Original Research

Establishment of neural stem cells from fetal monkey brain for neurotoxicity testing

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Impact statement

There are increasing demands to understand the potential developmental neurotoxicity (DNT) of chemicals/agents. Challenges persist with the current situation that laboratory animal data on DNT evaluation is required. To reduce animal use and provide DNT information on various chemicals more efficiently, relevant *in vitro* models could be an alternative and serve as a screening tool. Here, we established a monkey neural stem cell (NSC) model which can proliferate and differentiate *in vitro* and has shown sensitive responses to recognized neurotoxicants. Compared to NSCs from other laboratory animals, NSCs from monkey fetal brain may better reflect the human responses to an agent due to the closer similarities between monkey and human central nervous system development. Moreover, observations from monkey NSCs can be verified in animals *in vivo*.

Abstract

Neurotoxicity assessments are generally performed using laboratory animals. However, as *in vitro* neurotoxicity models are continuously refined to reach adequate predicative concordance with *in vivo* responses, they are increasingly used for some endpoints of neurotoxicity. In this study, gestational day 80 fetal rhesus monkey brain tissue was obtained for neural stem cells (NSCs) isolation. Cells from the entire hippocampus were harvested, mechanically dissociated, and cultured for proliferation and differentiation. Immunocytochemical staining and biological assays demonstrated that the harvested hippocampal cells exhibited typical NSC phenotypes *in vitro*: (1) cells proliferated vigorously and expressed NSC markers nestin and sex-determining region Y-box 2 (SOX2) and (2) cells differentiated into neurons, astrocytes, and oligodendrocytes, as confirmed by positive staining with class III β-tubulin, glial fibrillary acidic protein, and galactocerebroside, respectively. The NSC produced detectable responses following neurotoxicant exposures (e.g. trimethyltin and 3-nitropropionic acid). Our results indicated that non-human primate NSCs may be a practical tool to study the biology of neural cells and to evaluate the neurotoxicity of chemicals *in vitro*, thereby providing data that are translatable to humans and may also reduce the number of animals needed for developmental neurotoxicological studies.

Keywords: Neural stem cells, proliferation, differentiation, *in* vitro model, neurotoxicity, Rhesus macaque

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Introduction

There are increasing concerns and demands to understand the potential developmental neurotoxicity (DNT) of chemicals/agents. To address these concerns, establishment of relevant models would significantly aid in screening for xenobiotic-induced DNT. While laboratory animal data are required for DNT evaluations,[1] there is an increasing trend in the use of *in vitro* models to understand and predict *in vivo* results.[2–4] *In vitro* models provide a higher throughput platform for chemical screening with lower costs and could facilitate detailed mechanistic studies. It has been reported that many *in vitro* models responded to certain stimuli in a similar manner as observed using *in vivo* models. For example, the assessment of the DNT induced by general anesthetics using *in vitro* models has confirmed many *in vivo* observations in addition to providing more detailed mechanistic information.[5,6]

Among the *in vitro* models used in DNT evaluations, neural stem cells (NSCs) can recapitulate the basic developmental events occurring in intact animal brains.[7] Thus, DNT data from NSCs can provide valuable information and guidance for later laboratory animal studies and even human clinical trials. Given the differences in central nervous system (CNS) organization between humans and various animal species, an NSC model from a species that is very similar

to humans would provide an ideal alternative for screening chemicals with DNT potential and understanding the relevant mechanisms at a molecular level. Among experimental animals, the CNS of non-human primates (NHPs) is similar to the human CNS.[8,9] These similarities make NSCs from NHP a good candidate for an *in vitro* model and for possibly bridging the gap between animal study data and human data. Moreover, development of NSC models from NHP could reduce laboratory animal use, specifically NHP use in DNT studies. In this study, NSCs were harvested from the hippocampus of a gestational day (GD) 80 fetal rhesus monkey brain with the aim of establishing an *in vitro* model to screen for potential DNT compounds. Our results indicated that these NSCs were sensitive to known neurotoxicants. Overall, the model has demonstrated promise in predicting vulnerability of the NSCs to various insults during gestation.

Materials and methods

Cesarean-section surgery for fetal monkey delivery

All animal procedures were approved in advance by the National Center for Toxicological Research Institutional Animal Care and Use Committee and conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. A pregnant rhesus monkey (*Macaca mulatta*) was sedated with Telazol (8.0 mg/kg, intramuscular [IM]) on GD 80, intubated and maintained on 1.5–2% isoflurane in 70% nitrous oxide. After intubation, the monkey was administered glycopyrrolate (0.004 mg/kg, IM) and received a buprenorphine (0.03 mg/kg) injection for pain relief. An intravenous (IV) catheter was placed in the left saphenous vein for fluid administration (Lactated Ringer's Solution). During anesthesia, the monkey was monitored continuously for blood oxygen level, heart rate, and temperature (Supplemental Table 1). The monkey was positioned on a warm water heating pad, and the surgical site was disinfected with alcohol and chlorhexidine. Aseptic technique was used to expose the uterus and remove the fetus via mid-line laparotomy. All organs were returned to correct anatomical placement prior to abdominal and skin closure. The monkey was monitored until fully recovered from anesthesia. Postoperative analgesics and antibiotics were administered under veterinary direction and oversight during the postoperative recovery period. The fetus was euthanized immediately after cesarean delivery (within 1 min), and the fetal brain tissue was collected for NSC isolation.

Harvest of hippocampal NSCs

Briefly, after removal of the meningeal tissue from the GD80 NHP brain, the hippocampus was removed and mechanically dissociated in ice-cold Hank's calcium- and magnesium-free medium (Gibco, Gaithersburg, MD, USA) using a fire-polished Pasteur pipette and centrifuged for 10 min at 200*g*. The cell pellet was suspended and washed in Dulbecco's modified Eagle's medium (DMEM) (Gibco) followed by centrifuging for 10 min at 200*g*. Cells were then evenly distributed across laminin-coated petri dishes (MilliporeSigma; St. Louis, MO, USA) for cultures maintained in NSC growth medium (PhoenixSongs Biologicals, Branford, CT, USA).

NSC culture

NSCs were maintained in NSC Growth Medium (PhoenixSongs Biologicals) for proliferation, with culture medium changed every other day. When the NSCs were confluent, they were collected and cryopreserved in liquid nitrogen for storage. NSCs were guided to differentiate when cultured in Differentiation Medium (PhoenixSongs Biologicals).

Flow cytometry analysis of NSCs

Cultured NSCs were dissociated from culture dishes using Accutase™ Cell Detachment Solution (BD Biosciences, San Jose, CA, USA), washed with phosphate-buffered saline (PBS), filtered through a 70-µm Falcon® cell strainer (Life Sciences, Tewksbury, MA, USA), fixed in BD Cytofix Fixation buffer (BD Biosciences) and permeabilized with BD Phosflow Perm Buffer III (BD Biosciences). For single marker immunofluorescence labeling, the cells were incubated with primary rabbit polyclonal antibodies: anti-nestin (MilliporeSigma) or anti-sex-determining region Y-box 2 (SOX2) (Invitrogen, Carlsbad, CA, USA), followed by incubation with anti-rabbit secondary antibody conjugated with rhodamine (MilliporeSigma). Cells were then washed, resuspended in PBS at a concentration of 10 million cells/mL, filtered through a 35-µm cell strainer and analyzed on a four-laser FACSAria III Flow Cytometer (BD Biosciences). Rhodamine fluorescence was measured with a 561-nm yellow green laser, and light scatter was measured with a 488-nm blue laser. Since both anti-nestin and anti-SOX2 primary antibodies were of the same rabbit isotype, simultaneous detection of nestin and SOX2 in cell samples was not possible. Therefore, cells were labeled with each antibody and analyzed by flow cytometry separately. A total of 10,000 events were recorded on the flow cytometer for each labeling.

NSC proliferation assessment and immunocytochemical staining of nestin

Cell proliferation was assessed using a 5-ethynyl-2′-deoxyruidine (EdU) incorporation assay (Click-iT® EdU Alexa Fluor® High-throughput Imaging [HCS] Assay) (Invitrogen) as previously described.[10] Immediately after EdU labeling, cells were stained with primary rabbit polyclonal anti-nestin (MilliporeSigma) and anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) to visualize NSCs. NuclearMask™ HCS Blue (Invitrogen) working solution was employed to stain DNA in the dark at room temperature. Positively labeled cells were visualized and imaged using an Olympus IX71 fluorescence microscope (Olympus, Center Valley, PA, USA).

Immunocytochemical staining of NSC-derived neurons, astrocytes, and oligodendrocytes

Harvested hippocampal cells were cultured in NSC differentiation medium (PhoenixSongs Biologicals) for five days before they were labeled with antibodies of either anti-class III β-tubulin (1:200; Abcam, Cambridge, MA, USA), anti-glial fibrillary acidic protein (GFAP; 1:200, MilliporeSigma) or anti-galactocerebroside (GALC, 1:100; MilliporeSigma).[11]

Positively labeled cells were viewed using an Olympus IX71 fluorescence microscope (Olympus).

Cytotoxicity assessments in neurotoxicant exposure in undifferentiated NSC cultures

NSCs were treated with different concentrations of recognized neurotoxicants trimethyltin (1–10 µM TMT, MilliporeSigma)[12] and 3-nitropropionic acid (0.1–10 mM 3-NP, MilliporeSigma),[13] for 24 h. After treatment, NSC proliferation rate was assessed using EdU incorporation assay (Invitrogen); cell viability was determined using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (MilliporeSigma) and LDH (lactate dehydrogenase) assays (Roche Applied Science, Indianapolis, IN, USA), as previously described.[10,11] The treatments were repeated three times independently.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assays on differentiated neural cells

On the fourth day of NSC differentiation, the cells were treated with TMT and 3-NP for 24 h. After the treatment, the cells were fixed with 4% paraformaldehyde at 4°C overnight, and then proceeded to apoptotic cell labeling using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Andy Fluor™ 488 Apoptosis Detection Kit (ABP Biosciences, Rockville, MD, USA). Positive staining was imaged using an Olympus IX71 fluorescence microscope (Olympus).

Statistical analysis

Data graphs and statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean ± SE. LDH, MTT, and EdU incorporation assays were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. A P value of <0.05 determined significance for all analyses.

Results

Morphological features of the harvested hippocampal cells

On the second day of *in vitro* (DIV 2) culture, a monolayer of hippocampal cells was evenly distributed on the culture dish (Figure 1(A)). By DIV 4, the number of individual cells increased substantially (Figure 1(B)). Clusters of newly generated NSCs (i.e. neurospheres) were observed on DIV 6 (Figure 1(C)). By DIV 8, the confluent cells were collected for cryopreservation or experimentation (Figure 1(D)). The cryopreserved NSCs had a >95% viability rate and began to proliferate when thawed and returned to culture.

Characterization and confirmation of NSCs

The isolated hippocampal cells positively stained for the NSC marker nestin, confirming them as NSCs (Figure 2(A), (C) and (D)). Their cellular division/proliferation capacity was verified through EdU incorporation which revealed numerous densely EdU-stained nuclei (Figure 2(B) and (D)).

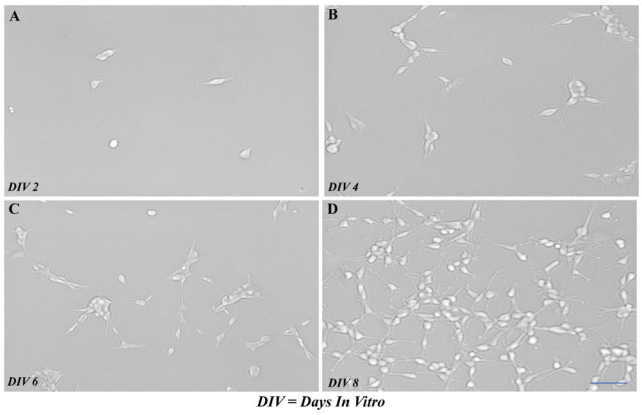

Figure 1. Representative micrographs of hippocampal NHP NSC proliferation (A to D). Harvested NSCs proliferated and formed clusters of cells in culture. Once confluent, NSCs were ready for experimental or cryogenic processing. Scale bar = 50 µm.

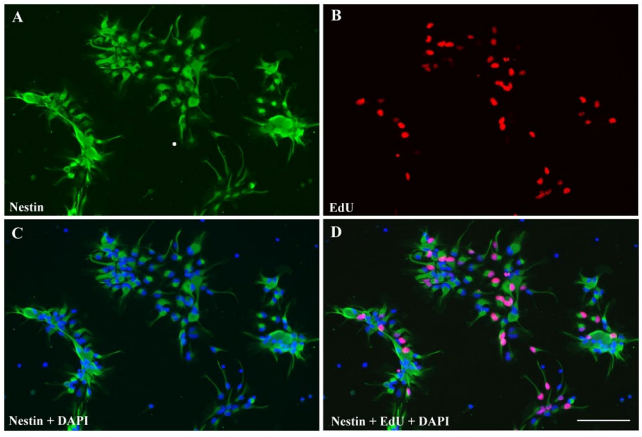

Figure 2. Representative micrographs of NSCs labeled with EdU and a polyclonal anti-nestin antibody. A majority of cells exhibited expression of the nestin protein (green; A). NSC proliferation was determined using EdU staining. Dividing cells were labeled by incorporation of EdU (red; B). DAPI was used to counterstain cell nuclei (blue; C). The merged image shows that numerous nestin-positive cells were also EdU-positive (purple; D). Scale bar = 50 µm.

Further confirmation of the cells as NSCs was conducted using flow cytometry. Cells were labeled with nestin or SOX2, two frequently used NSC markers. A total of 10,000 events were recorded during flow cytometry for each labeling. Figure 3(A) illustrates a negative control showing NSCs labeled with the secondary rhodamine-conjugated antibody only. When labeled with both primary and secondary antibodies, Figure 3(B) illustrates that 99.5% of harvested hippocampal cells were nestin-positive cells. Consistent with this finding, 98.6% of harvested single hippocampal cells were SOX2-positive (Figure 3(C)). Each distinct event (single NSC; gating strategy for single cells not shown) is represented as a single dot on the cytogram.

NSC differentiation

Figure 4(A) shows the morphological features of NSCs, and Figure 4(B) presents a general view of differentiated neural cells derived from the hippocampal NSCs. The differentiated

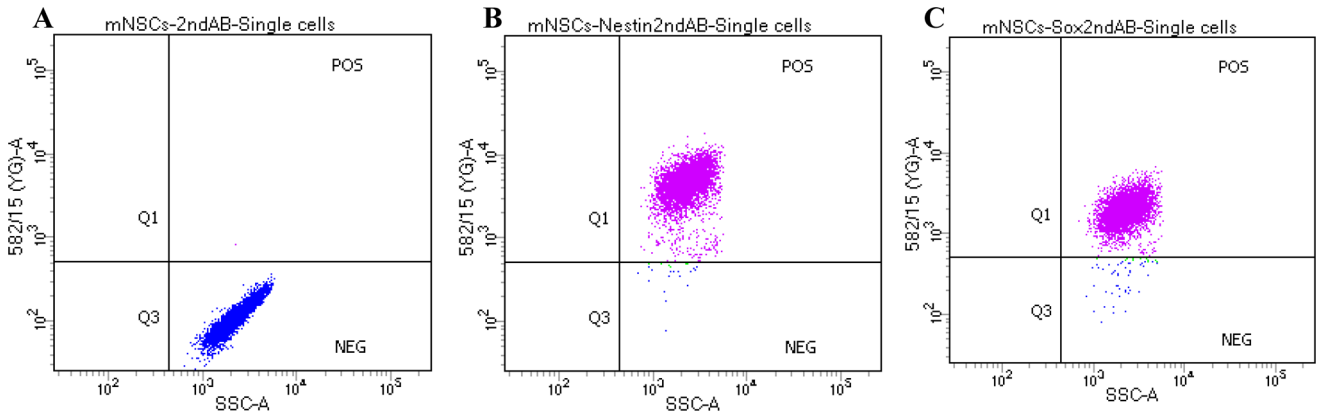

Figure 3. Representative flow cytometry plots of hippocampal NSCs. Cytogram (A) was generated using rhodamine fluorescence intensity (vertical axis) plotted against side-scatter intensity (horizontal axis) to indicate single NSCs that were labeled with secondary antibody only (negative control) were free from non-specific antibody binding. Cytogram (B) indicates that NSCs labeled with anti-nestin and the secondary antibody revealed that 99.5% of single cells were positively labeled. Cytogram (C) indicates that NSCs labeled with anti-SOX2 and the secondary antibody revealed that 98.6% of single cells were SOX2-positive NSCs. Each distinct event (a single NSC) is represented as a single dot.

cells demonstrated diverse morphological features with cellular network formations, and cellular interactions facilitated through contact between cellular protrusions.

The differentiated cells were confirmed to be neurons, astrocytes, or oligodendrocytes after five days of differentiation by labeling with their respective markers: class III β-tubulin for neurons, GFAP for astrocytes, and GALC for oligodendrocytes. Morphologically defined neurons were positively stained with class III β-tubulin (Figure 5(A)). Typical astrocytes were labeled with the anti-GFAP antibody (Figure 5(B)), and oligodendrocytes were identified with the monoclonal antibody anti-GALC (Figure 5(C)).

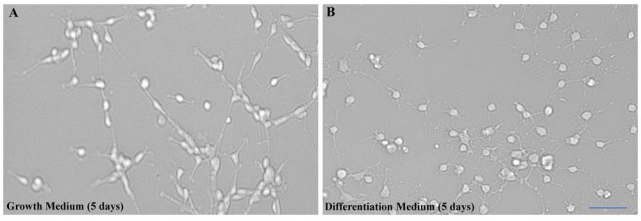

Figure 4. Representative micrographs of NSC proliferation and differentiation. The bipolar neural stem cells continuously proliferate (A) when the culture was maintained in NSC growth medium (contains growth factors and mitogens). (B) Differentiating neural cells with multiple processes and a clear neural network can be observed when these confluent NSCs were maintained in neural-differentiation medium. Scale bar = 50 µm.

Toxic effects of TMT and 3-NP on NSCs

TMT and 3-NP treatments for 24 h dose-dependently decreased MTT metabolism and increased LDH release of NSCs (Figures 6(A) and (B) and 7(A) and (B)), revealing toxic effects on NSCs, but the proliferation rate of NSCs was not significantly affected by the toxicant treatments (data not shown).

TUNEL assay

TUNEL assay indicated that TMT and 3-NP treatments for 24 h caused neural cell death, which was more obvious when their concentrations were increased (Figures 6(C) and 7(C)), suggesting that the viability of neural cells differentiated from NHP NSCs was subsequently reduced by defined neurotoxicants in a dose-dependent manner.

Discussion

At present, DNT data are missing for many chemicals and agents of concern as possibly being neurotoxicants. Studies involving laboratory animals for DNT evaluation is time-consuming and expensive. The use of *in vitro* models to screen agents with DNT potential would be practical and cost-effective. These models are also useful for understanding the underlying mechanisms and providing reference data to animal studies. In this study, we collected NSCs from the hippocampus of a fetal monkey brain with the aim to establish an *in vitro* model which was more human-relevant, bridging the gap between *in vivo* data and human data, which might reduce animal use in DNT research. Moreover, application of *in vitro* models in addition to *in vivo* models with biological, histological, and dynamic molecular imaging approaches should provide a well-rounded platform to increase confidence in translating preclinical data on DNT to humans.

The hippocampus is a primary region in the mammalian brain for neurogenesis.[14] In humans, NSCs are found in most areas of the fetal brain, and the hippocampus has the largest percentage of NSCs over other fetal brain regions.[15] NSC proliferation in the developing monkey brain was reported to be generally active in the cerebrum from GD 50 to GD 80.[16] After GD 120, however, the cerebral cortex showed decreased proliferation and an increased number of differentiated cells, and NSC proliferation was limited to the ventricular zone.[16] NSCs from different regions of the brain have different immunophenotypes and various proliferation and differentiation capabilities.[17] Transcriptomic analysis has provided additional evidence to support the varied properties of NSCs from different brain regions.[17] Since NSCs from the hippocampus were found to have the highest neuronal cell differentiation rate,[17,18] hippocampal NSCs were selected as a good candidate for establishing an *in vitro* model. In this

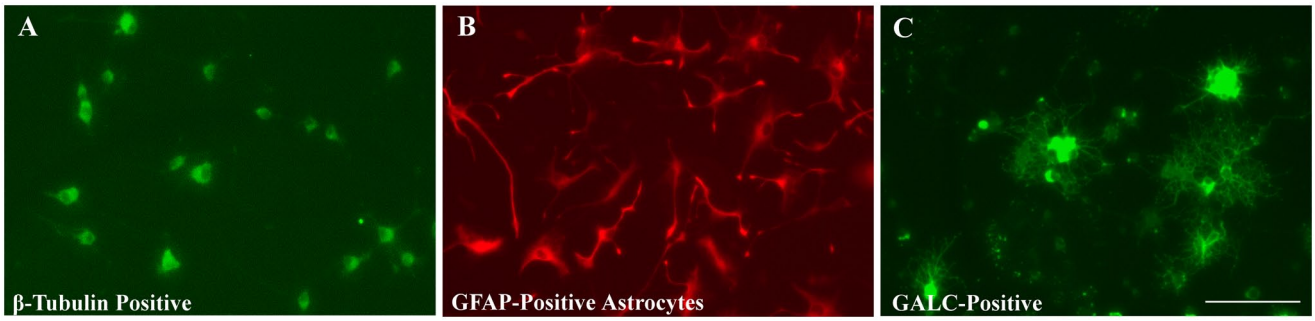

Figure 5. Immunofluorescence micrographs of neural cells derived from hippocampal neural stem cells. (A) Neuron-specific staining of cultured cells with β-tubulin shown by immunofluorescence of anti-mouse IgG conjugated to fluorescein isothiocyanate. (B) Astrocyte-specific staining of cultured cells with GFAP shown by immunofluorescence of anti-rabbit IgG conjugated to rhodamine. (C) GALC-positive cells show differentiated oligodendrocytes with typical oligodendrocyte morphology. Scale bar = 50 µm.

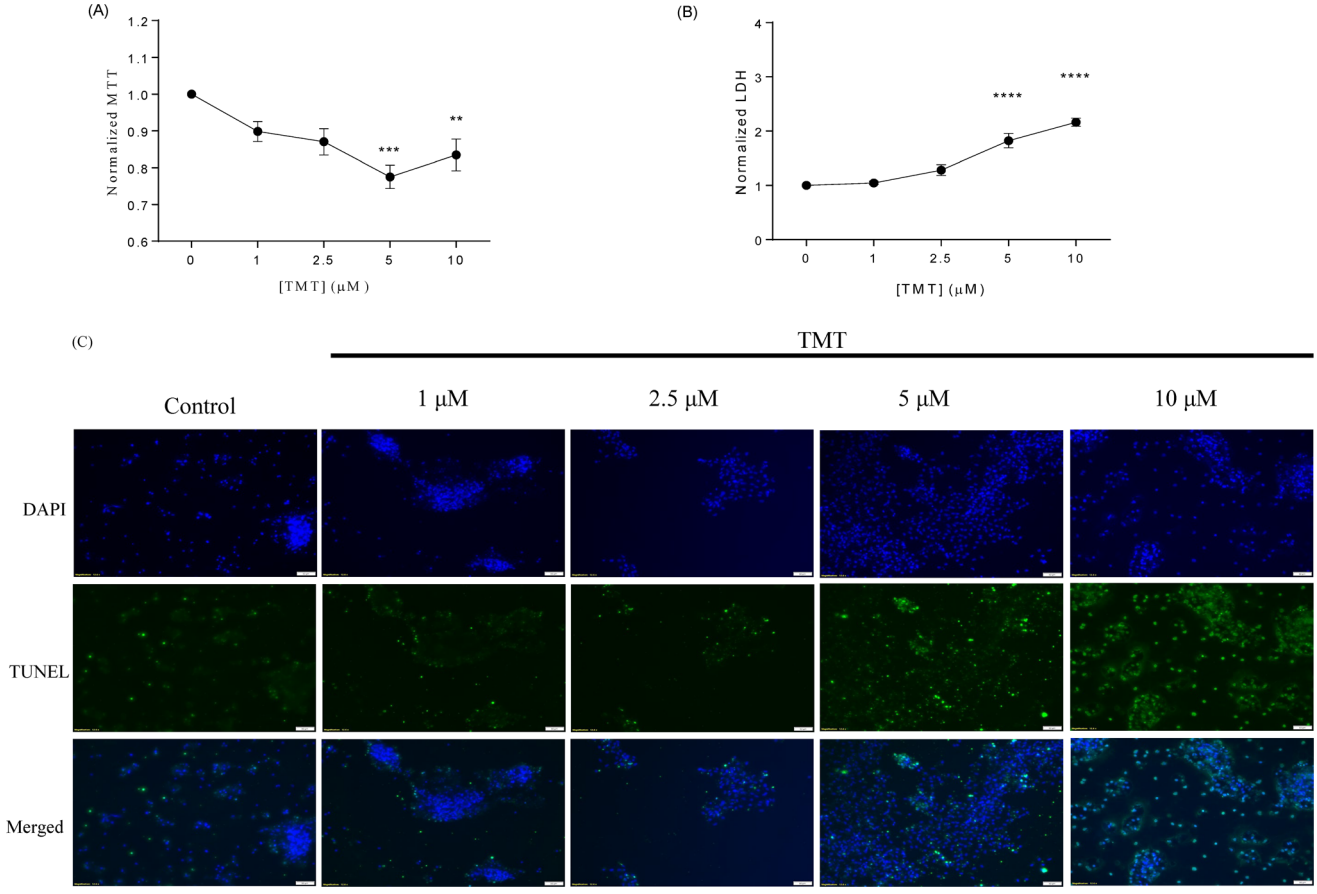

Figure 6. TMT exposure resulted in dose-dependent NHP neural stem cell death, as determined by MTT (A) and LDH (B) assays. Representative pictures of TUNEL assay (C) show TMT caused differentiated neural cell death. More cell death was observed when the cells were treated with higher concentrations of TMT. A one-way ANOVA with Dunnett's multiple comparison was used to determine significance. (A) $F(4, 81) = 5.183$, $P = 0.0009$ and (B) $F(4, 78) = 34.84$, $P < 0.0001$. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Scale bar = 50 µm.

study, we confirmed that harvested hippocampal NSCs can reproduce the most critical developmental processes *in vitro*: proliferation and differentiation.

With advances in stem cell technologies, NSCs can also be obtained through induction of embryonic stem cells (ESCs) or inducible pluripotent stem cells (iPSCs). Studies comparing human ESCs and iPSCs indicated that they have distinctive differentiation potentials *in vitro*.[19,20] If compared for neural-differentiation potential, human iPSCs have shown a potential similar to that of human ESCs, but with greater variability and reduced differentiation capability.[19] When ESCs and iPSCs are directed to differentiate to a neural lineage *in vitro*, there is also a chance of unwanted differentiation along other cell lineages due to their pluripotency. Moreover, ESC-derived NSCs and NSCs obtained from the fetal brain have shown different immunogenicity and immunomodulatory potentials.[21] In addition, NSCs harvested from the fetal brain maintained their region-specific gene expression

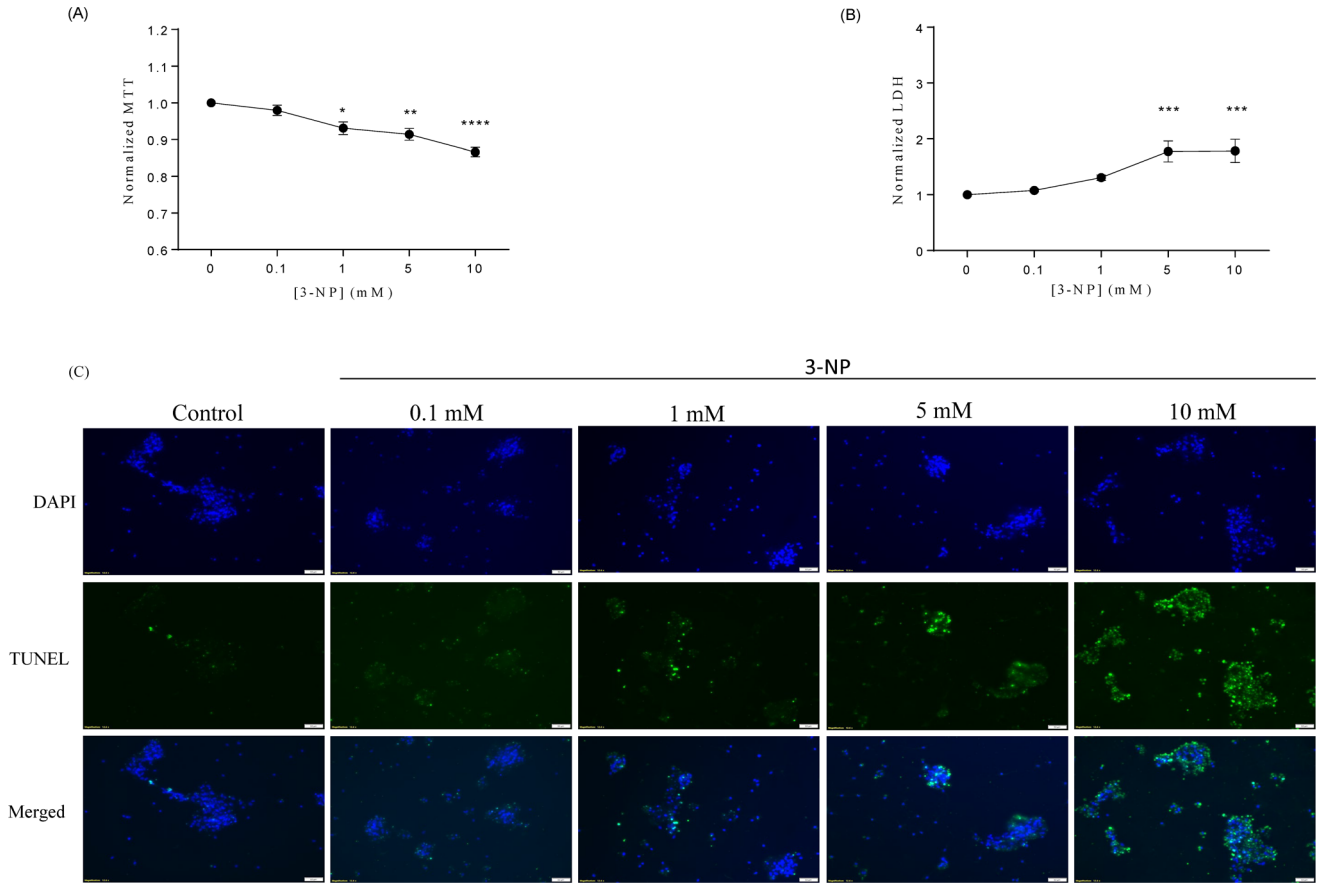

Figure 7. 3-NP exposure resulted in dose-dependent NHP neural stem cell death, as determined by MTT (A) and LDH (B) assays. Representative pictures of TUNEL assay (C) show 3-NP caused differentiated neural cell death. More cell death was observed when the neural cells were treated with higher concentrations of 3-NP. A one-way ANOVA with Dunnett's multiple comparison was used to determine significance. (A) $F(4, 78) = 11.10$, $P < 0.0001$ and (B) $F(4, 73) = 7.816$, $P < 0.0001$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Scale bar = 50 µm.

pattern and pathways that determine cellular function. Thus, their responses to stimuli should more accurately indicate the *in vivo* responses to stimuli, including neurotoxic insults. The hippocampus is a frequent target of various toxins (e.g. methylmercury, etc.).[22] Thus, hippocampal NSC cultures may reveal the effects of chemicals on neurogenesis and on neural cells differentiated from NSCs. NSCs from human fetal brain may better reflect the human response to an agent *in vitro* as compared to those from any other species. However, due to ethical concerns, human NSCs are not a generally available model. Alternatively, NSCs from fetal monkey brain, whose CNS development is closer to that of a human, can be utilized. The entire procedure for establishing a laboratory animal NSC model (e.g. monkey NSCs) is more cost-effective and accessible than NSCs from human fetal tissue. Moreover, observations from monkey NSCs can be verified in animals *in vivo*, making it a more practical model than NSCs from human brain.

As a primary cell model, monkey hippocampal NSCs may have limited proliferation capacity *in vitro*. To maximize their proliferation potential, monkey hippocampal NSCs have conventionally been cultured as a monolayer, taking into consideration that NSCs divide in a symmetric manner across numerous passages in an adherent monolayer culture.[23,24] These NSCs also have the advantage that they can be cryopreserved for long-term storage, their proliferation was still vigorous after more than 15 passages, and the cells retained the capacity for differentiating into neurons, astrocytes, and oligodendrocytes.

When treated with TMT and 3-NP, two well-known neurotoxicants, the monkey NSCs showed a dose-dependent decrease in metabolizing MTT and increased LDH release. A case study reported that 1.5 µM TMT was detected in blood of an intoxicated patient that resulted in neurological damage,[12,25] supporting that the concentrations selected in the experiments were relevant to human exposures. In this study, exposure to 1 and 2.5 µM TMT reduced NSC viability after a 24-h exposure. However, viability was not significantly decreased until exposure to 5 µM TMT (Figure 6). This reduced NSC viability following TMT exposure suggests that monkey NSCs possess similar sensitivity to TMT as that of humans. Dramatic cell death can result in reduced proliferation capability. In this study, however, the proliferation rate of NSCs was not substantially changed (data not shown) by either toxicant. This might be caused by the significant reduction of cell viability which stimulated a compensatory NSC proliferation in the primary cells during the 24-h treatments. This, in turn, counteracted any potential reduction in proliferation induced by TMT and 3-NP. After NSC differentiation, the TUNEL assay demonstrated that the viability of differentiated neural cells was also dose-dependently affected by TMT and 3-NP. More TUNEL positive cells were

observed in groups treated with higher concentrations of TMT and 3-NP. Such results suggested that monkey NSCs and neural cells that were differentiated from NSCs sensitively indicated the severity of neurotoxicity at early developmental stage (i.e. NSC stage) and later developmental stage (i.e. after differentiation).

In summary, hippocampal NSCs harvested from the fetal monkey brain are a promising *in vitro* model in that they: (1) mimic *in vivo* cellular development and simultaneously maintain the viability of neural cell types; (2) provide a continuous source of NHP NSCs; (3) provide a practical method for obtaining high yields of differentiated neurons, astrocytes, and oligodendrocytes *in vitro*; and (4) could be used to evaluate the effects of chemicals and other agents on NSCs and differentiated neural cells to predict human responses. In addition, NSCs can serve as a higher throughput platform than animal models, which more closely resembles the human condition for screening chemical-induced neurotoxicity. NSCs provide a tool for understanding the molecular and biochemical mechanisms underlying chemical-induced DNT and potentially identify biomarkers that can be confirmed *in vivo*. When *in vivo* experiments are inevitable, the application of monkey NSCs as a tier-one model would minimize animal use.

AUTHORS' CONTRIBUTIONS

All authors have participated in interpretation of the studies and reviewed the manuscript. LEL, VND, SL, CW, and FL conducted the experiments, analyzed the data, and drafted the manuscript. JT, JPH, and WSJ edited the manuscript, and JT also ensured animal welfare contract was maintained.

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DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Smirnova L, Hogberg HT, Leist M, Hartung T. Developmental neurotoxicity – challenges in the 21st century and in vitro opportunities. *ALTEX* 2014;**31**:129–56
2. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 2000;**18**:675–9
3. Wang C, Zhang X, Liu F. Application of advanced preclinical models and methods in anesthetic neurotoxicity research. *Neurotoxicol Teratol* 2017;**61**:1–6
4. Liu F, Wang C. *Neural stem cell biology and application to developmental neurotoxicity assessment: handbook of developmental neurotoxicology*. 2nd ed. Cambridge, MA: Academic Press, 2018, p.8
5. Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG, Wang C. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci* 2007;**98**:145–58
6. Slikker W Jr, Liu F, Rainosek SW, Patterson TA, Sadovova N, Hanig JP, Paule MG, Wang C. Ketamine-induced toxicity in neurons differentiated from neural stem cells. *Mol Neurobiol* 2015;**52**:959–69
7. Seto Y, Eiraku M. Human brain development and its in vitro recapitulation. *Neurosci Res* 2019;**138**:33–42
8. Belmonte JCI, Callaway EM, Caddick SJ, Churchland P, Feng G, Homanics GE, Lee KF, Leopold DA, Miller CT, Mitchell JF, Mitalipov S, Moutri AR, Movshon JA, Okano H, Reynolds JH, Ringach D, Sejnowski TJ, Silva AC, Strick PL, Wu J, Zhang F. Brains, genes, and primates. *Neuron* 2015;**86**:617–31
9. Phillips KA, Bales KL, Capitanio JP, Conley A, Czoty PW, 't Hart BA, Hopkins WD, Hu SL, Miller LA, Nader MA, Nathanielsz PW, Rogers J, Shively CA, Voytko ML. Why primate models matter. *Am J Primatol* 2014;**76**:801–27
10. Liu F, Rainosek SW, Sadovova N, Fogle CM, Patterson TA, Hanig JP, Paule MG, Slikker W Jr, Wang C. Protective effect of acetyl-l-carnitine on propofol-induced toxicity in embryonic neural stem cells. *Neurotoxicology* 2014;**42**:49–57
11. Liu F, Liu S, Patterson TA, Fogle C, Hanig JP, Wang C, Slikker W Jr. Protective effects of xenon on propofol-induced neurotoxicity in human neural stem cell-derived models. *Mol Neurobiol* 2020;**57**:200–7
12. Fabrizi C, Somma F, Pompili E, Biagioni F, Lenzi P, Fornai F, Fumagalli L. Role of autophagy inhibitors and inducers in modulating the toxicity of trimethyltin in neuronal cell cultures. *J Neural Transm* 2012;**119**:1295–305
13. Brownell AL, Chen YI, Yu M, Wang X, Dedeoglu A, Cicchetti F, Jenkins BG, Beal MF. 3-Nitropropionic acid-induced neurotoxicity – assessed by ultra high resolution positron emission tomography with comparison to magnetic resonance spectroscopy. *J Neurochem* 2004;**89**:1206–14
14. Rolando C, Taylor V. Neural stem cell of the hippocampus: development, physiology regulation, and dysfunction in disease. *Curr Top Dev Biol* 2014;**107**:183–206
15. Yin X, Li L, Zhang X, Yang Y, Chai Y, Han X, Feng Z. Development of neural stem cells at different sites of fetus brain of different gestational age. *Int J Clin Exp Pathol* 2013;**6**:2757–64
16. Toyoshima Y, Sekiguchi S, Negishi T, Nakamura S, Ihara T, Ishii Y, Kyuwa S, Yoshikawa Y, Takahashi K. Differentiation of neural cells in the fetal cerebral cortex of cynomolgus monkeys (*Macaca fascicularis*). *Comp Med* 2012;**62**:53–60
17. Fan Y, Marcy G, Lee ES, Rozen S, Mattar CN, Waddington SN, Goh EL, Choolani M, Chan JK. Regionally-specified second trimester fetal neural stem cells reveals differential neurogenic programming. *PLoS ONE* 2014;**9**:e105985
18. Kim HT, Kim IS, Lee IS, Lee JP, Snyder EY, Park KI. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp Neurol* 2006;**199**:222–35
19. Hu BY, Weick JP, Yu J, Ma LX, Zhang XQ, Thomson JA, Zhang SC. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc Natl Acad Sci U S A* 2010;**107**:4335–40

20. Narsinh KH, Plews J, Wu JC. Comparison of human induced pluripotent and embryonic stem cells: fraternal or identical twins? *Mol Ther* 2011;**19**:635–8
21. Liu J, Götherström C, Forsberg M, Samuelsson EB, Wu J, Calzarossa C, Hovatta O, Sundström E, Åkesson E. Human neural stem/progenitor cells derived from embryonic stem cells and fetal nervous system present differences in immunogenicity and immunomodulatory potentials in vitro. *Stem Cell Res* 2013;**10**:325–37
22. Heimfarth L, Delgado J, Mignori MR, Gelain DP, Moreira JCF, Pessoa-Pureur R. Developmental neurotoxicity of the hippocampus following in utero exposure to methylmercury: impairment in cell signaling. *Arch Toxicol* 2018;**92**:513–27
23. Sun Y, Pollard S, Conti L, Toselli M, Biella G, Parkin G, Willatt L, Falk A, Cattaneo E, Smith A. Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. *Mol Cell Neurosci* 2008;**38**:245–58
24. Reynolds BA, Vescovi AL. Brain cancer stem cells: think twice before going flat. *Cell Stem Cell* 2009;**5**:466–7; author reply 468–9
25. Yoo CI, Kim Y, Jeong KS, Sim CS, Choy N, Kim J, Eum JB, Nakajima Y, Endo Y, Kim YJ. A case of acute organotin poisoning. *J Occup Health* 2007;**49**:305–10

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