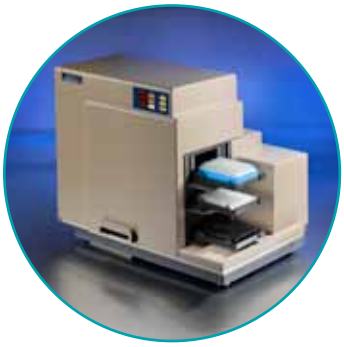
Molecular Devices

Stem Cell-Derived Human Neurons in CNS Drug Discovery

FLEXSTATION APPLICATION NOTE #7



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INTRODUCTION

Central nervous system (CNS) disorders affect many people worldwide. To increase the quality and number of drugs available for treatments of CNS disorders such as Alzheimer's disease, schizophrenia and anxiety, the success rate of CNS drug discovery programs must be improved. The challenge in drug discovery is to develop more relevant assay systems to identify lead compounds for further development. Cell-based screening assays currently used for CNS drug discovery and development involve primary cells or commercially available cell lines, both of which have many disadvantages.

MATERIALS & METHODS

Differentiation process

→ Undifferentiated stem cells were seeded by mechanical passaging of human embryonic stem cell (hESC) colonies onto Matrigel™ basement membrane matrix-coated IVF dishes drom BD Biosciences. Cells were differentiated to human neurons according to Gerrard *et al.*, 2005.

Immunocytochemistry

→ Cells were fixed and immunocytochemistry performed as described in Gerrard *et al*, 2005. Primary antibodies used were mouse anti-Oct4 (C-10; Santa Cruz) and Rabbit anti-Pax6 (Chemicon). Secondary antibodies were goat anti-mouse IgG Alexa Fluor 488 and goat anti-rabbit IgG Alexa Fluor 555 (Life Technologies, Inc.). Images were taken with a Zeiss Axiovert 100M confocal microscope using the 63X oil lens.

Western blot analysis

→ Cell lysates were prepared and approximately 30 µg of total protein was separated on a NuPage® Novex 4–12% Bis-Tris gel (Life Technologies, Inc.). Following transfer onto nitrocellulose, membranes were probed using rabbit anti-GAD65/67 antibodies (Chemicon). A goat anti-rabbit HPRT secondary antibody (Sigma) was applied and signal detected using an ECL detection substrate (Pierce).

Electrophysiology

→ The whole-cell patch-clamp technique was used to record ionic currents and action potentials at room temperature, with a physiological extracellular solution containing (mM): NaCl (140), KCl (20), $CaCl_2$ (2), $MgCl_2$ (1), glucose (10), HEPES (10), pH 7.4. The intracellular pipette solution contained (mM): KCl (30), HEPES (5), $MgCl_2$ (1), K-aspartate (110), Na_2ATP (4), NaGTP (0.4), EGTA (0.015), pH 7.3. The voltage dependency of currents was measured from a holding potential of -90 mV or from -40 mV to inactivate sodium current, with pulses of 100-ms duration at 1 Hz increasing in steps of 5 mV, up to +80 mV. The time-dependent effect of drugs (*e.g.* GABA), were measured by keeping the holding potential steady at -90 mV throughout the protocol. In current-clamp experiments, action potentials were evoked in each cell by stimulating repetitively with trains of current pulses, increasing progressively until it produced a stable and regenerative action potential.

GPCR drug screening assay development

→ Responses to G protein-coupled receptor (GPCR) activation were measured in the FlexStation® 3 Multi-Mode Microplate Reader using dye from the FLIPR® Calcium 4 Assay. For the 96-well format, cells were seeded into poly-D-lysine pre-coated, black-well, clear-bottom 96-well BioCoat plates (BD Biosciences), in 100 µL growth media 24 hours prior to assay. 100 µL of dye from the FLIPR Calcium 4

Assay containing 5 mM probenecid and 0.1% BSA was added to each well, and the plate was incubated at 37°C for 1 hour. 10 µM LPA (50 µL) agonist was added to the test plate using the "Flex" read mode and integrated pipettor of the FlexStation Reader, and the fluorescence was measured with excitation at 485 nm and emission at 525 nm.

RESULTS

Generation of hESC-derived neurons

Undifferentiated hESCs were seeded in neuronal inducing media containing Noggin and bFGF. Cells were maintained in culture for approximately three weeks, after which cells began to differentiate towards the neuronal lineage. This is evident from the induction of pax6 and nestin (Figure 1), which are markers associated with neuroprogenitors (NPs). Further neuronal maturation was achieved by withdrawal of bFGF and seeding at low cell density. Neuronal cells were identified by expression of MAP-2, beta-tubulin III, and PSA-NCAM. It was also shown that these neuronal cells express Gad 65/67, which is indicative of a GABAergic phenotype. (Figure 2) These neuronal cells were seeded into 96-well plates for analysis on the FlexStation® 3 Microplate Reader, or onto 10-mm coverslips for electrophysiology.

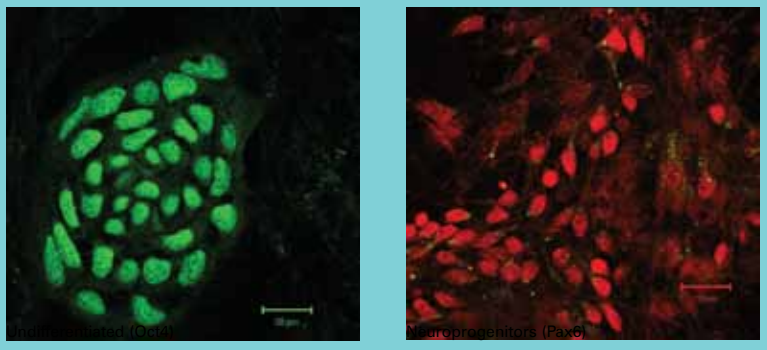
Electrophysiology

Whole-cell patch-clamp recordings were made from neuroprogenitors and mature neurons. Both types of cells exhibited the presence of potassium outward currents. However, only matured neurons exhibited inward sodium current, sensitive to tetrodotoxin (TTX) at 100 nM. (See Figure 3A.) The inward current generally coexisted with the outward current, with both currents displaying typical voltage dependence properties. (See Figure 3B.) Matured neurons developed typical whole-cell currents in response to application of Glutamate (100 µM) and GABA (1 mM) (Figure 4A) and displayed spontaneous firing activity. (Figure 4B) No such activity was observed in neuroprogenitor cells. Under current-clamp, cells were stimulated to elicit action potentials. Most of the matured neurons were able to fire overshooting action potentials (Figure 5A), consistent with the presence of sodium channels in this group of cells. However, no action potentials could be generated or recorded in neuroprogenitors stem cells. (Figure 5B)

GPCR calcium assays

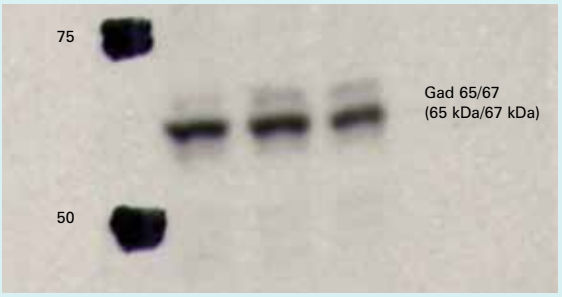
In this study, we show a calcium mobilization assay that can be used to screen candidate targets in a natural endogenous system. The results demonstrate that high quality data and assay performance can be obtained with the FLIPR Calcium 4 Assay and that this is comparable to other similar assay systems. In the present study, we verified this assay using selective antagonists against a HEK cell line overexpressing a GPCR target, and neurons (generated as above) endogenously expressing the same target. (Figures 6 and 7) Both the overexpressing stable cell line and the neurons endogenously expressing the GPCR target showed similar inhibitor profiles and selectivity. This would indicate the neuronal cell line as a good model to profile the GPCR target in its natural environment.

Neuronal Differentiation (Figure 1)



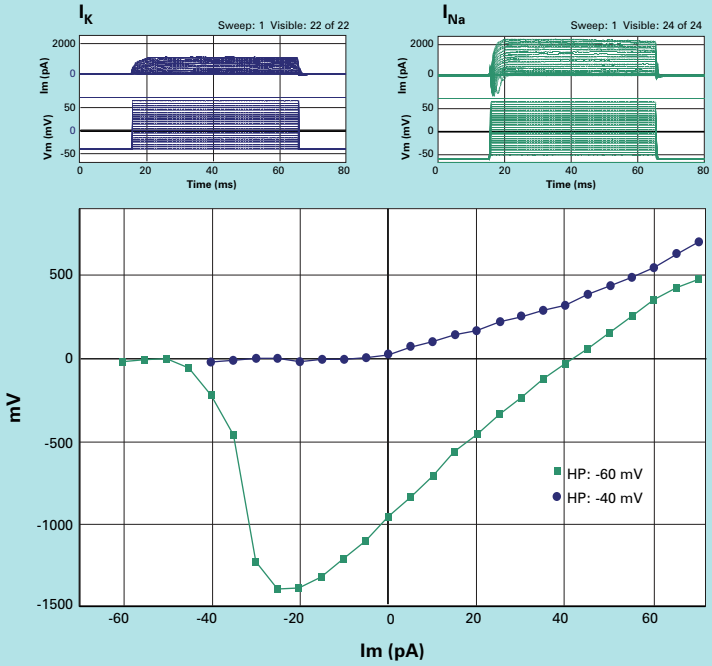
Differentiation process from undifferentiated stem cells to mature neurons. Upon neuronal differentiation cells lose expression of Oct 4 and express Pax6 and Nestin as the neuroprogenitor stage is reached. Cells then lose these markers upon further maturation and begin expression of Map2, β-tubulin III and PSA-CAM, markers of mature neurons.

Gad 65/67 Expression (Figure 2)



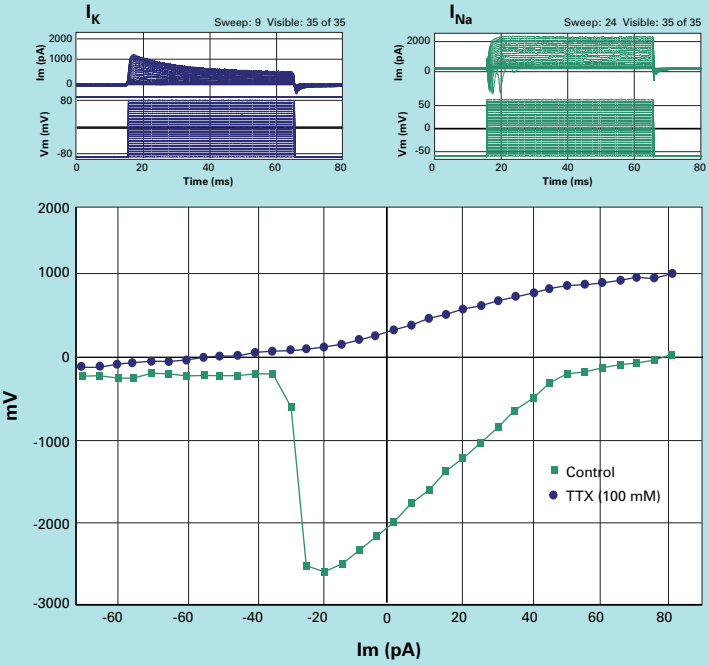
Expression of Gad 65/67 in three different batches of mature neurons. Batches produced are reproducible in expression of markers and functionality.

Whole-Cell Patch-Clamp Recordings— Current-to-Voltage Plot (Figure 3A)



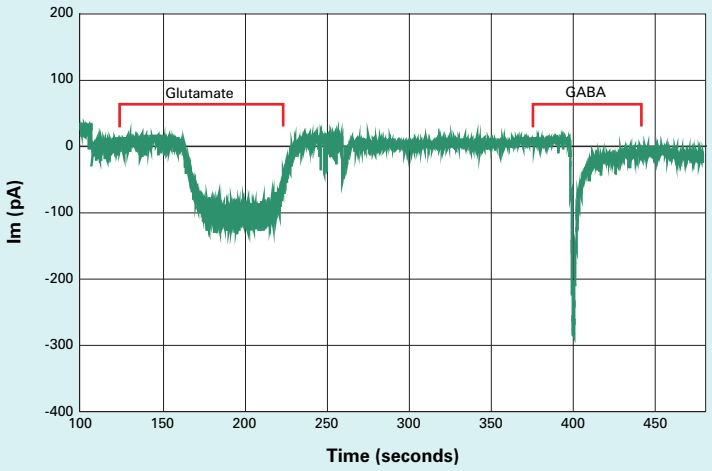
Current-to-voltage plot of inward and outward currents in the same mature neuronal cell reveal typical behaviour of voltage gated Na^+ (green square) and K^+ (blue circle) currents recorded at -60 and -40 mV, respectively. Whole-cell recordings are shown in the smaller graphs.

Whole-Cell Patch-Clamp Recordings— Whole-Cell Currents (Figure 3B)



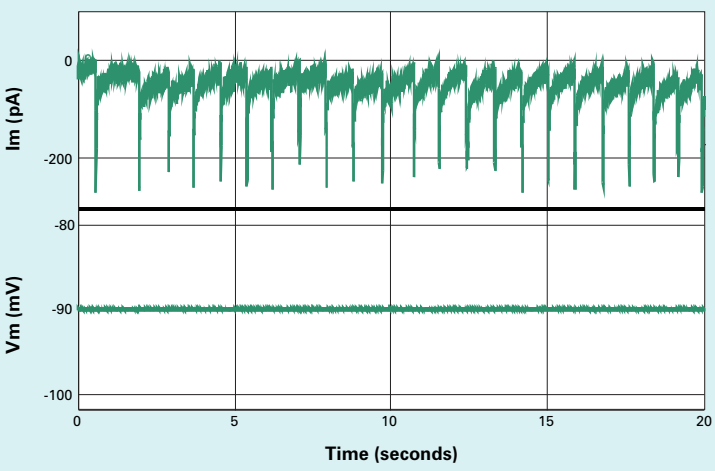
Current-to-voltage plot of inward and outward currents in the same mature neuronal cell reveal typical behaviour of voltage gated Na^+ (green square) and K^+ (blue circle) currents recorded in the presence and in the absence of TTX at 100 nM. Whole-cell recordings are shown in the smaller graphs.

Whole-Cell Patch-Clamp Recordings— Whole-Cell Currents (Figure 4A)



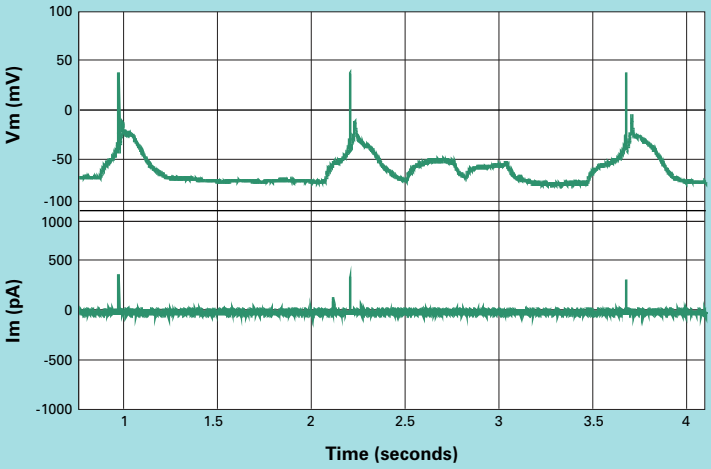
Transmitter-gated currents and spontaneous activity in neurons. Typical whole-cell currents evoked by Glutamate (100 μM) and GABA (1 mM) in a matured neuron.

Whole-Cell Patch-Clamp Recordings— Whole-Cell Currents (Figure 4B)

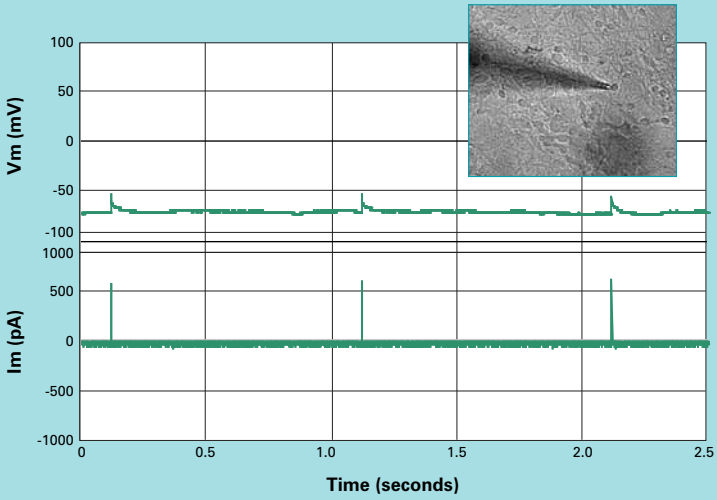


Transmitter-gated currents and spontaneous activity in neurons. Spontaneous firing activity recorded at a holding potential of -90 mV from a matured neuron.

Evoked Action Potentials in Mature Neurons and Neuroprogenitor Stem Cells (Figures 5A and 5B)

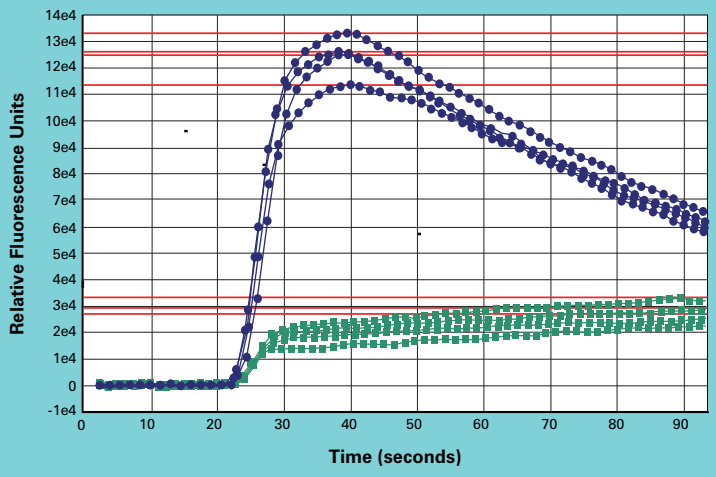


Depolarising current steps elicit overshooting action potentials (> 0 mV) in mature neurons.



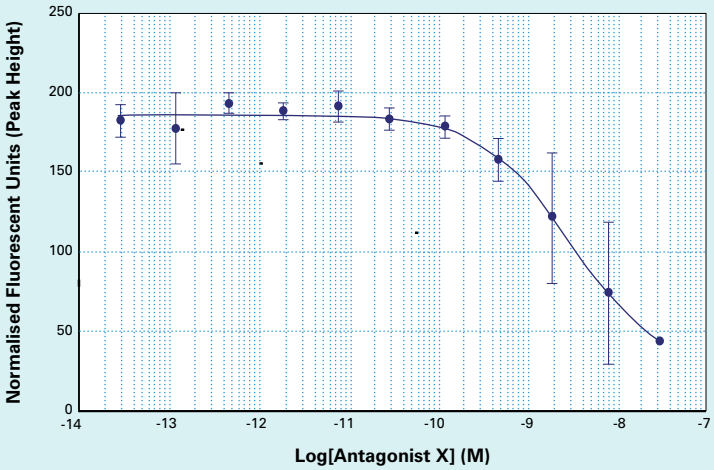
Depolarising current steps do not elicit overshooting action potentials (> 0 mV) in neuroprogenitor stem cells.

Calcium Assay Results (Figure 6)



FLIPR Calcium Assay performed on FlexStation 3 Reader: results from mature neurons stimulated with two concentrations of GPCR ligand. Kinetic traces are shown.

Dose Response Curve (Figure 7)



Dose response to identified hit compound from GPCR library screen (calcium assay) in stem cell-derived neurons.

DISCUSSION

We have generated reproducible batches of human neurons that will significantly impact and improve CNS drug discovery. The stem cell-derived neurons express all known markers typically associated with mature neurons and are functionally active. They express typical sodium (TTX-sensitive) and potassium currents and respond to saturating concentrations of the neurotransmitters GABA and glutamate. Furthermore, these cells have the ability to fire overshooting action potentials.

We are able to format the neurons for screening purposes and can measure calcium and membrane potential responses upon stimulation. We have ascertained an expression profile of transporters and receptors endogenously expressed in the neurons and are able to manipulate them to overexpress proteins of interest. This enables us to further enhance the superior screening systems that we can build, based around multiple CNS-related targets.

We have used these neurons in a recent screen to identify antagonists of a novel GPCR target implicated in schizophrenia. The neurons were shown to express this GPCR in high levels, and we generated a stable cell line over-expressing the GPCR for a comparitive study. We identified hit compounds from a focused library screen by measuring calcium responses. We found that the neurons gave us increased data content over the stable cell lines, which was proven to be data necessary for the successful outcome of the project.

REFERENCE

1. Gerrard, L., Rodgers, L. and Cui, W. (2005). Differentiation of human embryonic stem cells into neural lineages in adherent culture by blocking BMP signalling. *Stem Cells,* 23: 1234–41.

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Printed in U.S.A. 3/10 #0120-1497A