



# Comprehensive Gene Expression Analysis and Neurotoxicity Testing of Human iPSC-Derived Neural Progenitor Cells and Neurons

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## Abstract

Neurological models are critical to understanding the physiology of the nervous system and for assessing the neurotoxicity of pharmaceuticals and environmental compounds. In this study, we examined the ability of a novel medium to stimulate neural progenitor cells (NPCs) to differentiate into multiple types of neurons. To determine the suitability of the NPC culture system for use in toxicological studies, we investigated the sensitivity of the NPCs and dopaminergic neurons for various known toxic compounds.

## Introduction

Human induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) and neurons are an attractive in vitro model to study neurological development, neurotoxicity, and to model disease. However, there is a lack of validated NPC lines and media that support differentiation into multiple types of neurons for disease modeling, drug screening, and toxicity screening<sup>1-6</sup>.

New model systems that recapitulate the components of the nervous system are vital for novel neurobiological discoveries and for toxicological testing. Because the nervous system is one of the most complex organ systems, it is difficult to recreate in a culture dish. Neurons do not expand quickly or at all, neural differentiation occurs over months, and knowing when your neurons are ready for experiments can be challenging. Current in vitro models of nervous system typically consist of tissue slices or neurons isolated from cadavers and animals, neural cells derived from continuous cell lines (e.g., SH-SY5Y, Neuro-2a, and PC-12), and neural cells derived from iPSCs<sup>7-12</sup>. However, primary sources can suffer from donor variation and continuous cell lines may not faithfully represent the in vivo state. Additionally, it can take two or more months before iPSC-derived differentiated neurons are ready for experimentation<sup>13</sup>.

NPCs combine the best qualities of continuous cell line-, primary cell-, and iPSC-derived neurological models. NPCs can be expanded over ten passages and fully differentiate into neurons in as little as three weeks. In addition, NPCs do not suffer from donor variation because they are derived from a single clone. ATCC recently created a complete NPC system of cells and media supplements. This application note describes the characterization and validation of two of those cell lines, an expansion medium, and a dopaminergic neuron differentiation medium.

## Materials and Methods

For dopaminergic neuron differentiation, a normal human non-reporter NPC line (ATCC® ACS-5003™) and a MAP2- NanoLuc® HaloTag® reporter NPC line (ATCC® ACS-5007™) were seeded in CellMatrix Basement Membrane Gel (ATCC® ACS-3035™)-coated 12-well plates at a seeding density of 10,000 cells/cm<sup>2</sup> and cultured in NPC expansion media (ATCC® ACS-3003™) overnight prior to treating NPCs with Dopaminergic Differentiation Media (ATCC® ACS-3004™) for up to three weeks. For qRT-PCR, RNA was extracted from ACS-5003 and ACS-5007 NPCs treated with the dopaminergic differentiation media for 0, 1, 2, or 3 weeks and cDNA was synthesized for qRT-PCR analysis of neuronal gene expression by using TaqMan® probes (Thermo Fisher Scientific, Inc.) shown in Table 1. For neurotoxicity assays, the ACS-5003 and ACS-5007 NPCs were seeded in CellMatrix Basement Membrane Gel-coated 96-well plates at a seeding density of 40,000 cells/cm<sup>2</sup> and cultured in NPC expansion media for two days prior to adding multiple neurotoxicants at 1 μM, 10 μM, or 100 μM for two days. Neurotoxicity was assessed by using resazurin viability dye. To examine neurotoxicity of neurons using resazurin and high-content imaging methods, ACS-5003 and ACS-5007 NPCs were seeded in CellMatrix-coated 96-well and 24-well plates, respectively, at a seeding density of 10,000 cells/cm<sup>2</sup> and treated with dopaminergic differentiation media for three weeks prior to adding various concentrations of drugs for two days.

**TABLE 1.** TaqMan® probes used for gene expression analysis.

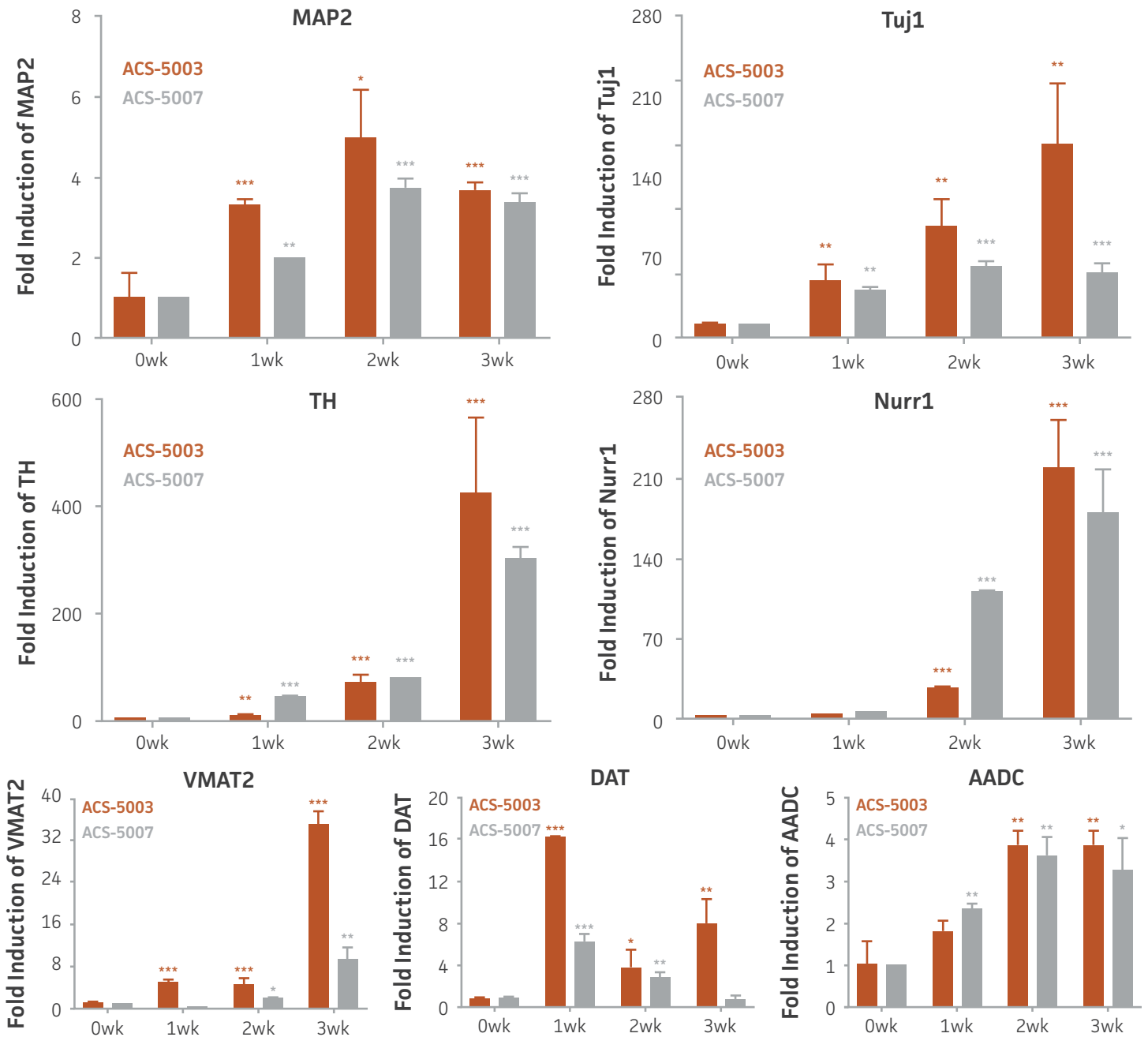
| Type of neurons       | Name of TaqMan probes       |
|-----------------------|-----------------------------|
| Early neurons         | MAP2, TUBB3 (Tuj1)          |
| Dopaminergic neurons  | TH, Nurr1, VMAT2, DAT, AADC |
| Glutamatergic neurons | GLS2, vGLUT1, vGULT2        |
| GABAergic neurons     | GABRB3 (GABA)               |
| Motor neurons         | EN1, LIM3, HB9              |
| Cholinergic neurons   | ChaT                        |

## Results/Discussion

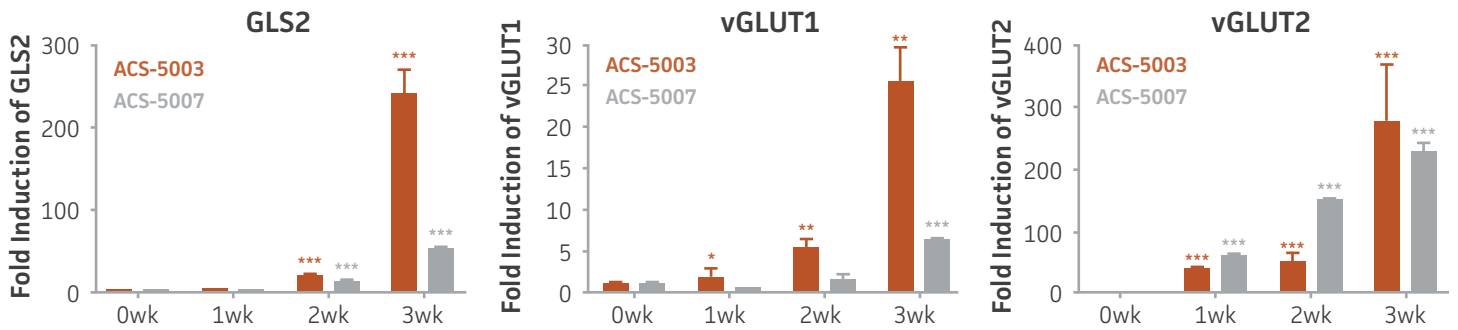
Here, we investigated the expression of genes associated with the differentiation of NPCs during three weeks in dopaminergic differentiation media. Known early neuron markers, MAP2 and Tuj1 genes reached peak expression at two weeks while expression of dopaminergic neuronal genes (TH, Nurr1, VMAT2, AADC) was significantly increased in a time-dependent manner ( $p < 0.05$ ) in two types of normal NPCs (Figure 1). Furthermore, expression of genes associated with glutamatergic (vGLUT1, vGLUT2, GLS2; Figure 2) and GABAergic (GABRB3; Figure 3) neurons was also induced and peaked at the end of three weeks. This shows that ATCC NPCs and dopaminergic differentiation media are capable of producing GABAergic and glutamatergic neurons, in addition to dopaminergic neurons. Noticeably, there was significant induction of motor neuron gene (EN1, LIM3, Hb9) expression in ACS-5003 NPCs while there was significant induction of cholinergic (ChAT) neuron gene expression in ACS-5007 NPCs, but not in ACS-5003 NPCs during dopaminergic differentiation (Figure 3). Moreover, expression of TH, Tuj1, Nurr1, GLS2, and vGLUT2 neuronal markers in both ACS-5003- and ACS-5007-derived neurons has been confirmed by immunocytochemistry (Figure 4). To validate that ATCC NPCs and dopaminergic neuron differentiation media are suitable for drug screening, we conducted neurotoxicity screenings in the two types of NPCs and NPC-derived neurons by using a resazurin viability assay and high-content imaging analysis. We found that paclitaxel, a microtubule-stabilizing chemotherapeutic agent, significantly induced neurotoxicity ( $p < 0.001$ ) in the two types of NPCs evaluated at a  $IC_{50}$  of  $\sim 1 \mu\text{M}$ , but not in NPC-derived neurons (Figures 5 and 6). Vincristine, amiodarone, and chlorhexidine significantly decreased viability of both NPCs and neurons, whereas piperine, cisplatin, and hydroxyurea did not induce any significant neurotoxicity in neurons (Figures 5 and 7). This study demonstrates that ATCC iPSC-derived NPCs and dopaminergic differentiation media are suitable for studying neurological development and neurotoxicity screening.

## Conclusions

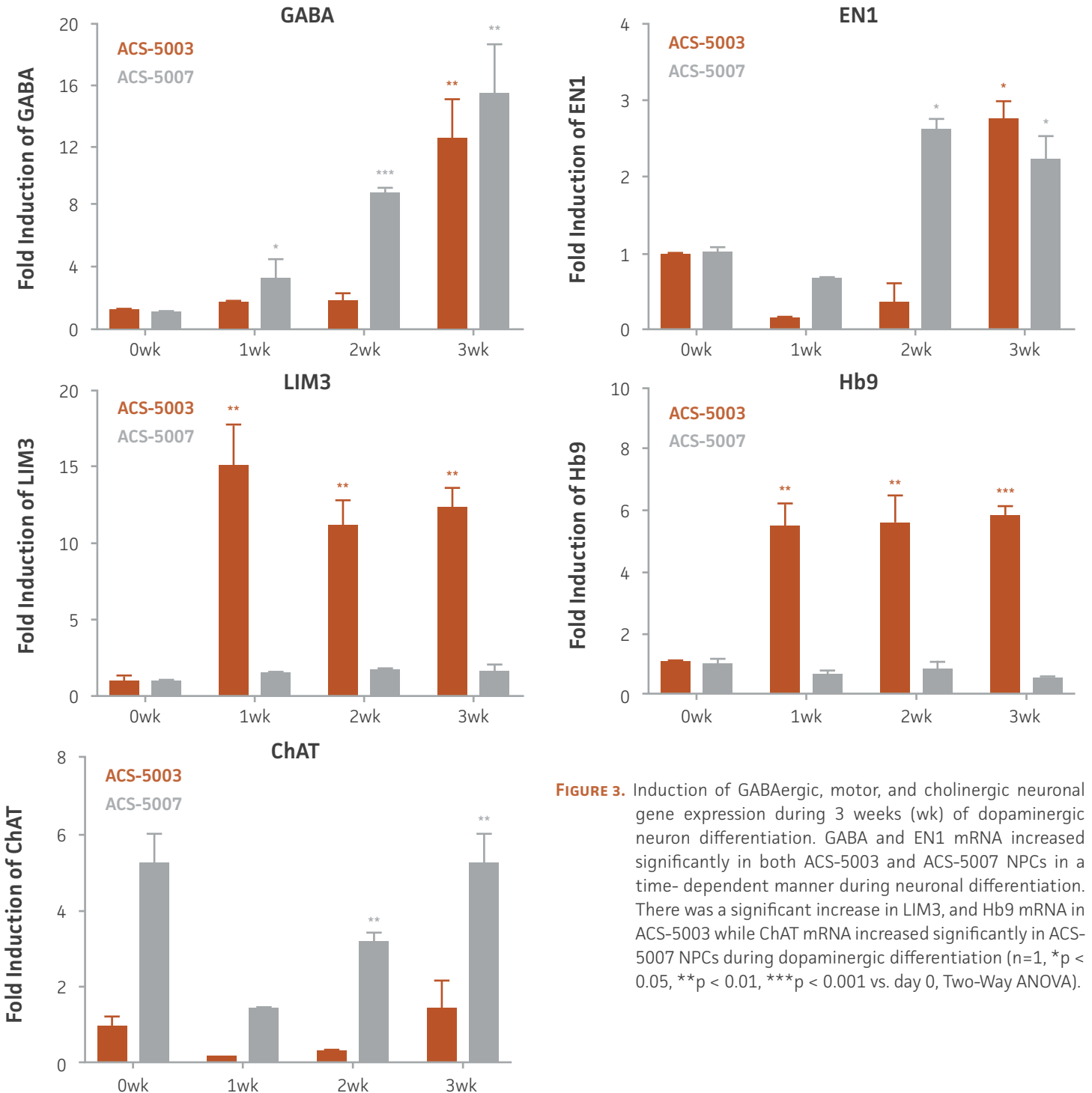
Human iPSC-derived NPCs and neurons provide an unlimited resource for in vitro disease modeling, toxicity screening, and drug screening. ATCC has developed normal and lineage-specific GFP or Nanoluc-HaloTag reporter NPCs. The normal and reporter-labeled NPCs have the potential to be differentiated into dopaminergic, GABAergic, glutamatergic, motor, and cholinergic neurons after treatment with ATCC Dopaminergic Differentiation Media. In addition, NPCs and NPC-derived neurons have been validated for drug screening with several neurotoxicants by using a resazurin viability assay and high-content imaging assessment. Therefore, ATCC NPCs, NPC Expansion Media, and Dopaminergic Differentiation Media are suitable for studying neurological development and neurotoxicity screening.



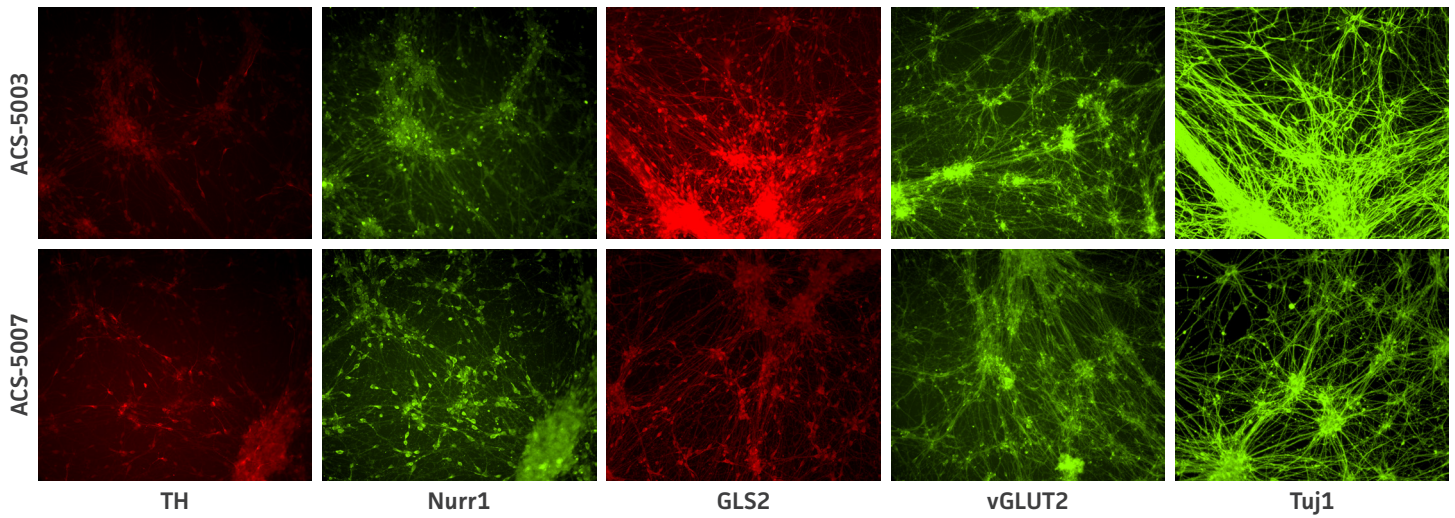
**FIGURE 1.** Induction of neuronal gene expression during 3 weeks of dopaminergic neuron differentiation. Expression of early neuronal genes (MAP2 and Tuj1) increased significantly in both ACS-5003 and ACS-5007 within a week (wk) of dopaminergic media treatment. In contrast, TH and dopaminergic neuronal transcription factor Nurr1 mRNA increased significantly and reached maximum level by the end of three weeks of differentiation. Related genes like VMAT2 and DAT with opposite functions also increased significantly. AADC also significantly increased in a time dependent manner in ACS-5003 and ACS-5007 during neuronal differentiation (n=1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. day 0, Two-Way ANOVA).



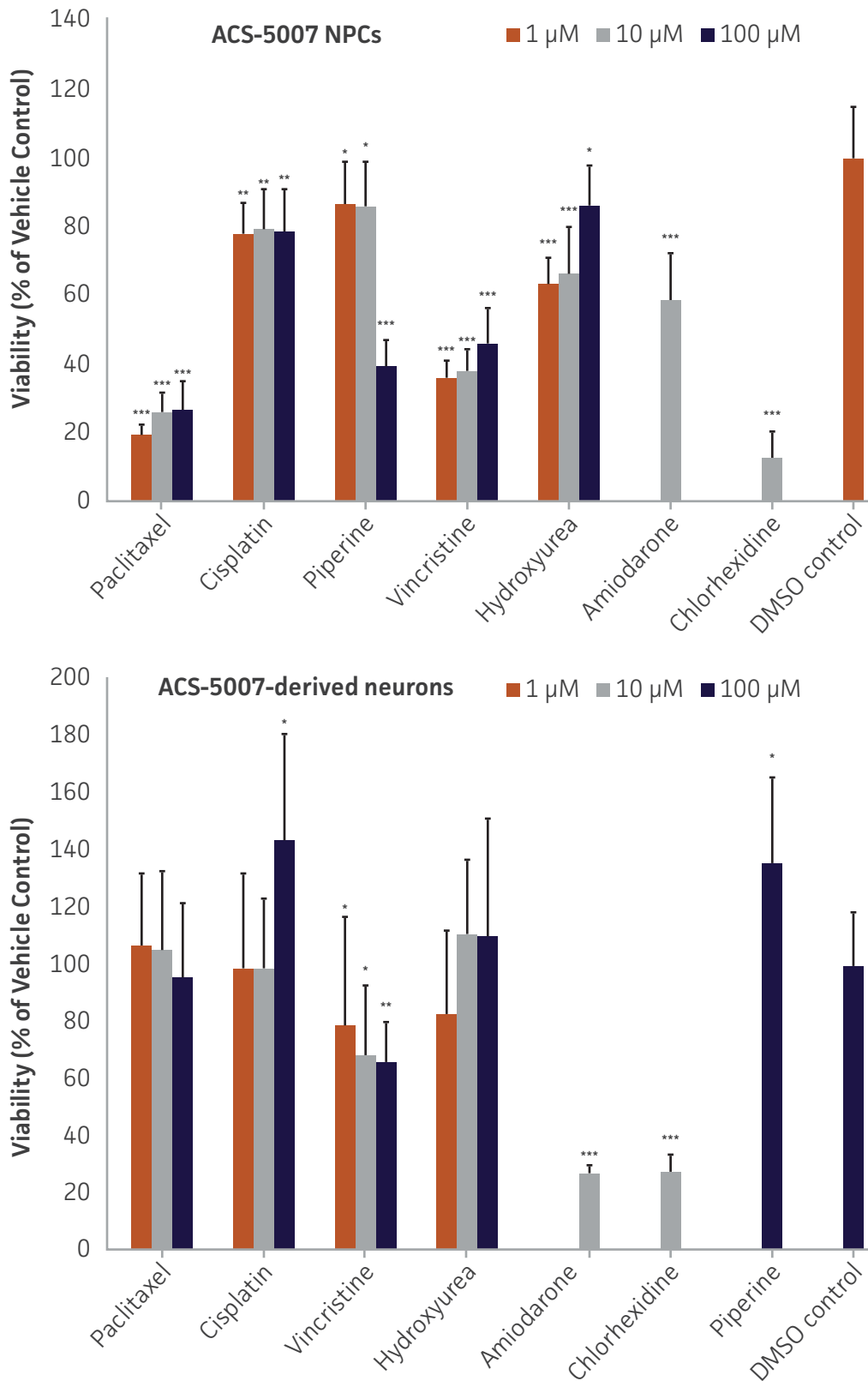
**FIGURE 2.** Induction of glutamatergic neuronal gene expression during 3 weeks of dopaminergic neuron differentiation. Expression of GLS2, vGLUT1, and vGLUT2 mRNA increased significantly in a time dependent manner during 3 weeks (wk) of dopaminergic media treatment in ACS-5003 and ACS-5007 NPCs (n=1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. day 0, Two-Way ANOVA).



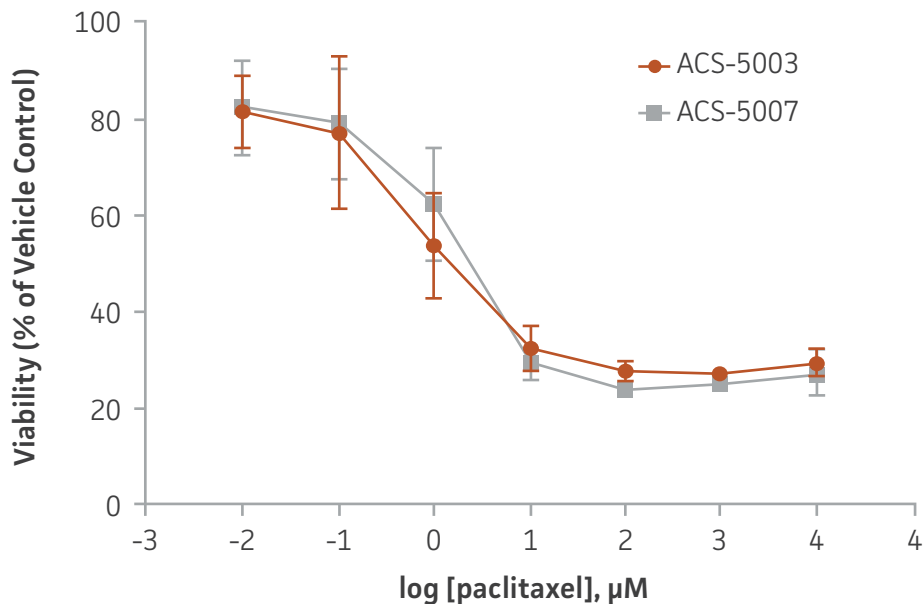
**FIGURE 3.** Induction of GABAergic, motor, and cholinergic neuronal gene expression during 3 weeks (wk) of dopaminergic neuron differentiation. GABA and EN1 mRNA increased significantly in both ACS-5003 and ACS-5007 NPCs in a time- dependent manner during neuronal differentiation. There was a significant increase in LIM3, and Hb9 mRNA in ACS-5003 while ChAT mRNA increased significantly in ACS-5007 NPCs during dopaminergic differentiation (n=1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. day 0, Two-Way ANOVA).



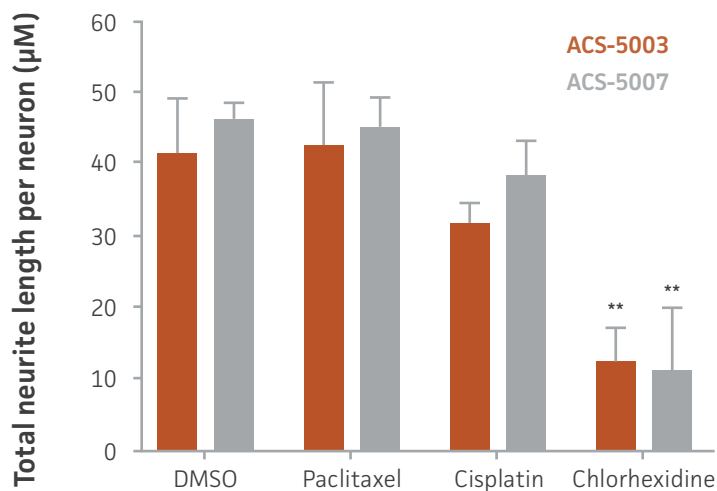
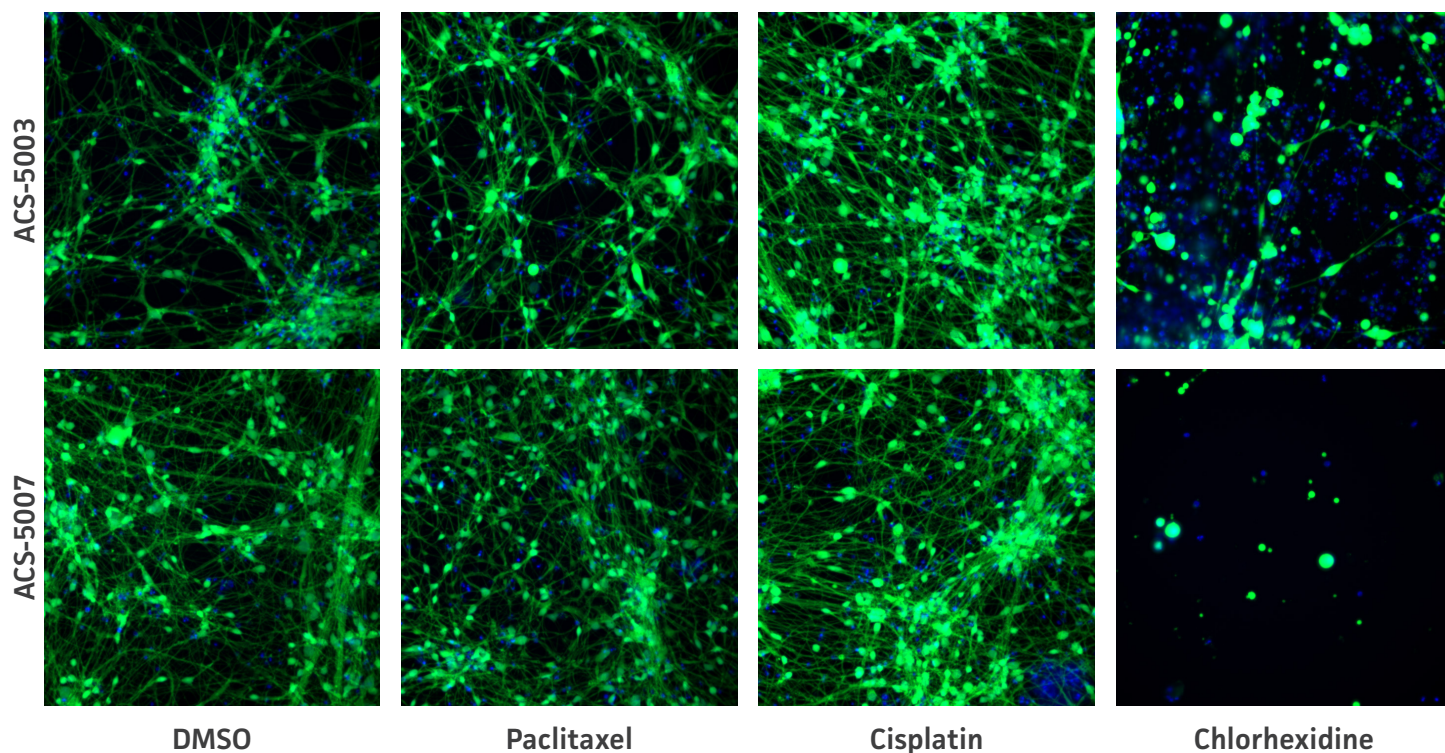
**FIGURE 3.** Immunocytochemistry of ACS-5003- and ACS-5007-derived neurons after 3 weeks of dopaminergic neuron differentiation. Consistent with qRT-PCR data, after three weeks incubation in dopaminergic differentiation media there was a marked increase in expression of TH, Nurr1, GLS2, vGLUT2, and Tuj1 neuronal markers. Original magnification, x20.



**FIGURE 4.** Dose-response curves for cell viability of ACS-5007 NPCs and ACS-5007 NPC-derived neurons treated with paclitaxel, cisplatin, piperine, vincristine, chlorhexidine, amiodarone, and hydroxyurea for two days. Paclitaxel, cisplatin, piperine, and hydroxyurea significantly induced neurotoxicity ( $p < 0.05$ ) in NPCs, but not in NPC-derived neurons. Vincristine, chlorhexidine, and amiodarone significantly decreased viability ( $p < 0.01$ ) of both NPCs and neurons ( $n=3$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. DMSO control, Student's T-test). The only concentration tested for amiodarone and chlorhexidine was 10  $\mu\text{M}$ . Similar neurotoxicity was observed in ACS-5003 cells (data not shown).



**FIGURE 6.** Dose-response curves for cell viability of ACS-5003 and ACS-5007 NPCs treated with paclitaxel for two days. The neurotoxic effect of paclitaxel was similar in both types of NPCs. IC<sub>50</sub> of paclitaxel on ACS-5003 and ACS-5007 NPCs was 0.9 μM and 1.6 μM, respectively (n=2).



**FIGURE 7.** High-content imaging of chemotherapeutic-treated NPCs supports cell viability experiments. ACS-5003 and ACS-5007 NPCs-derived neurons were incubated with 10 μM paclitaxel, cisplatin, or chlorhexidine for two days, stained with Calcein Green AM, and Hoechst 33342, and then subjected to high content imaging. Consistent with the viability data, chlorhexidine significantly inhibited the neurite outgrowth (n=2, \*\*p < 0.01 vs. DMSO, Student's T-test). Original magnification, x20.

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