Supplemental Information

One-step seeding of neural stem cells with vitronectin-supplemented medium for high throughput screening assays

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Content:

Supplemental Materials and Methods

Compounds. Twenty four compounds (**Table S2**) with reported neurotoxicity were selected for tests in cytotoxicity assays using NSCs differentiated from stem cells. All of the compounds were dissolved in DMSO as 10 mM stock solutions that were diluted in DMSO at a 1:3 ratio for eleven concentrations. AR-C 118925XX, Suraminutes hexasodium salt, Tolterodine L-tartrate, and Telenzepine dihydrochloride were purchased from R&D Systems; Ionomycin from Tocris; Carbachol from Sigma-Aldrich; and EGTA-AM and BAPTA-AM from Santa Cruz Biotechnology. All of the compounds were dissolved in DMSO as 10 mM stock solutions that were diluted in DMSO at a 1:3 ratio for eleven concentrations.

NSCs Induction from iPSCs. Wild type (GM23450, Coriell Cell Repositories) and iPSCs previous derived from NPC patient fibroblasts[1] were cultured under the condition as reported by Beers J, et al.[2] Briefly, Matrigel (354277, Corning Matrigel® hESC-Qualified Matrix, LDEV-Free, Corning) was diluted in Advanced DMEM/F12 medium (Life Technologies) to 200 μg/ml that was added to 6-well plates at 1 ml/well. After incubation for 1 hour at room temperature, the plates were washed once with medium. The iPSCs were suspended in Essential 8™ Medium (A1517001, Life Technologies) and seeded to 6-well plates coated with Matrigel as above. When cells reached 80-90% confluency, cells were digested with 0.5 mM EDTA and reseeded to Matrigel coated 6-well plates at 3 X 10^5cells/well for NSC differentiation. Neural Induction Medium kit (Life Technologies) was used for generation of neural stem cells from iPSCs. On Day 1 after iPSC splitting (15-25% confluency), the culture medium was replaced with the Neural Induction Medium containing the Neurobasal medium supplemented with Neural Induction Supplement. On day 7, the initial NSCs were dissociated with Accutase and plated for further expansion in neural expansion medium containing Neurobasal medium, Advanced™ DMEM⁄F-12 medium and 1X GIBCO Neural Induction Supplement (Catalog # 21103-049, 12634010 and A16477-01, Life Technologies). After 2 passages, the culture medium was switched to StemPro NSC SFM (A1050901, Life Technologies) containing knockout DMEM/F12, StemPro neural supplement, 20ng/ml bFGF, 20ng/ml EGF and 1X GlutaMAX. Immunohistochemistry staining with antibodies against Nestin, SOX1, SOX2, PAX6, Vimentin, and SSEA4 neural markers was utilized to characterize the NSCs induced from human iPSCs. The WT NSCs and NPC NSCs were cultured as previously described[1].

Microtiter plates pre-coating procedures. Polystyrene plates (384- and 1536-well, tissue-culture treated) were purchased from Greiner Bio-One (Monroe, NC) and Poly-D-Lysine (PDL) coated 384-well plates were purchased from Corning. As a control for assays using neural stem cells, Matrigel was freshly coated to assay plates before each experiment. Briefly, 5μl/well of 200μg/ml Matrigel in cold DMEM⁄F-12 medium was added to 1536-well plates and the plates were centrifuged for 5 minutes at 200g to allow

even coating on the bottom of plates followed by incubation at RT for one hour. Immediately before cell seeding, the plates were inverted and centrifuged for 2 minutes at 200g to remove the coating liquid. As a control for assays using neurons differentiated from neural stem cells, the poly-L-ornithine and laminin dual coating was used. Briefly, 384-well plates received 20µl/well of 20µg/ml PLO (P3655, Sigma) in distilled water and incubated overnight at room temperature followed by three plate-washes with distilled water. The plates received 20µl/well of 10µg/ml laminin in phosphate-buffered saline (PBS) without calcium or magnesium and incubated for 3 hours at 37 °C followed by one plate wash before use.

Immunofluorescence Staining of iPSC NSCs and Neuronal Cells. Immunohistochemistry staining of NSC markers (Nestin, SOX-1, SOX-2, PAX6, SSEA4, and Vimentin) and neuronal cell markers (β-III Tubulin (TUJ1), microtubule-associated protein 2 (MAP2) and Neurofilament L (NF-L)) as well as astrocyte marker (GFAP) were performed for characterizations of differentiated NSCs and neurons (**Table S4**). The NSCs and neuronal cells were stained in 1536- and 384-well plates respectively. All the liquid handling steps including aspiration, dispensing and washing were conducted in automated microtiter plate liquid handling system (GNF system). The volume for blocking, antibody incubation and nuclear staining was 5µL/well for 1536-well plates and 25µL/well for 384-well plates and was 10µL/well and 50µL/well for plate washing. The same cell staining buffer (420201, Biolegend) was used to block and dilute antibodies. Briefly, 8% paraformaldehyde was added to 1536- or 384-well plates at the equal volume with the culture medium in the plates and incubated with cells for 30 minutes to fix cells. After aspirating the fixation solution, 5µL/well and 25 µL/well of 0.3% Triton X-100 was added into 1536-well and 384-well plates respectively, and incubated for 20 minutes for cell membrane permeabilization followed by 1 hour blocking. The cells were then incubated with primary antibodies at optimized concentration at 4 °C overnight. After washing 3 times with PBS, correlated secondary antibodies were added. Cells were then stained with Hoechst 33342 (H1399, Life Technologies) for 20 minutes after 3 PBS washes and imaged on an IN Cell 2200 Imaging system (GE Healthcare Life Sciences) with a 40X objective lens and FITC or/and CY5 and DAPI filter sets.

Karyotyping of iPSC NSCs growing on rhVTN-N. The NSCs suspended in the medium added with 20µg/ml rhVTN-N were seeded to 6-well plates and the cells were cultured for at least 10 passages. The cells were sent to the cytogenetics Lab of the WiCell Research Institute (WI) for karyotyping analysis.

Data analysis and statistics. In the cytotoxicity assays, S/B ratio was calculated by dividing the luminescence or fluorescence signal readout from wells with cells added with DMSO control by those from wells with the highest concentration of cytotoxic compound (such as thapsigargin) or calcium

antagonists compounds. Z-factor was calculated using the following equation: $Z = 1 - ((3 \times s.d._{total} + 3 \times s.d._{basal})/(mean_{total} - mean_{basal}))$, where sd_{total} and $mean_{total}$ are the standard deviation and mean of DMSO treated wells, and $s.d._{basal}$ and $mean_{basal}$ are the standard deviation and mean of wells treated with cytotoxic compound (thapsigargin) or calcium antagonists compounds. CV (%) was calculated as (s.d./mean) x 100.

Unless otherwise noted, all values are expressed as the mean ± s.d. (n = 3). For the statistical analysis, results were analyzed using one-way or two-way ANOVA and differences were considered statistically significant if $p < 0.05$.

Figure S1

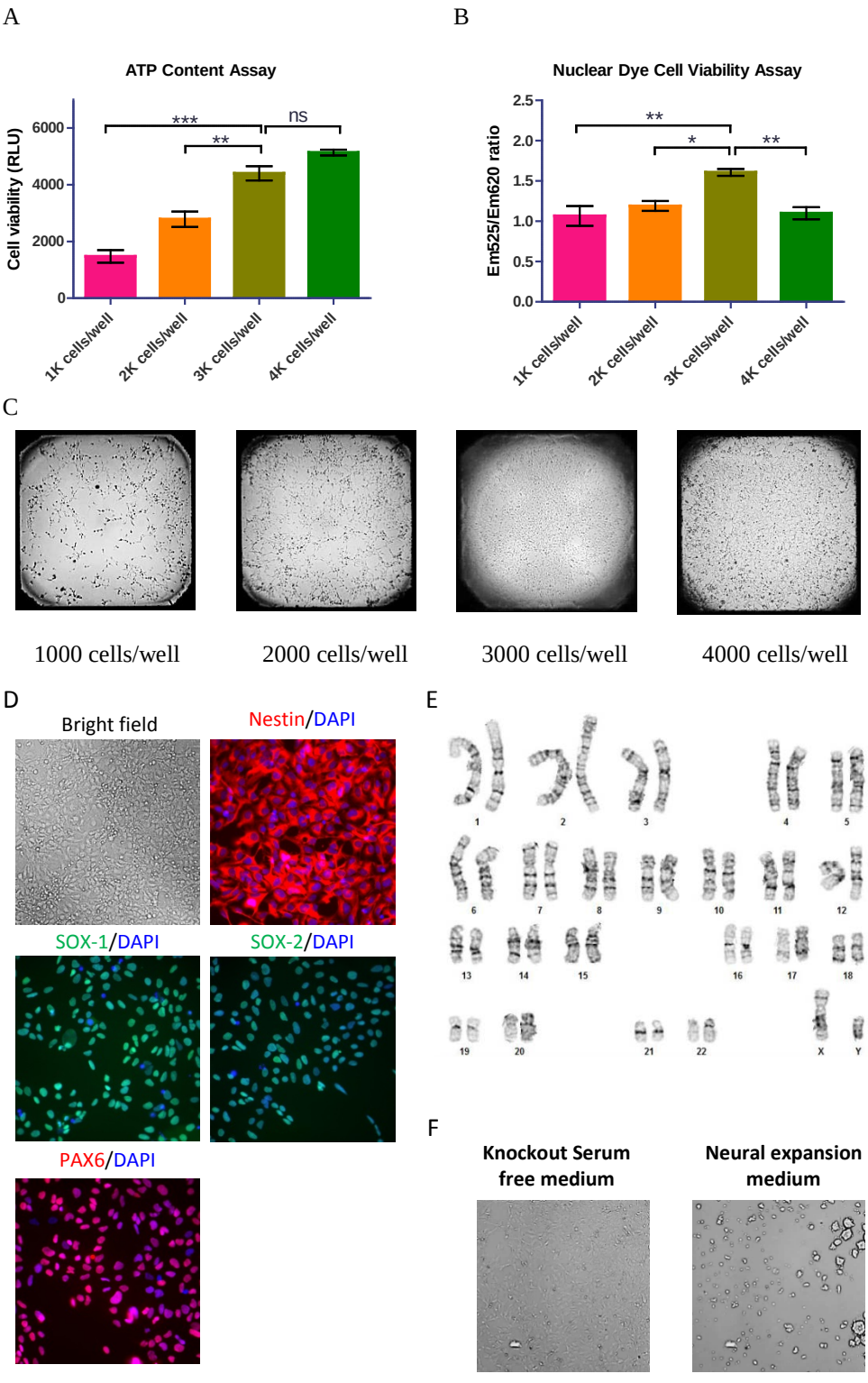
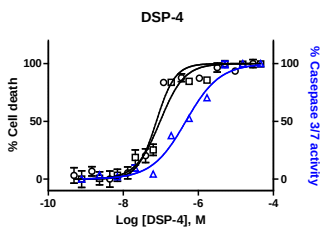
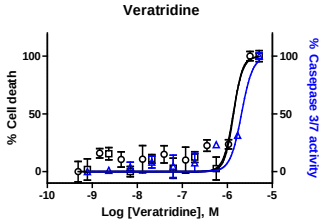

A
ATP Content Assay
Cell viability (RLU)
6000
4000
2000
0
***
**
ns
1K cells/well
2K cells/well
3K cells/well
4K cells/well
B
Nuclear Dye Cell Viability Assay
Em525/Em620 ratio
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
1K cells/well
2K cells/well
3K cells/well
4K cells/well
C
1000 cells/well
2000 cells/well
3000 cells/well
4000 cells/well
D
Bright field
Nestin/DAPI
SOX-1/DAPI
SOX-2/DAPI
PAX6/DAPI
E
F
Knockout Serum free medium
Neural expansion medium

Figure S1. Assay development and validation. NSCs cultured in 1536-well plates were first tested at densities of 1000, 2000, 3000 and 4000 cells/well in 1536-well plates using the ATP content and nuclear dye staining assays. The density of 3000 cells/well yielded more viable cells than 1000 and 2000 cells/well both in (A) ATP content and (B) nuclear dye cell viability assays (3000 cells/well vs. 1000 cells/well, $p<0.001$ in ATP content assay and $p<0.01$ in nuclear dye cell viability assays, 3000 cells/well vs. 2000 cells/well, $p<0.01$ in ATP content assay and $p<0.05$ in nuclear dye cell viability assays). Although there was no difference of the cell viability at 4000 cells/well in the ATP content assay ($p>0.05$), the relative fluorescence unit (RFU) ratio of the live cells to dead cells at the density of 3000 cells/well was significant higher than 4000 cells/well ($p<0.01$) in the nuclear dye cell viability assay. As a result, 3000 cells/well was selected for subsequent experiments. Data are represented as mean ± SEM of three replicates. (C) The panel of images showed the bright field pictures (10x) the neural stem cells in the 1536-well plates at different cell density. (D) Normal neural stem cells morphology and markers, as well as (E) normal 46XY karyotype observed in NSC directly seeded and cultured in regular 6-well plates for at least 10 passages with rhVTN-N-supplemented medium. (F) The WT NSCs could not attach the assay plates and unable to grow when the one-step seeding method was used with the neural expansion medium composed of Neurobasal® medium, Advance DMEM/F-12 medium and GIBCO® Neural Induction Supplement (Catalog # 21103-049, 12634010 and A16477-01, Life Technologies, as neural expansion medium) supplemented with rhVTN-N.

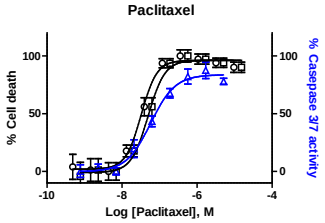
Figure S2

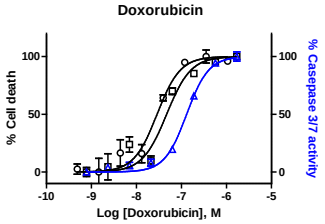
i. Toxin



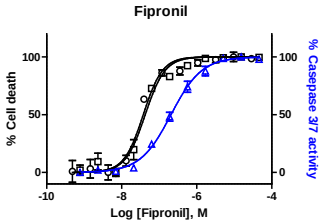


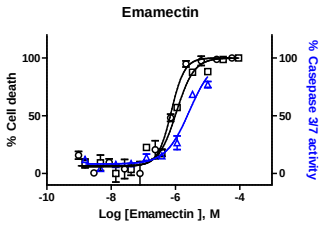
ii. Antineoplastic Agent



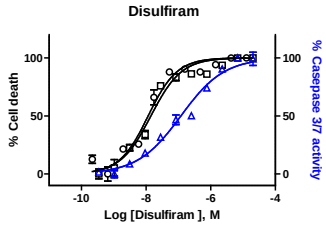


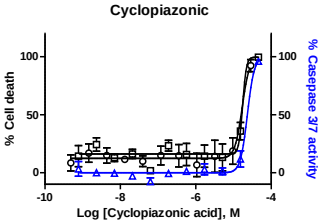
iii. Insecticide



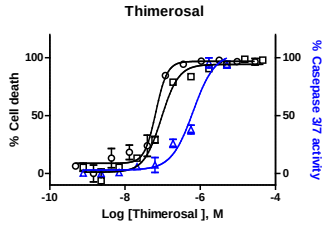


iv. Anti-infective Agent





v. Others



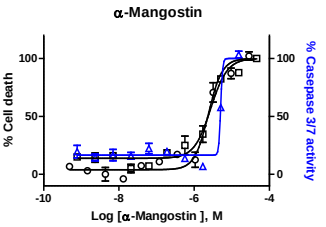


Figure S2. Concentration-response curves of representative cytotoxic compounds. The compound activities were determined in the ATP content assay (□), nuclear dye assay (○), and caspase 3/7 assay (Δ) using NSCs plated in 1536 well plates with rhVTN-N added medium. The IC_{50} values of all 24 compounds determined from these three assays were listed in **Table S2**. Data are represented as mean ± SEM of three replicates.

Figure S3

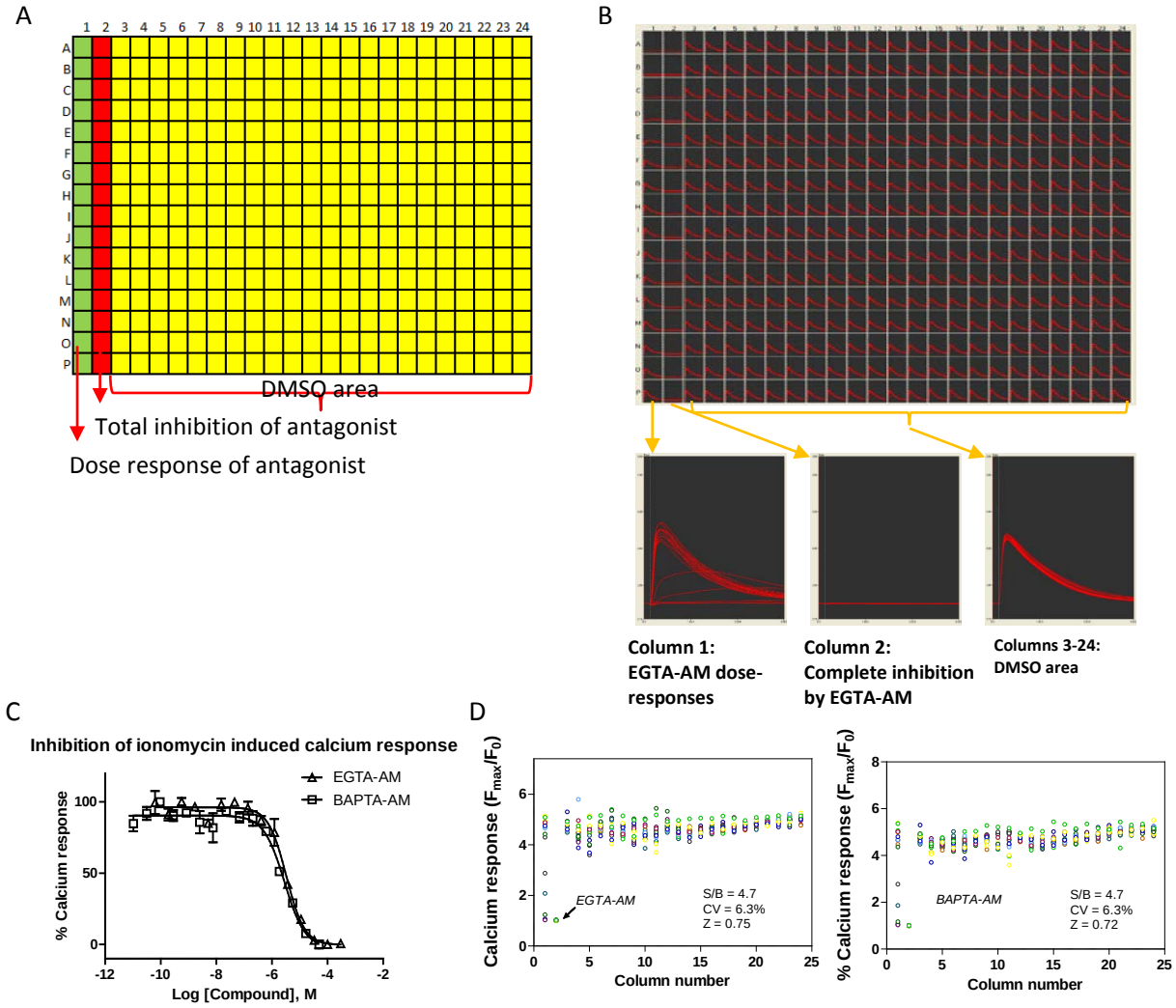


Figure S3. Results of homogenous calcium response assay using NSCs one-step seeded in 384-well plates with rhVTN-N-supplemented medium. (A) A plate map for the DMSO plate test with different antagonists. Column 1 was the concentration titration of antagonists, column 2 was the highest concentration of antagonists for complete inhibition, and columns 3 to 24 are wells that had DMSO added as a control. The whole plate was stimulated with the $EC_{50}80$ amount of agonists after taking the base line signal during the assay. (B) Tracers of kinetic calcium response determined in FDSS® µCELL kinetic fluorescence plate reader (Hamamatsu). Top panel: Ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (EGTA-AM) inhibition of ionomycin induced calcium response. Bottom panel: combined multiplex tracer of each column as indicated. (C) Concentration-dependent inhibition curves of EGTA-AM and 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) determined on 1 µM ionomycin induced calcium response in NSCs. The IC_{50} values for EGTA-AM and BAPTA-AM were 3.3 µM and 2.8 µM, respectively. Data are represented as mean ± SEM of three replicates. (D) Scatter plots of DMSO plate tests with the complete inhibition (0% response) was defined by 100 µM EGTA-AM or 50 µM BAPTA-AM. The S/B ratio, CV, and Z-factor were listed in the related plots.

Figure S4

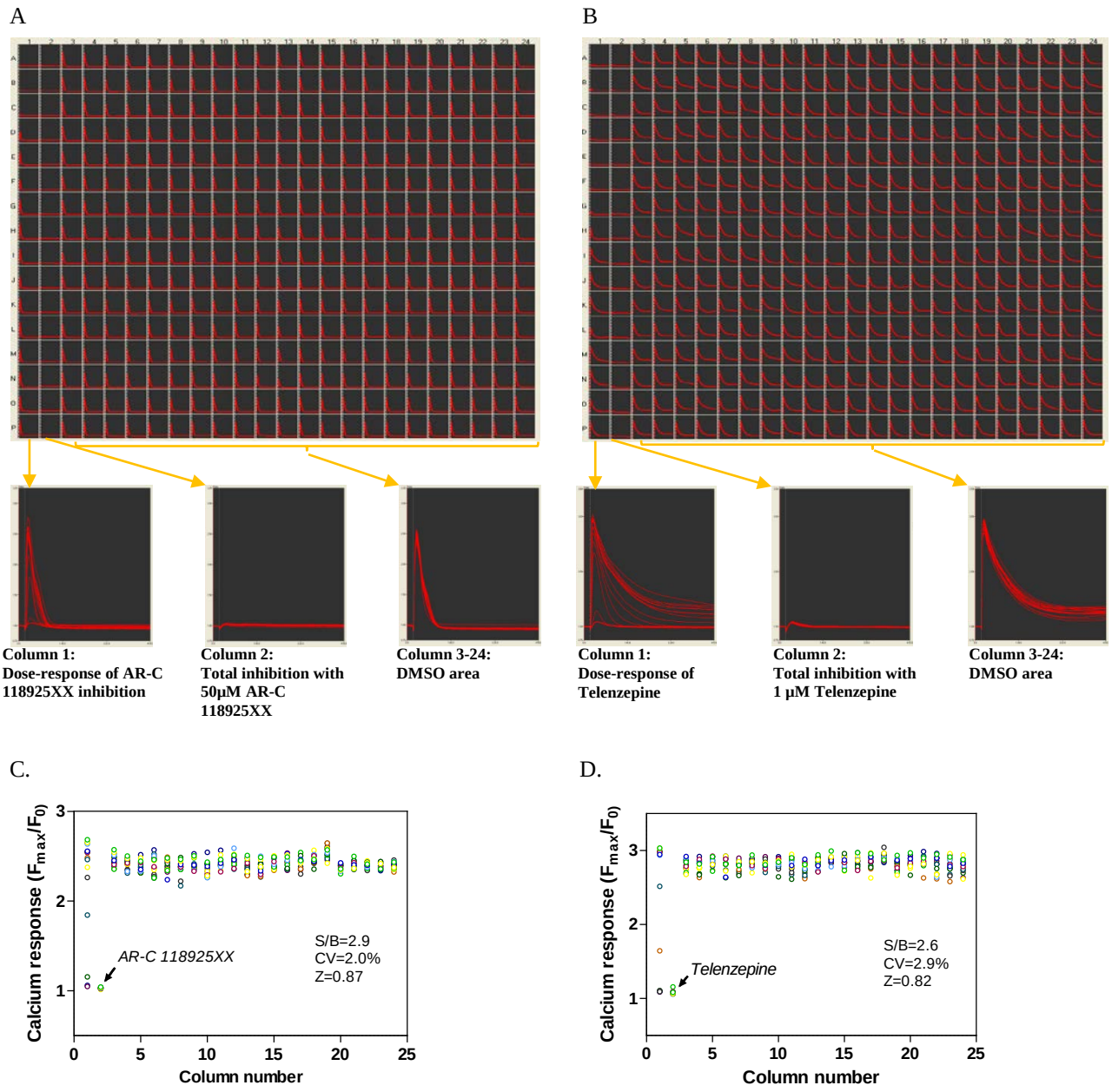


Figure S4. Results of calcium response determined in neurons differentiated from NSCs one-step seeded in commercial PDL-coated 384-well plate with rhVTN-N-supplemented medium. Whole-plate kinetic fluorescence intensity tracers of calcium responses in neurons stimulated by 0.1 μM ATP (EC_{80} concentration) through the purinergic receptor (A) and 1 μM carbachol through the acetylcholine muscarinic receptor (B). The column-1 added with a titration of related antagonist and column 2 added with a high concentration of antagonist that resulted 100% inhibition of calcium response. Using the response in wells with the high concentration of antagonist as 100% inhibition, the assay parameters (S/B ratio, CV and Z-factor) of the DMSO plate test were calculated and listed in the scatter plot (C) for ATP stimulated calcium response with AR-C 118925XX and (D) for the carbachol-stimulated calcium response with telenzepine dihydrochloride.

Figure S5

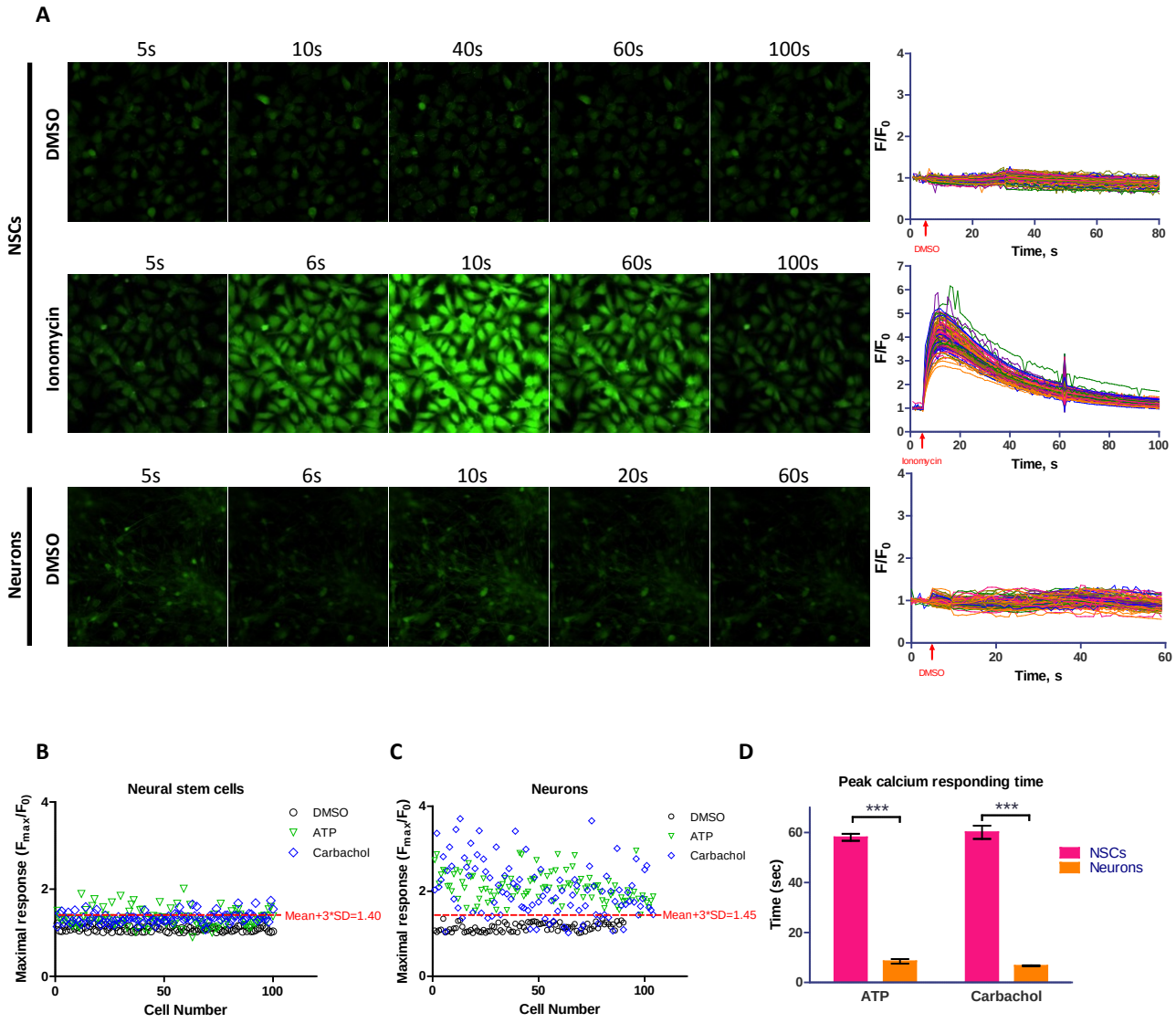


Figure S5. Results of single cell calcium responses in neural stem cells and neurons directly one-step in commercial 384-well PDL-plates with rhVTN-N-supplemented medium. (A) Serial fluorescence images and kinetic tracers of neural stem cells and neurons recorded in a single cell calcium response assay. Positive responding cells from populations of NSCs (B) and neurons (C). The calcium responses in NSCs and neurons were stimulated by DMSO control, 0.1 µM ATP, or 1 µM carbachol. The positively responding cells were identified as these with the maximal response equal to or above the average (F_{max}/F_0) of total control cells plus 3 times the standard deviations (sd) (mean of F_{max}/F + 3 X sd). The calculated cut-off ratios in NSC and neurons were 1.40 and 1.45, respectively. (D) Comparison of average times to reach the peak calcium response in NSCs and neurons. The average times to reach the peak response to the ATP and carbachol in NSCs were 58 and 60 seconds, respectively. But in neurons the times decreased to 8.5 and 6.7 seconds, respectively ($p<0.01$). The data suggested that neuronal differentiation from NSCs to neurons increased expression of receptors/channels. Data are represented as mean ± SEM of three biological replicates.

Figure S6

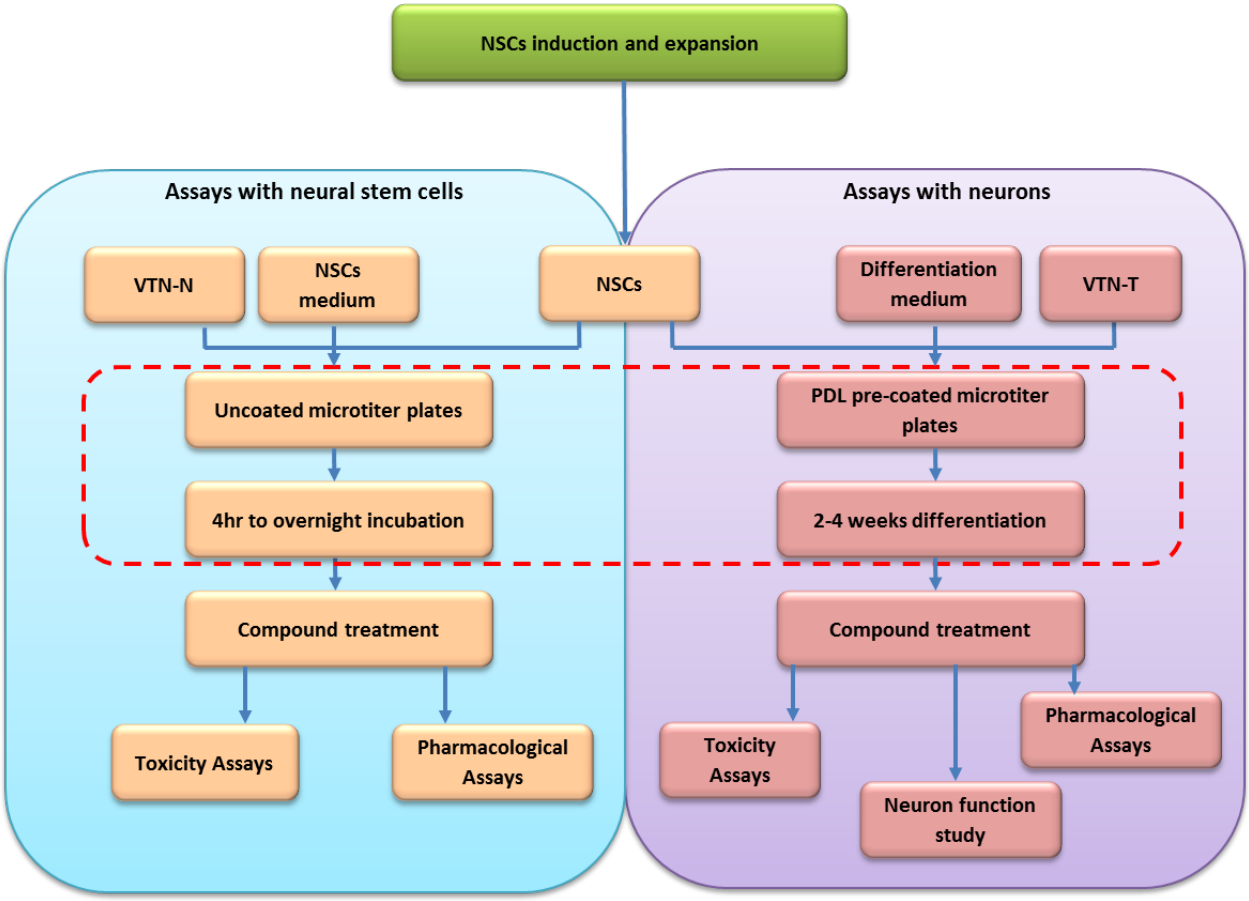


Figure S6. Flow chart of HTS assays using NSCs and NSC-differentiated neurons by directly plating NSCs with rhVTN-N-supplemented medium.

Table S1. Summary of substrates tested in the high throughput iPSC NSCs assays with one-step seeding.

Substrates Name	Final Concentration	Vendor	Catalog#
Matrigel	200μg/ml	CORNING INC.	354277
rhVTN-N	10μg/ml	Life Technologies	A14700
Fibronectin	10μg/ml	Life Technologies	PHE0023
Geltrex Matrix	1%	Life Technologies	A1413302
Laminin	10μg/ml	SIGMA	L4544
Poly-D-Lysine	20 μg/ml	SIGMA	P6407
Poly-L-Ornithine	20 μg/ml	SIGMA	P3655
Collagen Type IV	6μg/ml	SIGMA	C6745
Gelatin	50μg/ml	SIGMA	G9391

rhVTN-N: Truncated recombinant human vitronectin

Table S2. Summary of 24 cytotoxic compounds determined in the high throughput iPSC NSCs assays with rhVTN-N one-step seeding.

Category	Compound Name	ATP Content Assay		DNA Dye Assay		Caspase 3/7 Assay		Reported drug activity	Reported mechanism of action
		IC50 (μM)	Efficacy (%)	IC50 (μM)	Efficacy (%)	EC50 (μM)	Efficacy (%)		
Toxin	DSP-4	0.09	99.69	0.06	100.00	0.54	99.97	Adrenergic neurotoxin	Inhibit the uptake of noradrenaline [3]
	Veratridine	2.46	100.00	1.39	100.00	2.06	100.00	Steroid-derived alkaloid neurotoxin	Voltage-gated Na+ channel opener[4]
	MPTP	0.006	80.49	0.004	77.96	0.010	89.72	parkinsonian toxin	Damage to substantia nigra pars compacta dopaminergic neurons[5]
Insecticide	Diazinon	5.73	100.00	4.08	100.00	6.16	100.00	Organothiophosphorus insecticide	Cholinesterase inhibitor[6]
	Fenthion	2.12	77.87	2.20	77.42	4.54	77.20	Insecticide and acaricide	Cholinesterase inhibitor[7]
	Fipronil	0.04	98.50	0.04	99.59	0.21	100.00	Organochlorine insecticide	Inhibit GABAA receptors and glycine receptors[8]
	Emamectin	0.91	100.00	0.67	100.00	2.73	82.27	Insecticide	Chloride channel activation[9]
Antineoplastic agent	Genistein	6.29	100.00	4.03	100.00	6.31	100.00	Antineoplastic agent	Inhibit protein-tyrosine kinase and topoisomerase-II[10]
	Doxorubicin	0.05	95.96	0.03	100.00	0.13	100.00	Antineoplastic antibiotic	Anthracycline topoisomerase inhibitor[11]
	Cytarabine	1.15	100.00	0.23	100.00	1.34	90.46	Antimetabolite antineoplastic agent	Inhibit DNA synthesis[12]
	Paclitaxel	0.03	94.10	0.03	97.13	0.10	94.45	Antineoplastic agent	Microtubule Inhibitor[13]
	Oxaliplatin	1.43	92.76	1.48	90.12	4.29	103.79	Platinum-based antineoplastic agent	Inhibit DNA synthesis[14]
Anti-infective agent	Thimerosal	0.10	98.73	0.06	98.17	0.56	95.56	Antibacterial and antifungal agent	Inhibit sulfhydryl-dependent enzymes and proteins [15]
	Clarithromycin	0.004	80.29	0.003	100.00	0.003	115.01	Antibiotic	Inhibit protein synthesis [16]
	Clioquinol	2.28	99.14	1.57	95.13	5.26	69.14	Topical anti-intestinal antiamebic	Bacteriostatic, the precise mechanism of action is unknown[17]
	α-Mangostin	1.98	100.00	2.03	102.12	5.10	103.31	Antimicrobial, antioxidant	Induce apoptosis via the mitochondrial pathway[18]
	Zidovudine	4.05	79.22	3.52	76.43	31.15	56.41	Antiretroviral drug	Nucleoside analog reverse-transcriptase inhibitor [19]
	Eugenol	8.48	78.93	7.93	82.06	21.14	83.27	Local antiseptic and anesthetic	Increase Histamine Release and Cell-mediated Immunity[20]
Others	Disulfiram	0.014	100.00	0.012	100.00	0.12	99.16	Treatment of chronic alcoholism	Inhibitor of aldehyde dehydrogenase [21]
	Cyclopiazonic acid	17.05	92.97	16.95	99.61	23.29	96.12	Toxic fungal secondary metabolite	Inhibit sarcoplasmic reticulum Ca2+-ATPase.[22]
	Fenfluramine	13.37	79.19	8.61	82.02	14.13	62.53	Appetite Depressant	Block serotonin uptake, provoke transport-mediated serotonin release[23]
	Paraquat dichloride	0.004	76.59	0.003	85.38	0.010	80.02	Herbicide	Redox cycling and intracellular oxidative stress generation[24]
	Aluminum chloride	0.006	80.56	0.006	82.85	0.004	90.85	Antiperspirant, Astringents	Increasing effects on inflammation and ROS[25]
	3-Nitropropionic acid	16.47	59.05	16.22	81.02	19.60	54.90	Antihypertensive and Convulsant	Inhibit succinate dehydrogenase[26]

Table S3. Summary of published pre-coating substrates of neural cells

Substrate(s)	Disease	Cell type	Plate format	Throughput	Assay format	Readout(s)	Ref.
PLO	Spinal muscular atrophy	Motor neurons	384-well	high	Immunofluorescence	Cytoplasmic and nuclear SMN protein levels	[27]
PDL	Human iPSCs	Neurons	384-well	high	Fluorescence	neuronal cell viability, neurite outgrowth, Mitochondrial Membrane Potential Assay	[28]
Matrigel	Amyotrophic lateral sclerosis	Motor neurons	384-well	high	Immunofluorescence	ISLET1 and TDP-43 aggregates	[29]
	Rett syndrome	Neurons	dish	Low	Fluorescence, electrophysiology	Immunocytochemistry, neuronal morphology quantification, Cell cycle analysis, calcium imaging and electrophysiology	[30]
	Fragile X Syndrome	Neuronal Progenitors	384-well	High	Fluorescence	FMRP expression	[31]
Geltrex	Dermatology patients or healthy volunteers	Dopaminergic neurons	96-well	Medium	Fluorescence	Flow cytometry or immunostaining of neuronal markers	[32]
	Fragile X Syndrome	Neural Stem Cells	384,1536-well	High	TR-FRET	TR-FRET-Based FMRP Assay	[33]
	NCRM-1 iPSC line	NSCs	96-well	Medium	Absorbance	MTT cell viability assay	[34]
LN+PLO	Alzheimer disease	Neurons	384-well	high	Luminescence	ATP content cell viability	[35]
	Huntington disease	Neural stem cells	384-well	high	Luminescence	Derepression of reporter	[36]
	Human iPSCs	Neural Progenitors	384-well	High	Luminescence	TCF/LEF Luciferase Reporter Assay	[37]
	Human iPSCs	Neuroepithelial-like Stem Cells	96-well	Medium	Luminescence	ATP content cell viability	[38]
	NCRM-1 iPSC line	Neurons	96-well	Medium	Absorbance	MTT cell viability assay	[39]
LN+PLO+FN	Familial dysautonomia	Neural crest cells	384-well	high	qRT-PCR	WT IKBKAP	[40]
LN+PDL+FN	Human iPSCs	GABAergic neurons	4 or 8-well	Low	Fluorescence	hVGAT-mCherry expression	[41]

PLO, poly-L-ornithine; PDL, Poly-D-Lysine; LN, laminin; FN, fibronectin

Table S4. Summary of the antibodies

	Name	Isotype	Vendor	Catalog#	Dilution factor
Primary	Nestin	Mouse	Life Technologies	A24345	1:50
	SOX-1	Goat	Life Technologies	A24347	1:50
	SOX-2	Mouse	Cell Signaling	4900S	1:50
	PAX6	Rabbit	Life Technologies	A24340	1:50
	Vimentin	Rabbit	Cell Signaling	5741S	1:100
	SSEA4	Mouse	Cell Signaling	4755S	1:500
	β3-Tubulin	Rabbit	Cell Signaling	5568S	1:200
	MAP2	Rabbit	Cell Signaling	8707S	1:200
	Neurofilament-L	Rabbit	Cell Signaling	28374S	1:100
	GFAP	Mouse	Cell Signaling	3670S	1:200
Secondary	Anti-Rabbit IgG (H+L), Alexa Fluor 488 conjugate	Donkey	Life Technologies	A21206	1:200
	Anti-Mouse IgG (H+L), Alexa Fluor 594 conjugate	Rabbit	Life Technologies	A11062	1:200
	Anti-Goat IgG (H+L), Alexa Fluor 488 conjugate	Donkey	Life Technologies	A11055	1:200

Supplemental references

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