

# Cryopreserved Human Neuronal Cells Express SNAP-25 and Are Sensitive to BoNT/A

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## INTRODUCTION

To date, the large majority of mechanistic studies of Botulinum Neurotoxins (BoNT), as well as BoNT detection and potency assays utilize animals or animal tissues. For obvious reasons, studies using tissues of human origin are limited. hN2™ Cells (ArunA Biomedical, Inc. Athens, GA) are a source of cryopreserved, differentiated human neuronal cells derived from renewable, genotypically stable, neural progenitor stem cells. In this poster, we present evidence that SNAP-25 protein, the intracellular target of BoNT/A, is endogenously expressed in Aruna's hN2™ Cells. Further, we present preliminary evidence that this SNAP-25 is cleaved by exposure to 1nM BoNT/A within 48 hrs after plating hN2™ Cells, a clear indication that these human neuronal cells are sensitive to BoNT/A. Based on these preliminary findings, we propose that 'thaw and use' hN2™ Cells are ideally suited for feasibility studies to develop human cell-based bioassays for assessment of BoNT detection, potency, and antagonism. There are clear advantages of using human neuronal cells in general, and hN2™ Cells in particular, for these bioassays. Human nerve endings are exquisitely sensitive to BoNT/A, and are the clinically relevant targets of the BoNT/A. Thus, a human neuronal cell-based functional assay closely mimics the clinical situation. Second, a neuronal cell-based assay incorporates the same mechanistic steps employed by BoNT/A following human exposure, i.e., membrane receptor binding and uptake, translocation, and target protein inactivation. Further, the currently employed mouse bioassays that are used for BoNT detection or potency assessment are labor intensive, subject to variability, require large numbers of animals, and do not allow for unit standardization. Thus, there is a compelling need to develop alternative bioassays that possess the sensitivity of rodent assays, but also provide greater consistency, allowing both basic scientists and clinicians to assess BoNT/A activity with greater confidence. Our preliminary findings suggest that 'thaw and use' hN2™ Cells may fill this critical gap.

## BACKGROUND

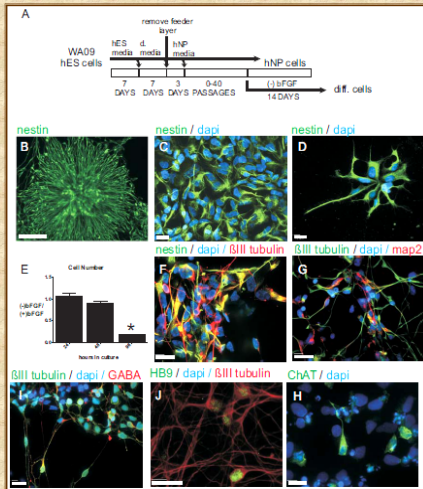
The hN2™ Cells are derived from adherent cultures of well characterized human neural progenitor cells. The adherent cell process, first developed at the University of Georgia and now Aruna, was originally published in 2006, and has been granted a U.S. patent licensed by Aruna Biomedical for commercialization. Briefly, pluripotent hESC are grown to a desired level of confluency on feeder cells, and then the growth media is replaced by serum-free media that still contains bFGF. Once the stem cells form rosette structures, the feeder cells are removed and the media is switched to neural inductive media containing neural basal medium, B27 supplement, bFGF and LIF. The derivation method is uniquely suited for the formation of adherent NP cell cultures with >95% neuronal populations within less than three weeks. Further differentiation for 2 weeks yields hN2™ Cells. In addition, although hN2™ Cell cultures are a mix of different neuronal phenotypes, it is possible to push the cells to certain towards specific phenotypes such as motor neurons by manipulating the culture conditions.

## Origin and Derivation of hN2™ Cells

Table 1 compares two NP cell lines generated from WA09 HESCs (Oct-4 positive) that were characterized for Nestin, Sox2, and Oct-4. Both lines were >90% Nestin- and Sox2-positive and <2% Oct-4, regardless of how the cells were counted (microscopy vs flow cytometry). Using analysis of variance, there was no statistical difference between the hNP lines for Nestin. Aruna's hNP lines are mostly Oct-4-negative (eESC marker) and their doubling time is 36 hrs. These NP cell lines can be continuously propagated as NP cells without altered karyotype, allowing for the expansion of cell numbers within a short period of time, and providing a continuous supply of neural progenitors that differentiate into cells such as hN2™ Cells.

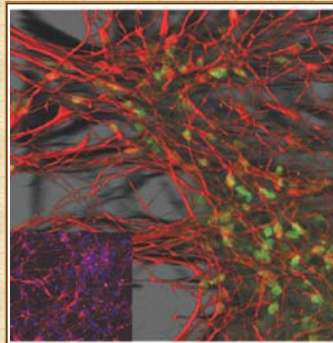
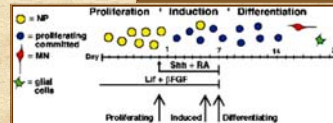
Table 1: % Positive hNPs

| Cell Line  | IF microscopy (>1000 cells counted) |                     |                     | flow cytometry (>10,000 cells counted) |                     |
|------------|-------------------------------------|---------------------|---------------------|--|---------------------|
|            | % Nestin <sup>+</sup>               | % SOX2 <sup>+</sup> | % OCT4 <sup>+</sup> | % Nestin <sup>+</sup>                  | % OCT4 <sup>+</sup> |
| hNP line 1 | 99 ± 1                              | 92 ± 2              | 2 ± 0.5             | 99.2                                   | 0.097               |
| hNP line 2 | 98 ± 1                              | 94 ± 2              | 2 ± 0.5             | 99.4                                   | 0.34                |
| Renal      |                                     |                     |                     | 0.5                                    | NA                  |
| HUVEC      |                                     |                     |                     | NA                                     | 0.01                |



A. Protocol used for derivation of nestin-positive neural progenitors (NP) from H9 human embryonic stem cells. B. Nestin-positive neural rosettes formed after removal of feeder cells. C. Cells maintain a neuroepithelial phenotype initially. D. After passaging onto laminin-coated plates cells, and extensive expansion up to 100 passages, cells still maintain normal phenotype and karyotype. E. Removal of bFGF slows proliferation, promoting differentiation by 96 h. F. Differentiated cells are initially nestin-positive. G. By 2 weeks in culture, cells become  $\beta$ -III tubulin-positive (hN2 cells). hN2 Cells are mainly  $\beta$ -III tubulin- and MAP2-positive. I-J. At this point they are mixed population of neuronal cells, some of which are (J) HXB9 positive (motor neuron marker).

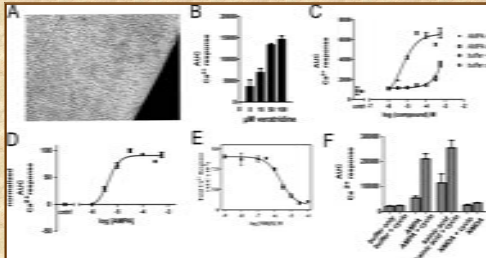
## Derivation Of Motor Neurons from Human NP Cells



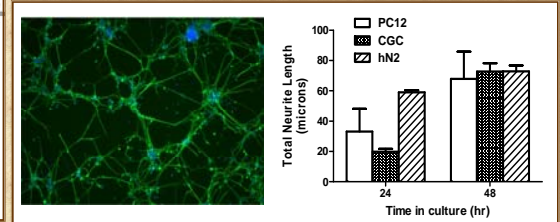
Motor neuron phenotype marker expression in differentiated neuroepithelial cells. A. Islet1 (green) and TuJ1 (red). Inset ChAT (red) and DAPI (blue). Magnification 200X.

## hN2™ Cells in Fast Cell-Based Assays

The hN2™ Cell cultures are a mix of several neuronal phenotypes but the cells have proven to provide uniform results in several cell-based assays. Aruna has cryopreserved differentiated cultures of hNPs which will send out neurite projections within hours of thawing, and are highly MAP2 and  $\beta$ III tubulin positive. These can be shipped overnight on dry ice. hN2™ Cells are ideal for functional assays because they form an adherent monolayer of neuronal cells when thawed, and can be used within 48 h in high content cell-based assays, avoiding the complexities and/or uncertainties associated with assays utilizing cultures of primary or immortalized cells. Two very different assays using these cells are illustrated below.



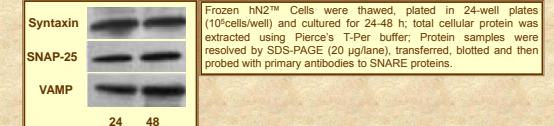
Pharmacology of hN2™ Cells using FLIPR assay. (A) hN2™ Cells were plated into 96-well plates where they formed a monolayer. (B) Increasing doses of veratridine, a Na<sup>+</sup> channel activator, resulted in an increased calcium response. (C) The differentiated human neurons respond to compounds that potentiate AMPA responses. Cyclothiazide (50  $\mu$ M) a compound known to potentiate 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 EC50 of 4.5  $\mu$ M (in the presence of 50  $\mu$ M cyclothiazide). (E) NBQX (AMPA/kainate receptor antagonist) blocks the glutamate-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub> in hN2™ Cells. (F) Responses to both AMPA and kainic acid but not NMDA could be detected and potentiated with 50  $\mu$ M cyclothiazide. (collaborative study with Dr. Thomas Murray, Creighton University)



Aruna thaw and use hN2™ Cells in a traditional Cellomics neurite outgrowth assay. Left, hN2™ Cells plated in 96-well plates, fixed and stained for  $\beta$  III tubulin 48 hours post-thaw. Right, Cultures are amenable to quantification with Cellomics software and provide consistent results; in this study, neurite extension was apparent earlier in hN2™ Cells than in PC12 cells and cerebellar granular cells (CGC) (courtesy of B. Mundy, EPA).

## hN2™ Cells Express SNARE proteins

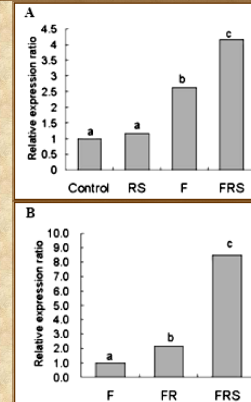
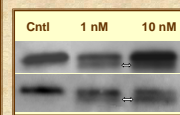
SNARE proteins are integral in the fusion machinery necessary for transmitter release, and are the intracellular targets for the different serotypes of BoNT. In this study, the SNAREs SNAP-25, VAMP and syntaxin are expressed in hN2™ Cells and were detected using standard western blotting protocols as early as 24 hours post-plating.



Frozen hN2™ Cells were thawed, plated in 24-well plates (10<sup>6</sup> cells/well) and cultured for 24-48 h; total cellular protein was extracted using Pierce's T-Per buffer; Protein samples were resolved by SDS-PAGE (20  $\mu$ g/lane), transferred, blotted and then probed with primary antibodies to SNARE proteins.

## BoNT/A Cleaves SNAP-25 in hN2™ Cells

Aruna hN2™ Cells were thawed, plated on ornithine-laminin coated 6-well plates and grown for 48 h at 37°C in a humidified CO<sub>2</sub> incubator. Purified BoNT/A (Metabolics, Inc., Madison WI) was then added to the cultures at 1, 10 nM concentrations. Cultures were harvested at 8 h post-toxin and probed for SNAP-25 cleavage using Millipore's SNAP iD™ western blotting system. SNAP-25 cleavage is indicated by a decrease in intact SNAP-25 and/or the appearance of a second, slightly lower cleaved SNAP-25 band (~24 kDa); in this case, the presence of cleaved SNAP-25 bands is indicated by white arrows.



## SUMMARY

Aruna's hN2™ Cells are derived from stable human NP cells that have the potential to produce motor neurons in an adherent culture system. SNAP-25 protein, the intracellular target of BoNT/A, is endogenously expressed in Aruna's hN2™ Cells. Further, our preliminary findings indicate hN2 cell SNAP-25 is cleaved by exposure to 1nM BoNT/A within 48 hrs after thawing/plating, a clear indication that these human neuronal cells are sensitive to BoNT/A. Based on these preliminary findings, we propose that 'thaw and use' hN2™ Cells are ideally suited for feasibility studies to develop human cell-based bioassays for assessment of BoNT detection, potency, and antagonism.