# Assessing the Developmental Effects of Fentanyl (Anesthetics/Analgesics) Using Neural Stem Cell Models

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## Abstract

The utilization of highly relevant preclinical models, such as neural stem cells derived from humans, might serve as a "bridging" model to evaluate the vulnerability of the developing nervous system and address FDA's regulatory needs.
In the present study, human neural stem cells (NSCs) were used to determine dose-effects and the temporal relationships between fentanyl exposures and neural stem cell health, differentiation, and viability. Although 24-hour fentanyl exposure of NSCs at concentrations of 0.5, 1.5, 3.0, 10 or 100 µM resulted in a slight dose-related increase in the release of LDH into the cell culture medium, no MTT reduction (indicating mitochondrial health) was observed compared with controls. Additionally, no significant changes were detected in LDH release and MTT uptake when the neuronal cells derived/differentiated from NSCs were exposed for 24 hours to any of these concentrations (5) of fentanyl. Our preliminary data indicate that PSA-NCAM immune-reactivity was clearly expressed in both control and fentanyl-exposed neurons after five days of differentiation. Substantial elevation of PSA-NCAM expression was observed on the neuronal surface and their processes in fentanyl-exposed neurons compared with control neurons.
These data suggest that fentanyl exposure did not induce dose-dependent adverse effects on the human NSCs over a 24-hour period. Measuring PSA-NCAM cell surface levels can provide sufficient information associated with anesthetic-induced neurotoxicity, neuronal viability, and plasticity.

## Introduction

Fentanyl is a potent and short-acting opioid medication that is often given to pediatric patients during surgery to relieve pain and as an adjunct to an anesthesia. While it is difficult to verify the adverse anesthetic effects on human infants and children, the utilization of highly relevant preclinical models, such as neural stem cells derived from humans, might serve as a "bridging" model to evaluate the vulnerability of the developing nervous system and address FDA's regulatory needs.

Suitable human neural stem cells (NSCs) were used to study fentanyl related anesthetic regimens and to determine relationships between fentanyl exposures and neural stem cell health, differentiation, and viability. Markers of mitochondrial health [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT)] and cell death/damage [lactate dehydrogenase (LDH)] were monitored to determine the dose response effects of fentanyl on neural stem cell/neuronal cells' viability.

Polysialylation of the neural cell adhesion molecule (PSA-NCAM) is a specific neuronal marker and can be detected on the synaptic contacts of both pre- and post-synaptic membranes. Thus, PSA-NCAM immune-reactivity on human neural stem cell-derived neurons was also monitored.

We hypothesize that: 1) application of in vitro neural stem cell models and/or differentiated neuronal cells should be able to provide data that can inform clinical interventions and preclinical toxic studies; 2) fentanyl-induced neural damage depends on the dose given and the duration of exposure.

## Materials and Methods

Commercially available, de-identified (Phoenixsongs Biologicals; *www.**phoenixsongs**bio.com/*) ***human neural stem cells*** [Hip-009 (Hippocampus Brain)] were utilized because the data from the systems will help determine the stem cell responses and the sensibility of differentiated neural cell to anesthetic-induced neurotoxicity. Cultured neural stem cells and/or cells differentiated from them exposed to fentanyl anesthetic regimens, including 0.5, 1, 3, 10 and 100 µM [32], for 24 hours.

***Cytotoxicity Detection Assay (LDH):*** neuronal cell death was measured by monitoring the LDH levels in the culture medium (see details in McInnis et al. 2002).

***MTT*** *metabolism* is an indicator of mitochondrial function/cell viability. The MTT assay was performed as previously described.

***Immunocytochemistry:*** a mouse monoclonal antibody to polysialic acid neural cell adhesion molecule (PSA-NCAM; 1:500; Miltenyi Biotec Inc, Auburn, CA, USA) was used to identify developing neurons. The cells were fixed with ice-cold 4% paraformaldehyde and permeabilized with 0.5% bovine serum albumin/Triton X-100 in PBS for 1 h. The cells were incubated with primary antibody at 4° C overnight. Bound antibodies were revealed with FITC-conjugated second antibody. DAPI, a nuclear dye, was used in the mounting medium to determine total cell counts in the cultures. Cells will be viewed using an Olympus FV1000 microscope (Olympus).

## Results and Discussion

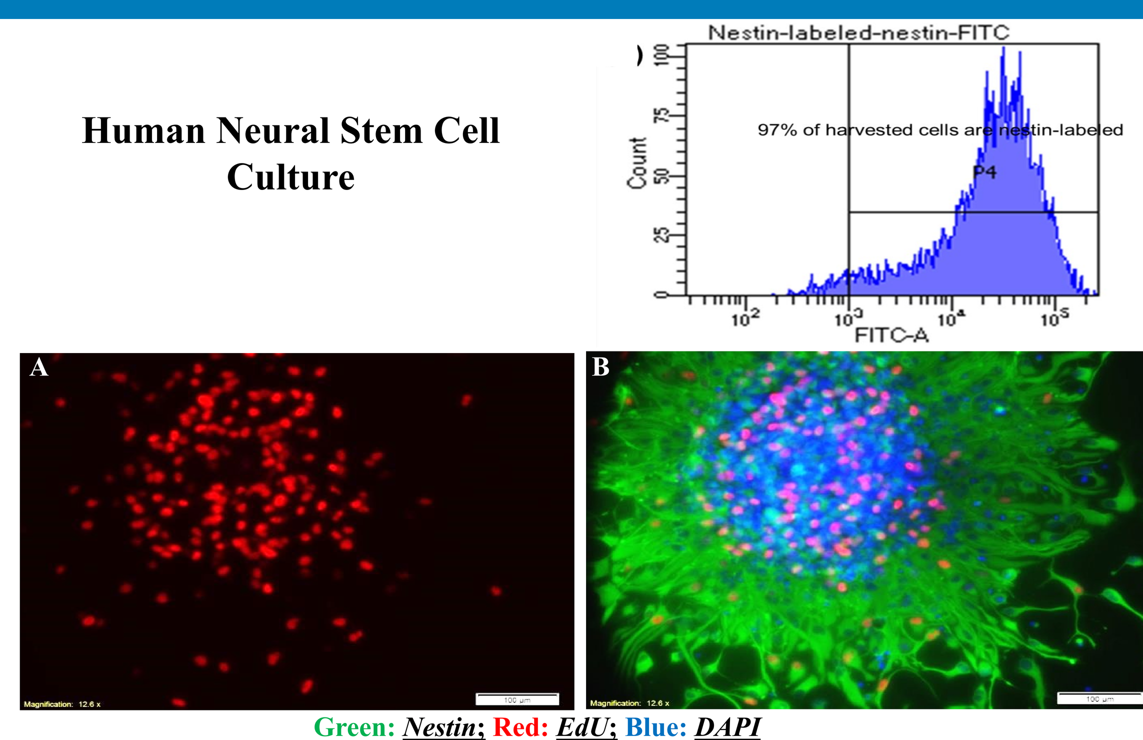


**Figure 1.** A typical human NSC sphere at the center and confluent and evenly distributed cells on the periphery can be observed. These cells were undifferentiated neural stem cells, when the cells are maintained in the growth medium. Nestin, NSC marker, is an intermediate filament protein expressed during the development. Most of the cells are stained by nestin (B). Flow cytometry data (C; figure in the upper right) demonstrated that as many as 97% harvested cells are nestin-positive. Neural stem cell proliferation was determined based on EdU staining (A). EdU, like BrdU, is a thymidine analog that is incorporated into cells only during the S-phase of cell division and is used to assess cellular proliferation. Dividing cells were labeled by incorporation of EdU. In this control culture, numerous nestin positive neural stem cells were EdU-positive.

## Results and Discussion

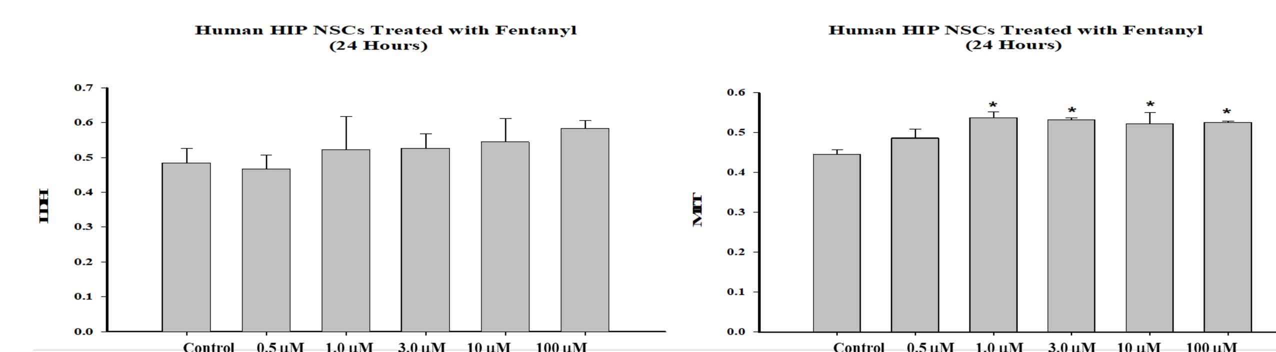


**Figure 2.** Markers of cell death/damage [releasing lactate dehydrogenase (LDH) into the culture medium] and mitochondrial health, MTT, were used/monitored to determine the dose response effects and temporal relationships of fentanyl on neural stem cell viability. 24-hour fentanyl exposure of NSCs at concentrations of 0.5, 1.5, 3.0, 10 or 100 µM resulted in a slight dose-related increase (not significant) in the release of LDH into the cell culture medium, compared with controls. No MTT reduction (indicating mitochondrial health) in fentanyl exposure of NSCs was observed, compared with controls. In contrast, 24-hour fentanyl exposure at concentrations of 1.5, 3.0, 10 or 100 µM even caused a certain level of elevation of MTT uptake.

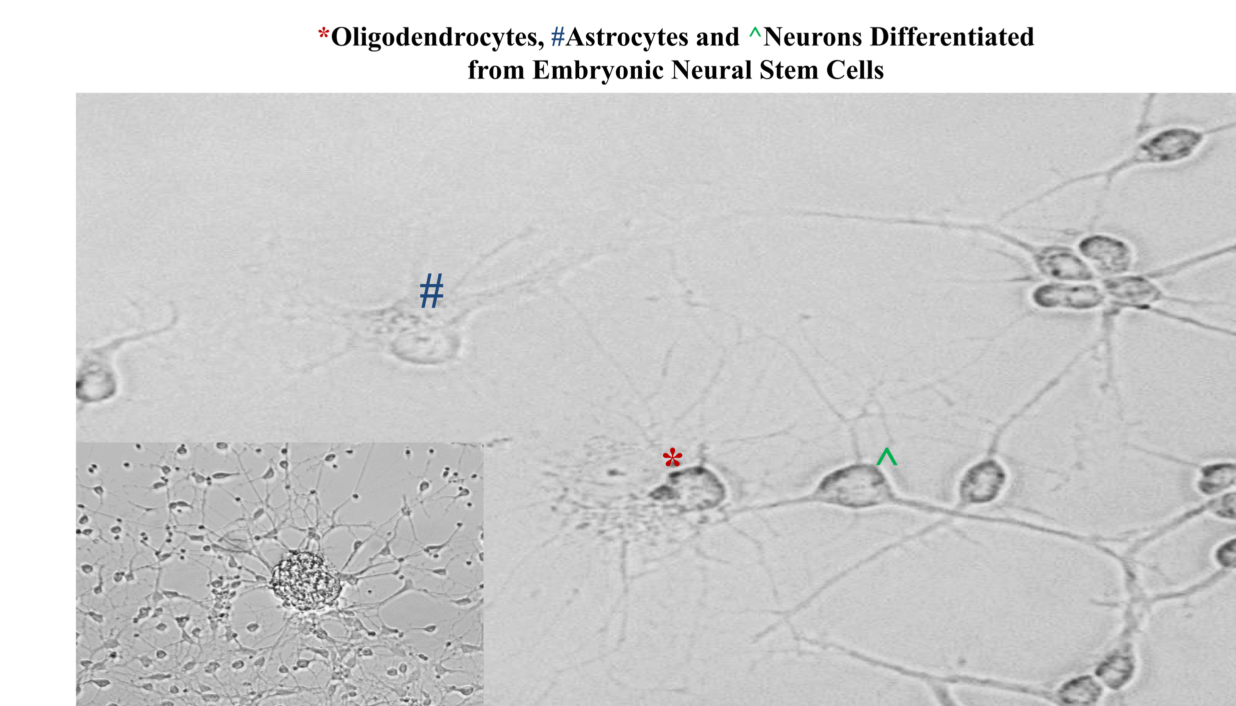


**Figure 3.** This is a general view of the different neural cells differentiated from the same neural stem cells. Most NSCs are differentiated with multiple processes and a clear neural network is formed (inset in the lower left corner), when these neural stem cells are maintained in differentiation medium. Based on their morphology, ^ represent a typical neuron with an axon and multiple dendrite processes; * represents a differentiated oligodendrocyte and # indicates an astrocyte.

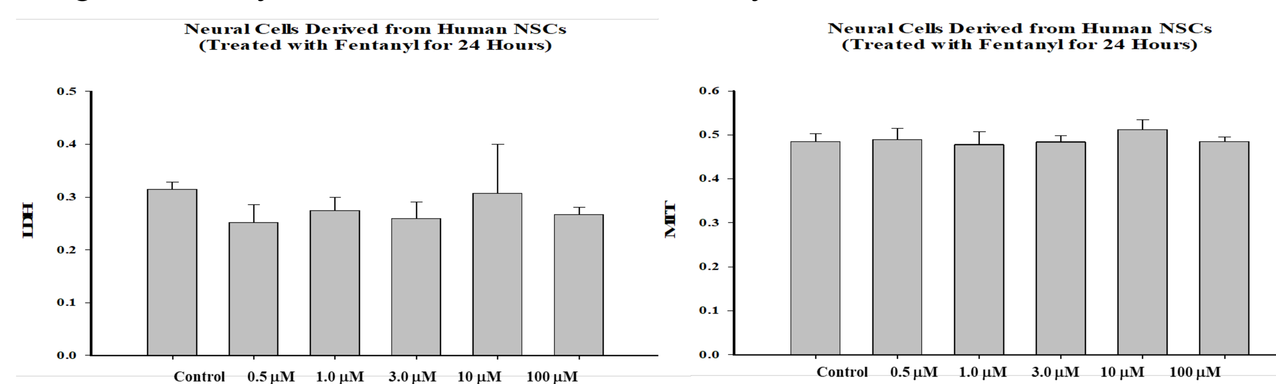


**Figure 4.** Markers of cell death/damage, LDH, and mitochondrial health, MTT, were used/monitored to determine the dose response effects and temporal relationships of fentanyl on the neuronal cells (differentiated from NSCs) viability. No significant changes were detected in LDH release and MTT uptake when the neuronal cells were exposed (24 hours) to any of those concentrations (5) of fentanyl. Each treatment condition was assessed at least in triplicate, and experiments were repeated three times independently.

## Results and Discussion

**PSA-NCAM (Neuronal Marker) Immuno-staining**

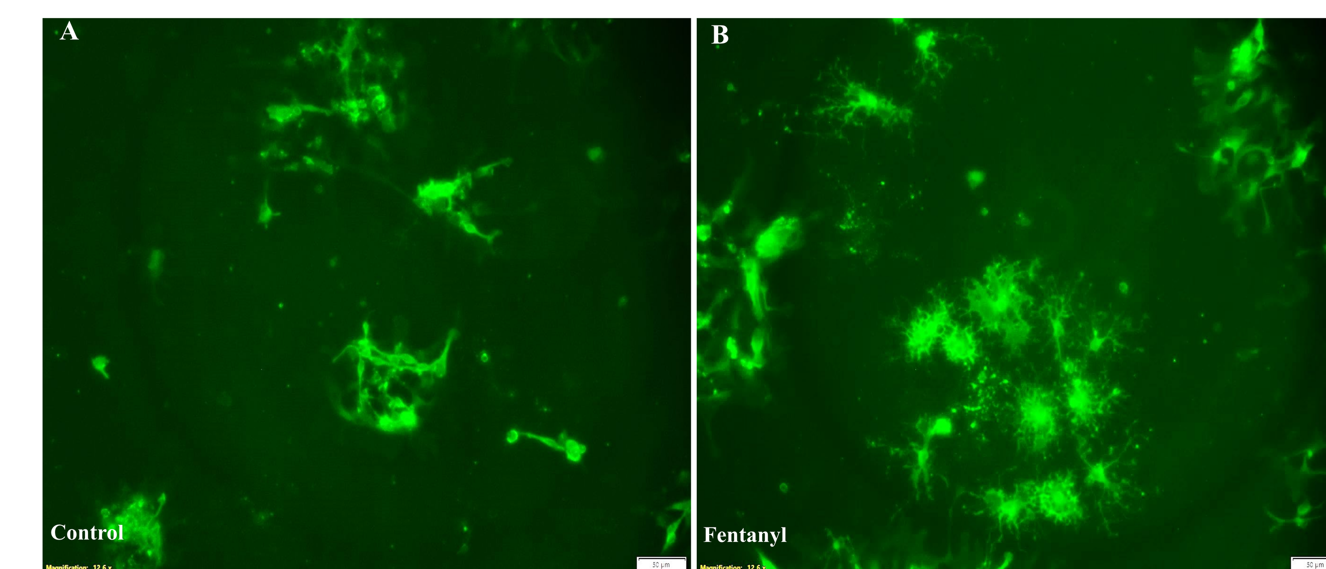


**Figure 5.** Polysialylation of the neural cell adhesion molecule (PSA-NCAM) is a specific neuronal marker for developing neurons. And it can also be detected on the synaptic contacts of both pre- and post-synaptic membrane. As a target molecule, the PSA-NCAM immune-reactivity on human neural stem cell-derived neurons was monitored.
The cultures were maintained in differentiation medium for 5 days.
Our preliminary data indicated that typical neurons can be defined by monoclonal anti-PSA-NCAM. PSA-NCAM immune-reactivity was clearly expressed in both control and fentanyl-exposed neurons, after five days of differentiation. And substantial elevation of PSA-NCAM expression (including size, form, and intensity) was observed on the neuronal surface and their processes in fentanyl-exposed neurons compared with control neurons.

## Conclusion

Neural stem cells have some identifying characteristics and can change their fate after exposure to environmental cues, and neural stem cell models can recapitulate some major events of CNS development *in vivo*.

Clinically relevant concentration of fentanyl exposure did not induce dose-dependent adverse effects on the human neural stem cells and neuronal cells over a 24-hour period.

Measuring/monitoring PSA-NCAM cell surface levels can provide sufficient information associated with anesthetic-induced neurotoxicity, neuronal viability, and plasticity.

**Reference**

Fritz, H.G., et al., *The effect of mild hypothermia on plasma fentanyl concentration and biotransformation in juvenile pigs.* Anesth Analg, 2005. **100**(4): p. 996-1002.

McInnis, J., et al., *The role of superoxide and nuclear factor-kappaB signaling in N-methyl-D-aspartate-induced necrosis and apoptosis.* J Pharmacol Exp Ther, 2002. **301**(2): p. 478-87.

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