinsed with water and treated with 0.3 ml of 10 μg/ml fibronectin in PBS. The fibronectin solution was left on the arrays for 1 h at room temperature before being aspirated off the arrays. Cells were also plated onto similarly treated 35 mm plastic dishes for further passaging. After seeding, cultured networks were maintained at 37 °C in NB/B27 medium containing bFGF for 5 days, followed by the addition of brain-derived neurotrophic factor (BDNF; Intergen, Purchase, NY) or the cell adhesion molecule N-CAM (Abcam, Inc., Cambridge, MA) at a concentration of 100 ng/ml for 3–4 days. Afterward, the culture medium was depleted and replaced with NB/B27 without bFGF. The medium was renewed once or twice a week. The networks were stored in a 5% CO_2 incubator until use.

2.2. Passaged cultures of neural stem and progenitor cells

Cultures were either used immediately (primary culture or passage 0; p0) or after being passaged one time (passage 1; p1). Passaging of neural precursor cells was carried out after 9 days. After growing for 9 days in 35 mm plastic dishes coated with PDL and fibronectin, the neural progenitor cells were incubated in 2 ml trypsin-ethylenediamine tetraacetic acid (EDTA) (0.5% w/v trypsin, 5.3 mM EDTA; Invitrogen) for 10 min at 37 °C. Next, 0.2 ml of trypsin inhibitor (Invitrogen) was added and the cells were centrifuged for 10 min at 1000 × *g*. After centrifugation, the cells were resuspended in 2 ml NB/B27 containing 0.5 mM L-glutamine and 60 ng/ml bFGF. Viable cells were counted by trypan blue exclusion. Cells were plated onto MEAs coated with PDL and fibronectin. Cells were then treated with either BDNF or N-CAM as described in the above section.

2.3. Immunocytochemistry

To evaluate possible differentiation of precursor cells along neuronal and glial lineages, cells were incubated with a mouse monoclonal antibody against microtubule associated protein 2 (MAP2; a marker for neuronal lineage; 1:500; Sigma, St. Louis, MO) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, a marker for astrocytes; 1:500; Millipore, Billerica, MA) and followed by an incubation with rhodamine-conjugated donkey anti-mouse IgG and FITC-conjugated anti-rabbit IgG (Jackson Immunological Research, West Grove, PA). Immunostained cultures were examined with a Nikon Eclipse E800 epifluorescence microscope equipped with a Sony DKC-5000 digital photo camera.

2.4. Extracellular recording from MEAs

Electrophysiologic measurements were performed using the portable microelectrode array recording system developed at the Naval Research Laboratory (NRL). In brief, the system integrates temperature and fluidics control with on-line monitoring and recording of neuronal extracellular potentials derived from MEAs in a portable format. The technical specifications of the portable recording system are described in detail elsewhere (Gray et al., 2001; Pancrazio et al., 2003). The MEAs used by the system have been previously described (Gross, 1979; Gross et al., 1985). Briefly, they consist of a 2 in. square glass substrate with 64 indium–tin oxide (ITO) electrodes at the center. The electrodes form a grid 0.8 mm^2 and are 10 μm in diameter. ITO traces lead from the electrode pads to two opposing edges of the glass substrate, where connections are made to the amplifier system. Over the ITO traces and glass substrate is an insulating layer of a polysiloxane; the insulting layer over the electrode pads having been removed by laser.

Prior to an experiment, a MEA containing a p0 or p1 culture was placed into a stainless steel recording cartridge filled with a solution consisting of minimum essential medium (MEM; HyClone, Logan, UT) supplemented with 25 mM glucose, 40 mM N-[2-hydroxyethyl]-piperazine-N-[2-ethanesulfonic acid] (HEPES; Sigma), and 26 mM $NaHCO_3$ at pH 7.4. This cartridge containing the culture was then placed into the portable recording system and the culture was perfused at a flow rate of 1 ml/min with the same MEM solution in a recirculating bath with a reservoir such that the total recirculating media volume was 40 ml. The cell recording chamber temperature was maintained within the range of 37 ± 0.5 °C. Control medium perfusion usually continued for 30–40 min in order to establish stable baselines of electrophysiological parameters for a network before testing of toxin sensitivity began. Test compounds were added directly to the recirculating bath reservoir and included tetrodotoxin (TTX; 1 μM; Sigma), bicuculline (Bic; 5 μM; Sigma),

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM; Sigma), 2-amino-5-phosphonopentanoic acid (APV; 50 μM; Sigma).

2.5. Neuronal network data analysis

Spike activity from cultured neuronal networks was quantified using mean spike rate. Raw data were sampled at a rate of 40 kHz per channel. Spikes were detected using an offline threshold crossing algorithm with a variable threshold determined based on the noise level of the channel (minimum threshold of 25 μV) and a minimum time above threshold of 100 μs. Mean spike rate for the network was calculated in 1 min intervals by first computing the spike rate per channel and then generating the mean spike rate across all active channels. All data are presented as mean ± standard deviation.

3. Results

3.1. Both primary and passaged cultures of neuroepithelial cells expand and differentiate into neurons and glial cells on microelectrode arrays

Neuroepithelial cells isolated from embryonic day 13 rat cortex were expanded in serum-free medium containing bFGF and in some cases passaged one time. Both primary and passage 1 cells rapidly proliferated. Phase-contrast photomicrographs of representative fields of cells in the cultures at days 1, 5, 7 and 12 show that suspended individual progenitor cells divided rapidly and formed multi-cellular clusters at day 5 (Fig. 1). The robustly growing primary and passaged cells had flat, multiple-shaped cell bodies with short processes and became confluent on MEAs at day 7. To assess the extent to which primary and passaged cultures of neural precursor cells could be expanded, cell counting was carried out at day 7 in primary and passaged cultures of neural precursor cells. Results from cell counting showed that the number of cells increased by 10-fold in passage 1. Cells in the passaged cultures exhibited a similar immature morphology as in the primary cultures. To test whether the passaged cells maintain immature morphology and proliferative properties, immunocytochemistry for nestin, an intermediate filament protein characteristic for central nervous system precursor cells (Lendahl et al., 1990), was carried out in 7-day-old primary and passage 1 cultures. Nestin staining showed that most cells, if not all, in both primary and passage 1 cultures were nestin$^+$ (data not shown), indicating they were still proliferative precursor cells.

3.2. Effects of bFGF + BDNF and bFGF + N-CAM on differentiation of neural stem and progenitor cells

Neurotrophins and cell adhesion molecules are known to affect differentiation of neural progenitor cells dissociated from E16 hippocampus (Mistry et al., 2002). In order to optimize culture conditions, under which cortical progenitor cells generate functional neuronal network activity on MEAs, we examined neuronal and glial production under influence of bFGF alone, or together with BDNF or N-CAM in 4-month-old cultures. To quantify the percentage of neural stem and progenitor cell-derived neurons (MAP2$^+$), cell counting was performed from cultures double-immunostained for MAP2 and GFAP, together with nuclear DAPI counterstaining on microelectrode arrays from at least three independent experiments. MAP2$^+$ cells were manually counted and were expressed as a percentage of the total DAPI$^+$ cells. At least 2000 total cells were evaluated per array. Results showed that bFGF + BDNF treated cultures generated a 3.8-fold increase in the percentage of MAP2$^+$ neurons than bFGF-treated cultures and a 1.6-fold increase over treatment with bFGF + N-CAM. Neurite outgrowth in bFGF + BDNF treatment appears to be most active (Fig. 2). GFAP$^+$ astrocytes, as support cells, grew well under all three conditions.

Four-week-old passage 1 cultures treated with bFGF + BDNF were examined using double-immunostaining for the neuronal marker MAP2 and astrocyte marker GFAP. These cultures showed mature neurons intermingled with astrocytes on the MEAs (Fig. 3).

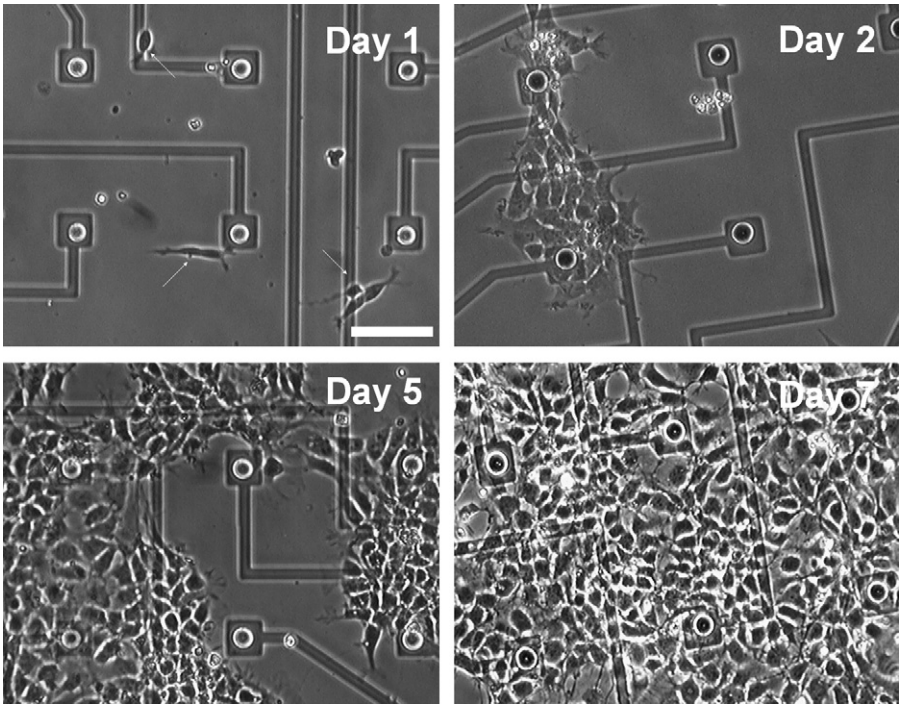

Fig. 1. Neuroepithelial cells isolated from embryonic day 13 cortex expand and differentiate into neurons and astrocytes over microelectrode arrays. Phase contrast micrographs showing continuous expansion of primary neuroepithelial cells from 1, 3, 5 to 7 days on MEAs. Scale bar: 50 μm.

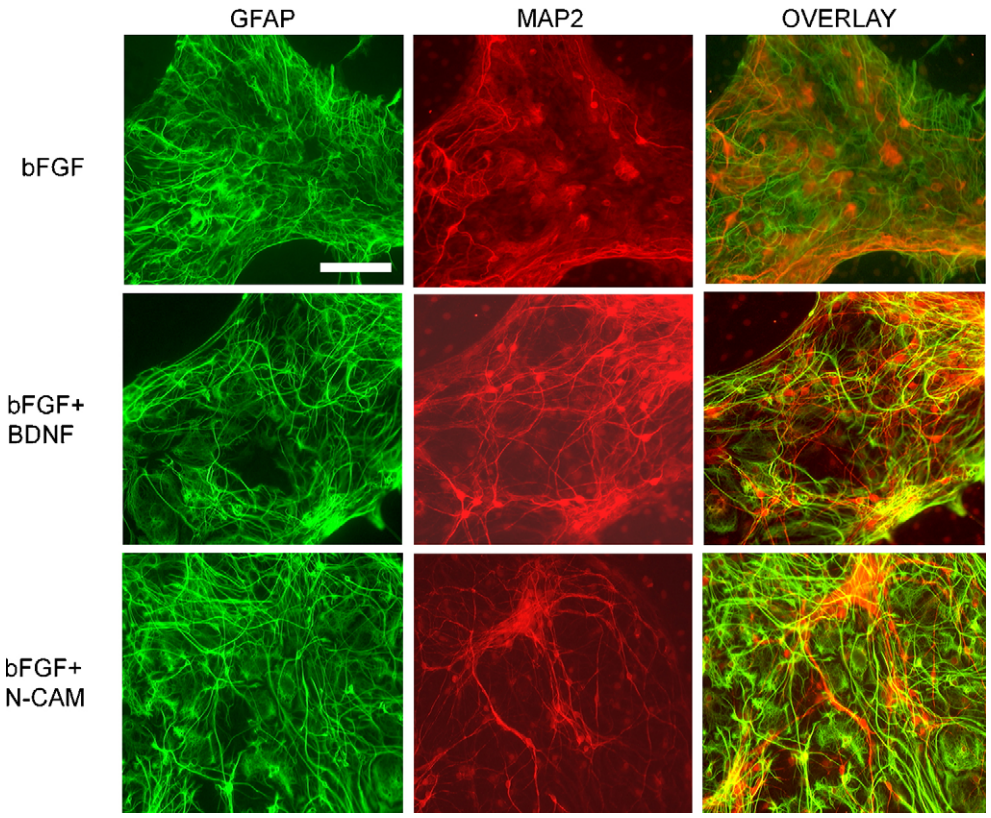

Fig. 2. Effects of bFGF, BDNF and N-CAM on neural stem and progenitor cell differentiation. Fluorescence photomicrographs of neurons ($MAP2^+$) and astrocytes ($GFAP^+$) differentiated from neural stem and progenitor cells cultured under different conditions for 4 months. $GFAP^+$ astrocytes are well developed under all conditions, but the treatment with bFGF + BDNF produces more neurons and longer processes and seems to be the most promising set of conditions tested for neuronal network generation. Scale bar: 50 μm.

3.3. *Functional neuronal networks derived from primary or passaged neural stem and progenitor cells*

Microelectrode arrays containing cultures of primary neural stem and progenitor cells treated with bFGF alone, bFGF + N-CAM, or bFGF + BDNF were tested for spontaneous action potential generation using the NRL portable microelectrode array recording system. Action potentials were confirmed by the ability of 1 μM TTX to abolish all of the detected spike activity. The results are summarized in Table 1. Of the three treatments, bFGF + BDNF was the most likely to result in a neuronal network expressing spontaneous action potentials. Treatment with bFGF + N-CAM failed to produce any active networks, while treatment with bFGF alone resulted in one of five of the networks expressing a low level of activity. Treatment with bFGF + BDNF resulted in six of eight of the networks treated producing spontaneous action potentials. For this treatment condition anywhere from 1 to 22 of the 64 electrodes on the MEAs showed evidence of action potentials. While the average age of the cultures tested was 32 ± 7 days, cultures showing activity started appearing as early as 21 days after being plated onto the MEA.

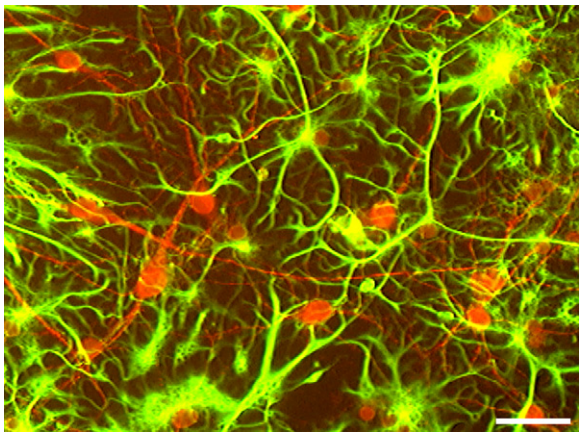

Fig. 3. Passaged neural stem and progenitor cells differentiate into neurons and glia. Double-immunostaining for MAP2 and GFAP of passaged progenitors grown on MEAs for 2 months using the bFGF + BDNF treatment shows differentiation of cells into neurons (red) and astrocytes (green). Scale bar: 30 μm.

Based on these results, two passage one cultures were seeded onto MEAs and treated using the bFGF + BDNF regime. One of the two networks showed spontaneous action potential activity with 12 active channels. The p1 networks were tested 39–40 days after being seeded onto the MEAs.

Networks with spontaneous action potentials were tested for synaptic activity by application of the $GABA_A$ antagonist bicuculline, the non-NMDA glutamate antagonist CNQX, and in a few cases, the NMDA glutamate inhibitor APV. All of the p0 bFGF + BDNF networks responded to bicuculline by an increase in mean spike rate ($96 \pm 104\%$), followed by a decrease in mean spike rate ($86 \pm 13\%$) when CNQX was added to the bath (Fig. 4). In addition, two of the networks were challenged with APV, which further decreased mean spike rate to almost zero. These results indicate the presence of both NMDA and non-NMDA excitatory synapses in the neuronal networks, as well as inhibitory $GABA_A$ synapses. The two bFGF only active networks also responded to both bicuculline and CNQX, however, the responses were not as consistent, with one of the two networks showing a decrease in mean spike rate in response to bicuculline. Since these networks had only one to two active electrode channels each, there is insufficient data to conclude anything

Table 1
Comparison of network activity between the three treatment groups.

Treatment	Passage	# networks	Age (days)	# active networks	Age (days)	# active channels per network
bFGF only	0	5	32 ± 7	2	28 ± 1	2 ± 1
bFGF + N-CAM	0	3	26 ± 5	0	–	–
bFGF + BDNF	0	8	32 ± 6	6	30 ± 6	6 ± 8
bFGF + BDNF	1	2	40 ± 1	1	40	12

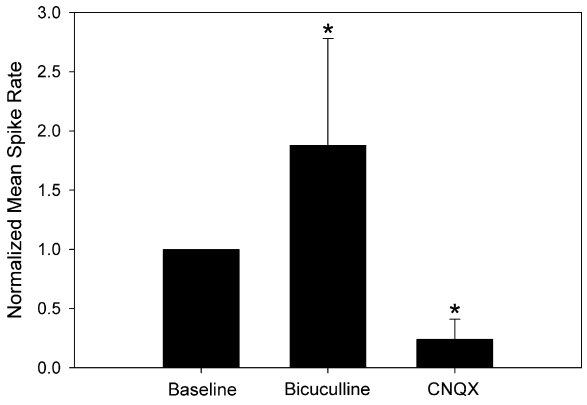

Fig. 4. Neuronal networks derived from stem and neural progenitor cells respond to neurotransmitter receptor antagonists. The six active networks derived using the bFGF + BDNF treatment (see Table 1) were tested for synaptic activity by first adding 5 μM bicuculline to the recirculating bath, followed by 20 μM CNQX. This figure shows the normalized average mean spike rates for 6 networks at baseline and after addition of the two neurotransmitter inhibitors. Mean spike rates were normalized to the baseline firing rate of each network. Data are presented as mean ± S.D. * indicates a significant difference ($P < 0.05$) between the treatment and control via one-way ANOVA.

beyond the presence of $GABA_A$ and non-NMDA glutamate synapses (no testing was done for NMDA glutamate receptors). Finally, the p1 bFGF + BDNF network responded to bicuculline (245% increase in mean spike rate), followed by APV (69% decrease), and CNQX (98% decrease; Fig. 5).

4. Discussion

The present study has shown there is considerable promise for using neural stem and progenitor cells as a renewable source of neuronal networks for a portable sensor system like the NRL portable neuronal network-based biosensor which has been demonstrated to provide rapid detection of environmental threats (O'Shaughnessy et al., 2004). Neural stem and progenitor cells in both primary and passaged cultures generate functional neuronal networks on MEAs. With culture medium containing bFGF and BDNF, the neural progenitor-derived neuronal networks showed both spontaneous action potentials and robust synaptic activity. These data suggest that neural stem and progenitor cells plated on MEAs could provide a suitable tool for detection of neuroactive substances. Neural stem and progenitor cells were expandable in culture with both the primary and passaged progenitors appearing equally effective in generation of functional networks, thus reducing dependence on animals for cell supply.

We attempted to understand the influence of several molecular signals on neural stem and progenitor cell differentiation on MEAs. Growth factors, neurotrophins and cell adhesion molecules are known key regulators in determination of neural stem cell fate (Hosomi et al., 2003; Panchision and McKay, 2002; Zheng et al., 2004). In hippocampal progenitor cell cultures, continued presence of bFGF, along with N-CAM and BDNF over 12 days of culture greatly increased the number of progenitors and neurons (Mistry et al., 2002). This study found N-CAM and BDNF appear equally efficacious in neural progenitor differentiation in neuronal production. In our cortical progenitor cell cultures, bFGF was added to the culture medium together with either BDNF or N-CAM. We found that continued bFGF presence and additional BDNF in culture yielded more neurons and more active networks than other treatments in both primary neuroepithelial cells and passaged cells. Active networks were seen as early as 21 days in culture.

Neural stem and progenitor cells dissociated from E13 cortex, as well as passaged cells generate a large number of glial cells, for example, astrocytes. Such mixed cultures appear to have advantages over pure neuronal cultures since astrocytes and astrocyte-conditioned saline are known to promote neuronal survival, differentiation and physiological maturation (Liu et al., 1998). Indeed, consistent spike activity from neuron-glial co-cultures has been demonstrated over a culture lifetime of up to 9 months (Gross and Rhoades, 1995).

While all three treatments resulted in cultures with neurons and glia, the key to producing viable neuronal networks for use as biosensor elements is to generate neuronal networks with active synaptic connections and spontaneous action potentials. A combination of bFGF and BDNF proved the most successful at generating

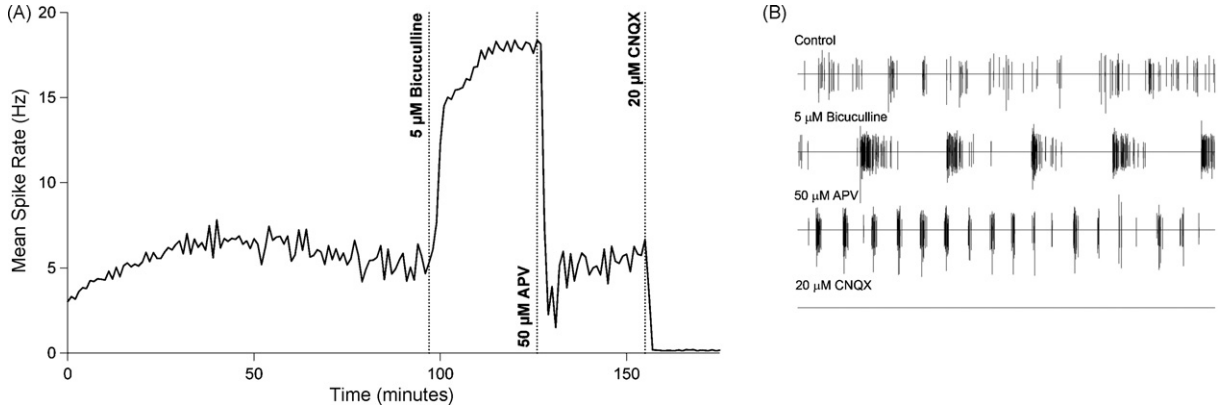

Fig. 5. A network derived from passaged stem and neural progenitor cells demonstrated spontaneous action potentials and synaptic activity. (A) Mean spike rate calculated on a per minute basis for the network in response to various neurotransmitter antagonists. (B) Spike trains from a representative electrode channel of the network corresponding to several treatments from panel A. Results in this figure are for the single active network derived from p1 neural stem and progenitor cells (see Table 1).

these types of networks. These stem and neural progenitor cell-derived networks had properties similar to primary culture murine cortical cultures (Keefer et al., 2001; Selinger et al., 2004) responding to antagonists of $GABA_A$, NMDA glutamate and non-NMDA glutamate receptors. The number of active electrode channels was lower than that previously reported for primary culture neuronal networks, which typically have from 8 to 25 active electrode channels (Pancrazio et al., 2003; Stenger and McKenna, 1994). However, one stem and neural progenitor cell-derived network did have 22 active electrode channels, suggesting that these networks can attain higher levels of active channels. For an electrode to be active, at least one neuron must be in close proximity to it. Network density plays a role in determining this. By altering the plating density of the original stem and progenitor cells, it may be possible to improve the number of active electrode sites.

That a neuronal network was derived from stem and progenitor cells that had been in culture and passaged is significant. This suggests that functional neuronal networks can be derived from stem and neural progenitor cells in continuous culture and not just from primary sources. As such, the stem and neural progenitor cells represent the potential for a renewable supply of neuronal networks for biosensing that is not dependant on obtaining primary culture cells directly from animals. There is a limitation in the present culture that must be overcome to make this a truly viable alternative. At present, the stem and neuronal precursors used here are prevented from differentiating by the presence of bFGF, however, they can be maintained in culture for only a limited number of passages because eventually they begin to differentiate despite the bFGF inhibition (unpublished observation). By augmenting the bFGF with other signaling molecules, it may be possible to maintain the stem and progenitor cells in proliferative culture for a substantially longer period of time as has been previously demonstrated for human neural precursor cells (Dhara et al., 2008).

5. Conclusions

This work has demonstrated a method for generating functional neuronal networks from both primary and passaged neural stem and progenitor cells. Treatment of rat neural stem and progenitor cells with bFGF + BDNF led to functional networks consisting of both neurons and glia. The intrinsic electrical activity of these networks was similar to that seen from primary culture murine neuronal networks. While active networks were formed, not all treated neural stem and progenitor cultures form active networks and the active networks formed often had a low number of active channels on the MEAs. Future work needs to focus on refining the treatment protocols in order to improve upon the production of active networks with higher densities of active channels. Ultimately, a renewable cell-based sensing element for a portable neuron-based biosensor represents a major step forward in overcoming the logistical issues present with having such a device accepted for field use.

Acknowledgments

The views expressed here are those of the authors and do not represent those of the U.S. Navy, the U.S. Department of Defense, or the U.S. Government.

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