

HUMAN NEURAL CELL-BASED BIOSENSOR FOR ENVIRONMENTAL TOXINS

D. W. MACHACEK AND S. K. DHARA AND S. L. STICE

ABSTRACT

Environmental toxicants represent a growing concern for both military and civilian agencies. While traditional, specific detector technologies are excellent at finding and quantifying known threats, there is a need for generic sensor technologies that utilize functional biology as the sensor element to detect both unknown toxicants as well as hazardous combinations of otherwise non-threatening compounds. The Naval Research Laboratory (NRL) has previously demonstrated a prototype biosensor that utilizes networks of mammalian neurons on microelectrode arrays (MEAs) as the sensor element. These neuronal cultures are derived from primary dissections of mice and consist of a mixture of neurons and support cells. The neuronal cultures, composed of terminally differentiated cells, have a limited lifespan (approximately 2 weeks in a sealed cartridge) in the field making frequent replacement a necessity. In order for a neuronal network-based biosensor to be viable, a renewable source of networks is needed. Here we have developed techniques to control the differentiation of human stem cells (from approved cell lines) into networks of neurons along with the necessary support cells.

INTRODUCTION

Neurons initiate and send electrical signals that control movement, sensation, memory, learning and countless other activities that we take for granted in our daily lives. Currently, neurotoxicity assays and sensors utilize harvested primary and transformed animal cells almost exclusively; however, they are not equivalent to human cells and can lead to erroneous results. Although stem cells are not as complex as whole animals, human embryonic stem cells (hESC) have been validated for use in toxicology applications and have proven to be highly accurate compared to *in vivo* models (Davila et al., 2004). Additionally, hESC tests have already been validated in comparison to *in vivo* results in an international double blind collaboration study (Bremer and Hartung, 2004). Other investigators have concluded that primary neural or stem cell culture models can and should be included in the early stages of risk assessment since they allow the ready identification of substances or proposed drugs with high neurotoxicity potential (Silva et al., 2005). Stem cells will likely be more informative than immortalized cell lines and can circumvent problems associated with the limited availability of primary neural cells. The similarity of an experimental system to the *in vivo* situation is generally directly related to the degree of complexity of the *in vitro* model. Immortalized cell lines are the least complex system and also least representative of the *in vivo* condition (Tahti et al., 2003). An animal source of primary neurons is less than optimal, because the surgical techniques can be difficult and the numbers and quality of recovered neurons vary widely. These cells also have a limited life span in culture, so new animals must continually be purchased and sacrificed. More important, cellular signaling differs between human and mouse cell lines (Williams et al., 1988 and Thomson et al., 1998). Few commercial

sources of primary human neural cells are available for use in cellular assays, and none are available for biosensing. These cells are unproven in their ability to meet customer/user requirements. Use of human neural cells is hampered because there is still no adequate source of human neural progenitor cells (hNPs) to produce bulk quantities of human neural cells for cell-based sensors. The goal of this study was to expand and differentiated hESC derived hNP cells for biosensor use.

Table 1. Advantages and disadvantages of neural stem cells*.

Source	Advantage	Disadvantage
hESC-derived neurons	Relatively easy to expand (derived neural cells). Unlimited cell types (<i>i.e.</i> , motoneurons and other cells; see Preliminary Studies). Genetically manipulated (reporter genes and homologous recombination). The hESC cells are banked so any rederivations result in identical genetic background	Potentially less pure, may contain non-neural cells, (see Preliminary Studies on recent advances). Potential loss of differentiation potential over time (see Preliminary Studies on recent advances) may need to re-derive neural stem cells from ESCs
Fetal neural stem cells	Relatively easy to expand Give rise to large numbers of neurons Already specified as neural (<i>i.e.</i> , pure neural stem cells)	Limited neuronal phenotypes Homologous recombination not demonstrated Rederiving cells from new tissue results in variation in genetic background
Adult neural stem cells	Already specified as neural Can culture cells from an affected person	Limited neuronal phenotypes Few neural cells generated <i>in vitro</i> Difficult to expand to high numbers (senesce after a few passages)

* adapted in part from Jakel et al., 2004.

MATERIALS AND METHODS

Standard preparation of culture dishes. Solutions of 0.2% gelatin (Sigma, St. Louis, MO), 20 µg/ml polyornithine (Sigma), and/or 5 µg/mL laminin (Sigma) will be prepared in sterile distilled water. **Media.** All media used in this study will be supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg /ml each of streptomycin and 2-ME (Gibco, Rockville, MD). WA09 hESC medium: DMEM/F12 medium (Gibco) supplemented with 20% KSR (Gibco), 1X MEM, and 4 ng/ml FGF2. MEF medium: DMEM (Gibco) supplemented with 20% fetal bovine serum (FBS, Hyclone), 1X MEM, 10 ng/ml hLIF, 0.1 mM β-mercaptoethanol (Gibco), and 4 ng/ml FGF2. Derivation medium: hESC

growth medium lacking FBS and Knockout Serum Replacer (KSR), but supplemented with N2 (Gibco). Proliferation medium: Neurobasal medium (Gibco) supplemented with 20 ng/ml FGF2 (Sigma) and 10 ng/ml LIF (Chemicon). Induction medium: Proliferation medium supplemented with 100 ng/mL SHH and 1 μ M RA. Differentiation medium: Proliferation media without FGF2, LIF, SHH, or RA. Co-culture medium: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and FBS, 10% (v/v).

MEF proliferation and inactivation. Mouse embryonic fibroblast (MEF) feeder layers will be derived from E13.5 random-bred mouse fetuses by standard procedures in MEF medium. Cells will be seeded on gelatin-treated dishes at 1.2×10^5 cells/cm². Approved by UGA IACUC.

Feederless adherent derivation of hNPs. WA09 hESCs are harvested with trypsin and plated on poly-ornithine/laminin-coated (5 μ g/ml) tissue culture dishes. The cells are cultured in defined hESC medium without FGF2 for 1 week, followed by 1 week in derivation medium. Finally, the cells are cultured and expanded with proliferation medium for 1 week. At about 10 days of derivation, neural rosettes (*in vitro* representation of neural tubes) start appearing and eventually cover the entire plate.

Flow cell cytometry. Cells will be fixed in 2% paraformaldehyde/2% sucrose for 15 min, washed twice with PBS and held at 4°C until all samples are collected. Fixed cells will be blocked in PBS supplemented with 1% BSA, then incubated with primary antibodies for 2 h. Cells will be washed 3 times for 20 min in PBS/0.5% TritonX-100. This process will be repeated for secondary antibodies and propidium iodide. Cells will be analyzed with a flow cytometer, and at least 10,000 events will be counted per sample.

IF microscopy analysis. We will follow a Stice lab protocol well-suited to cells in multi-well chambers. Cells will be washed with PBS/Triton X 0.5%, fixed with 4% paraformaldehyde in PBS for 20–30 min. Cells will be washed 3 times in PBS/Triton X 0.5% and blocked in 3% BSA in PBS/0.5% Triton X-100 for 20 min. Cells will be incubated in blocking solutions containing primary antibodies at a concentration determined empirically to show staining without excessive background signal. After washes, this strategy will be used to stain cells with blocking solution containing secondary antibodies. Cells will be stained with the chromatin fluorescent dye DAPI as well as another dye that can be detected with a laser (Molecular Probes). Chambers will be disassembled and mounted in Prolong-Gold Antifade medium (Molecular Probes).

RESULTS AND DISCUSSION

We derived cell cultures containing $\geq 90\%$ hNPs with the expected number of chromosomes and expected immunoreactivity (**Fig 1, table 2**). Two cell lines of neural progenitors were generated from WA09 hESCs and were fully characterized for Nestin, Sox2, and Oct-4 staining, using IF microscopy and flow cytometry for Nestin and Oct-4, and IF microscopy for Sox2. Both lines were $>90\%$ Nestin- and Sox2-positive and $\leq 2\%$ Oct-4 (**Fig. 1 and Table 2**), regardless of how the cells were counted. Using analysis of variance in a linear statistical model (GLM procedure in SAS), there was no statistical difference between hNP lines for Nestin. hNP lines are mostly Oct-4-negative (**Fig. 1C**).

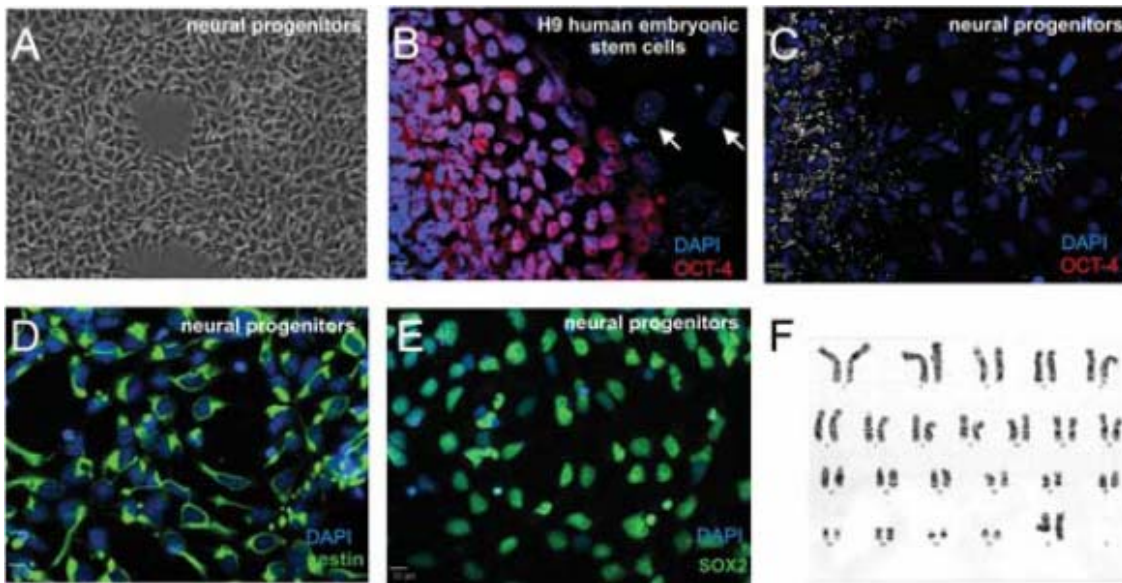
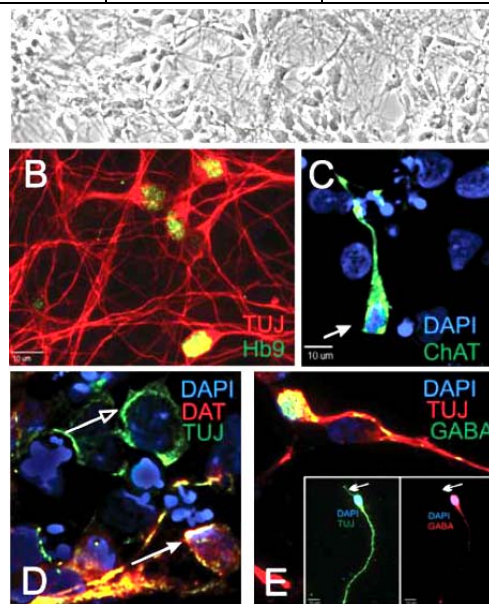


Figure 1: hNPs demonstrate the expected immunoreactivity and chromosome number. (A) Adherent hNPs at 95% confluency. (B) WA09 hESC are Oct-4 positive, while mouse feeder cells are negative (arrows). (C) hNP lines are Oct-4-negative. (D) hNP line labeled for Nestin immunoreactivity. (E) hNP line labeled for Sox2 immunoreactivity. (F) Karyotype from hNP line.

Table 2: % Positive hNPs.

Cell Line	Using IF microscopy (>1000 cells counted)			Flow cytometry	
	% Nestin ^{+ve}	% SOX2 ^{+ve}	% OCT4 ^{+ve}	% Nestin ^{+ve}	% OCT4 ^{+ve}
hNP 1	99 ± 1	92 ± 2	2 ± 0.5	99.2	0.097
Renal				0.5	NA
HUVEC				NA	0.01

Figure 2. Neural phenotypes derived from hNP lines. (A) Phase contrast image of differentiated culture. (B) Network including post-mitotic motoneurons (HB9). (C) Cholinergic neuron. (D) Tuj-1 positive cells that are DAT-positive (dopamine transporter; closed arrow) and DAT-negative (open arrow). (E) Gabaergic neurons, inset illustrates GABA in axon, but not the dendrites (arrow).



We developed differentiation protocols and demonstrated that our cells could produce multiple neuronal phenotypes (**Fig. 2**) and glial phenotypes (**Fig. 3**) that would be relevant to multiple nervous system disease models (**Table 4**). Including growth factors and/or genetic markers will no doubt be useful for enrichment of neural cell phenotypes, including motoneurons and dopaminergic cells. These techniques will be implemented in this proposal for enriched and robust populations of neural cells.

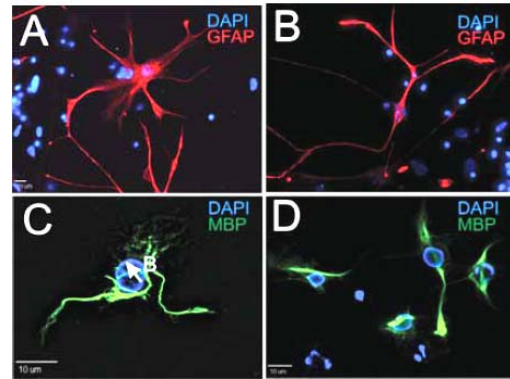


Figure 3. Glial cells. (A & B) GFAP-positive cells. (A) represents the morphological characteristics of astrocytes whereas (B) represents the structure of radial glia. (C & D) Mature oligodendrocytes are defined by expression of basic myelin protein.

CONCLUSIONS

We have successfully demonstrated that hNPs can be reliably quantified using immunocytochemistry, flow cytometry and electrophysiological analysis. The results were uniform for the two WA09 human ESC-derived NP cell lines. In the future, an automated human neural cell based biosensor would be used to detect environmental toxins in the field. The culture techniques necessary to grow the human stem cells into electrically active neuronal networks on the MEAs will be determined. The human stem cell-derived networks will be tested against a panel of toxicants and their responses compared to the primary culture mouse networks. Being of human origin, it is expected that the stem cell-derived networks will respond to the toxicants at least as well as the mouse neurons, if not better, based on relevance to known human toxicity.

LITERATURE CITED

- Bremer, S. and T. Hartung, The use of embryonic stem cells for regulatory developmental toxicity testing in vitro--the current status of test development. *Curr Pharm Des*, 2004. 10(22): p. 2733-47.
- Davila, J.C., et al., Use and application of stem cells in toxicology. *Toxicol Sci*, 2004. 79(2): p. 214-23.
- Jakel, R.J., B.L. Schneider, and C.N. Svendsen, Using human neural stem cells to model neurological disease. *Nat Rev Genet*, 2004. 5(2): p. 136-44.
- Pancrazio, J. J., S. A. Gray, et al. A portable microelectrode array recording system incorporating cultured neuronal networks for neurotoxin detection.(2003). *Biosensors & Bioelectronics* **18**(11): 1339-1347.

Reubinoff, B.E., et al., Neural progenitors from human embryonic stem cells. *Nat Biotechnol*, 2001. 19(12): p. 1134-40.

Silva, R.F., et al., Dissociated primary nerve cell cultures as models for assessment of neurotoxicity. *Toxicol Lett*, 2005. 163(1): p. 1-9.

Tahti, H., H. Nevala, and T. Toimela, Refining in vitro neurotoxicity testing--the development of blood-brain barrier models. *Altern Lab Anim*, 2003. 31(3): p. 273-6.

Thomson, J.A., et al., Embryonic stem cell lines derived from human blastocysts. *Science*, 1998. 282(5391): p. 1145-7. Williams, R.L., et al., Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*, 1988. 336(6200): p. 684-7.

Zhang, S.C., et al., In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol*, 2001. 19(12): p. 1129-33.