ArunA Biomedical, Inc. Electro Phys Data

Supplemental technical data- STEMEZ™ hN2 Human Neurons Discovery Kits

Demonstration of electrically active membranes of hN2 cells.

We have been able to show that adherent hN2 cells in differentiation media for 1 to 2 weeks have developed voltage-gated inward currents under whole cell voltage clamp. These currents can be blocked by 1 µM tetrodotoxin (n = 4 cells) and have kinetics and inactivation properties similar to classical voltage-gated sodium channels **(Fig.4B-E).** Under current minimal conditions, these cells give between 40 pA–1.5 nA of inward current. Cells yielding currents of at least 400 pA can also yield an overshooting action potential when stimulated by current injection **(Fig. 4F)**. Overall, we have recorded from 53 cells yielding inward currents >40 pA out of 83 cells total (64%). However, over the last 6 experiments, 40 out of 45 cells have yielded inward currents. This can mainly be attributed to two factors: An increase in the concentration of laminin used for plating these cells to 1 mg/ml and our ability to recognize a morphological phenotype that has a high probability of yielding current. The increase in the concentration of laminin yielded cultures with increased longevity which could then be successfully assayed after 20 to 30 days after removal of FGF. Increased longevity might be related to increased adherence to the substrate. hN2 cells have significant neurite outgrowth and thick soma such that they were phase bright, **(Fig. 4A)** had an increased probability of yielding significant inward current in comparison to the more prevalent thin (phase dark) cells.

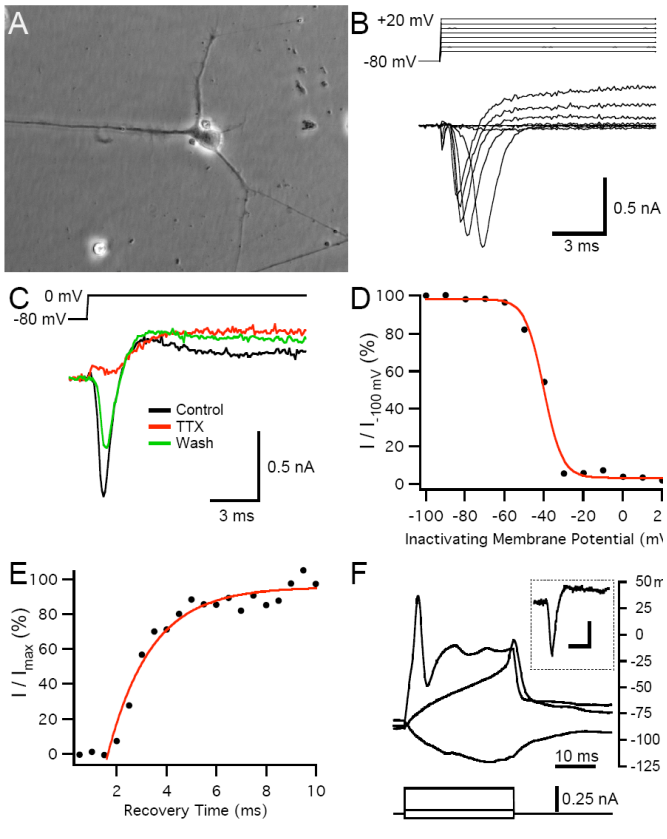

Figure 4. hN2 cells can produce inward currents that generate action potentials. (**A)** Isolated hN2 with significant neurite growth 1 week after plating . This cell was subjected to whole cell voltage clamp utilizing a potassium gluconate based intracellular solution. (**B)** Voltage gated inward and outward currents were elicited from this cell with depolarizing voltage steps. (**C)** Inward currents from another cell (potassium gluconate intracellular) were abolished by local application of 1 µM tetrodotoxin (*red trace*) while outward currents remained. Inward current recovered as TTX washed out of the region (*green trace*). (**D)** A different cell which exhibited voltage activated inward currents that inactivated in response to a 50 ms prepulse at different membrane potentials. The experiment was done 27 days after the removal of bFGF. A cesium gluconate based intracellular solution was used for this experiment to block outward potassium currents. The membrane potential for half maximal inactivation by standard Boltzman fitting (*red line*) was -40.1 mV with a slope of 4.7. (**E)** Recovery from fast inactivation utilizing a paired pulse protocol in the same cell as C. The single exponential time constant for recovery of inactivation was 1.7 ms (*red line*). (**F)** A different cell which elicited an overshooting action potential upon current injection under whole cell current clamp utilizing a potassium gluconate based intracellular solution. *Inset*: Response of the same cell under voltage clamp to a change in membrane potential from -80 mV to -10 mV elicited a peak current of 457 pA. Scale bars for inset: 5 ms, 0.2 nA.

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In collaboration with Dr. Murray at Creighton University, we demonstrated glutamatergic pharmacology on a population of differentiated hN2 using a high-throughput assay of $[Ca^{2+}]_I$. hN2 seeded into confluent 96-well plates **(Fig. 5A)**. Veratridine, a sodium channel activator, evokes an increase in $[Ca^{2+}]_I$, at high concentrations (300 μM) which was partially reversible with addition of TTX (p=.02), an inactivator of sodium channels. However this veratridine concentration is considered relatively high, and others have reported an EC50 of for veratridine of 22.2 μM using FLIPR voltage sensitive dyes [24].

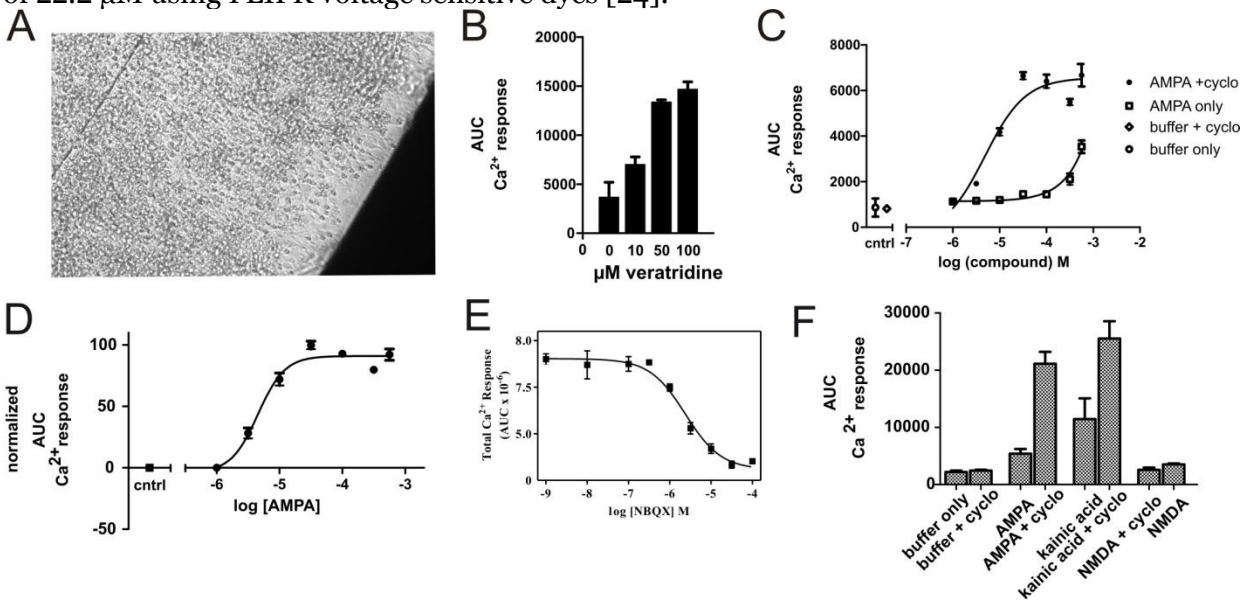

Figure 5. Pharmacology using FLIPR assay. (A) hN2 can be plated into 96 well plates. **(B)** Increasing doses of veratridine, a Na+ channel activator, result in an increased calcium response. **(C)** hN2 cells can identify compounds that potentiate AMPA responses. Cyclothiazide (50 μM) a compound known to potentate AMPA responses facilitates the population response to AMPA. **(D)** Normalized and pooled data from 3 separate experiments demonstrate a dose response curve to AMPA with an EC_{50} of 4.5 μM (in the presence of 50 μM cyclothiazide). **(E)** NBQX (AMPA/kainate receptor antagonist) blocks the glutamate-induced elevation of $[Ca2+]_I$ in hN2 cells. **(F)** Responses to both AMPA and kainic acid but not NMDA could be detected and potentiated with 50 μM cyclothiazide.

Recently we have shown that our cells have calcium responses at 10, 50 and 100 μM veratridine **(Fig 5B).** Importantly, we demonstrated that under basal differentiation protocols, these cells express AMPA/kainite receptors whose pharmacology and dose-response relationships can be elucidated **(Fig 5C-F)**. The data generated from such hN2 assays is novel and their response likely to be physiologically relevant in that they contain a heterogeneous combination of cell types.

Real time PCR was used to estimate relative fold change values using ΔΔCt quantification method (3 replications) of hN2, with their neural progenitors cells used as a normalizer sample. The data showed up regulation of ion channel subunits and proteins important to neural networks as well as confirming the diverse phenotype of cells (**Fig. 6)**. The HERG channel, important for toxicity screens expression was relatively high in neural cells. This data is part of our ongoing characterization of receptors and ion channels expression in hNP cells and differentiated (hN2 cells) so that further functional assays can be tested in a systematic manner. **More information on ion channel and GPCR gene expression characterization is available upon request.**

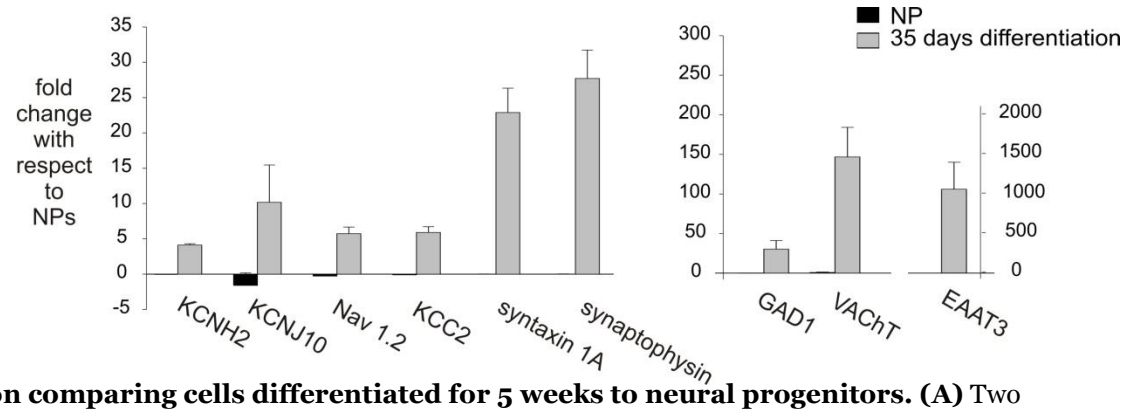

Figure 6: Relative fold change of mRNA expression comparing cells differentiated for 5 weeks to neural progenitors. (A) Two potassium channels KCNH2, or the HERG K+ channel, and KCNJ10 predominately found on glia showed upregulated gene expression as did NAV 1.2, a sodium channel and KCC2, a Cl transporter important in the development of inhibitory transmission. Two synaptic proteins syntaxin 1A and synaptophysin were also upregulated. **(B)** GAD1, VAChT and EEAT3 were all upregulated consistent with GABAergic, cholinergic and glutamatergic phenotypes respectively.