● RESEARCH ARTICLE

Monosialoganglioside 1 may alleviate neurotoxicity induced by propofol combined with remifentanil in neural stem cells

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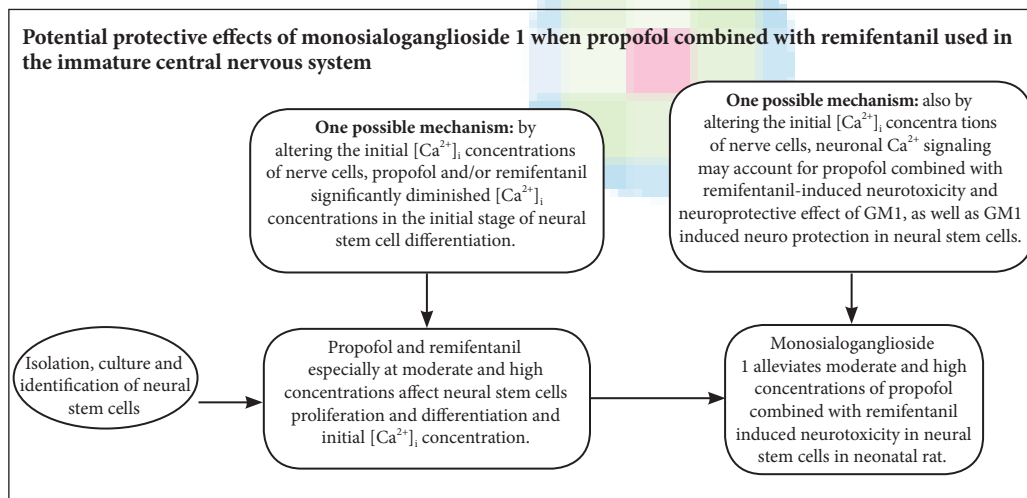4 Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China
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Graphical Abstract

Potential protective effects of monosialoganglioside 1 when propofol combined with remifentanil used in the immature central nervous system

One possible mechanism: by altering the initial $[Ca^{2+}]_i$ concentrations of nerve cells, propofol and/or remifentanil significantly diminished $[Ca^{2+}]_i$ concentrations in the initial stage of neural stem cell differentiation.

One possible mechanism: also by altering the initial $[Ca^{2+}]_i$ concentrations of nerve cells, neuronal Ca^{2+} signaling may account for propofol combined with remifentanil-induced neurotoxicity and neuroprotective effect of GM1, as well as GM1 induced neuro protection in neural stem cells.

Isolation, culture and identification of neural stem cells → Propofol and remifentanil especially at moderate and high concentrations affect neural stem cells proliferation and differentiation and initial $[Ca^{2+}]_i$ concentration. → Monosialoganglioside 1 alleviates moderate and high concentrations of propofol combined with remifentanil induced neurotoxicity in neural stem cells in neonatal rat.

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Abstract

Monosialoganglioside 1 (GM1) is the main ganglioside subtype and has neuroprotective properties in the central nervous system. In this study, we aimed to determine whether GM1 alleviates neurotoxicity induced by moderate and high concentrations of propofol combined with remifentanil in the immature central nervous system. Hippocampal neural stem cells were isolated from newborn Sprague-Dawley rats and treated with remifentanil (5, 10, 20 ng/mL) and propofol (1.0, 2.5, 5.0 μg/mL), and/or GM1 (12.5, 25, 50 μg/mL). GM1 reversed combined propofol and remifentanil-induced decreases in the percentage of 5-bromodeoxyuridine(+) cells and also reversed the increase in apoptotic cell percentage during neural stem cell proliferation and differentiation. However, GM1 with combined propofol and remifentanil did not affect β-tubulin(+) or glial fibrillary acidic protein(+) cell percentage during neural stem cell differentiation. In conclusion, we show that GM1 alleviates the damaging effects of propofol combined with remifentanil at moderate and high exposure concentrations in neural stem cells *in vitro*, and exerts protective effects on the immature central nervous system.

Key Words: nerve regeneration; monosialoganglioside 1; propofol; remifentanil; neural stem cells; neurotoxicity; neuroprotection; proliferation; differentiation; apoptosis; $[Ca^{2+}]_i$; neural regeneration

Introduction

A recent clinical study found that children exposed to general anesthesia from 0 to 4 years old may suffer from a higher risk of cognitive or functional limitations, especially in aspects of language and abstract reasoning (Lee et al., 2009; Atkins and Mandel, 2013; Beers et al., 2014; Wang et al., 2014; Gleich et al., 2015; Zhang et al., 2015; Sun, 2016; Ward et al., 2016; Jevtovic-Todorovic et al., 2017; McCann and de Graaff, 2017; Walters and Paule, 2017). Consequently, neurotoxicity of anesthetic agents, especially at moderate or high concentrations is potentially a concern for neonatal anesthesia. Propofol and remifentanil are widely used drugs in total intravenous anesthesia, because they potentiate each other for effectively controlling intraoperative anesthesia responses and facilitating rapid emergence from anesthesia (Hogue et al., 1996; Kuroyanagi et al., 2015; O'Connor et al., 2016). However, combined application of propofol and remifentanil in general anesthesia of infants may still be a concern in terms of potential adverse neurodevelopmental effects (Lee et al., 2009; van Kralingen et al., 2011; Wei et al., 2011; Atkins and Mandel, 2013; Wang et al., 2014; Zhang et al., 2015; Satomoto and Makita, 2016; Sun, 2016; Ward et al., 2016; Cho et al., 2017; Dalton et al., 2017; Jevtovic-Todorovic et al., 2017; McCann and de Graaff, 2017; Scott et al., 2017; Vutskits and Davidson, 2017; Walters and Paule, 2017). Our previous studies and those of others have shown that remifentanil, propofol, and other general anesthetic agents can have damaging effects on the immature central nervous system, including neuronal apoptosis and changes in dendritic morphology, particularly at high exposure concentrations (Davidson, 2011; Braz et al., 2012; Creeley et al., 2013; Li et al., 2014; Van Biesen et al., 2015; Satomoto and Makita, 2016; Ward et al., 2016; Earley et al., 2017; Jevtovic-Todorovic et al., 2017; McCann and de Graaff, 2017; Vutskits and Davidson, 2017; Walters and Paule, 2017; Zanghi and Jevtovic-Todorovic, 2017).

Monosialoganglioside 1 (GM1) is a major ganglioside subtype that is abundant in the outer leaflet of the plasma membrane of neuronal cells of the central nervous system (Skaper et al., 1991; Baek et al., 2010; Colsch et al., 2011; Ji et al., 2015; Dalton et al., 2017). Further, GM1 exhibits neuroprotective properties in the central nervous system (Baek et al., 2010; Kreutz et al., 2011; Bilotta et al., 2013; Kreutz et al., 2013; Scheller, 2014; Ji et al., 2015; Schneider et al., 2015), with GM1 preventing lead-induced impairment of synaptic activity and oxidative damage in the hippocampus *in vivo* (She et al., 2009). Hence, we determined whether GM1 alleviates the neurotoxicity of propofol combined with remifentanil, especially at moderate and high exposure concentrations, in the immature central nervous system using an *in vitro* model of hippocampal neural stem cells (NSCs) derived from neonatal rats.

Materials and Methods

Ethics statement

Fourteen specific-pathogen-free Sprague-Dawley rats (7 males and 7 females, postnatal day 1) were provided by Hu-

bei University of Medicine and Affiliated Hospital of Taihe, China (license No. SCXK (E) 2005-0008). This manuscript was prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals" by the International Committee of Medical Journal Editors. The experimental procedures were approved by the Animal Ethics Committee, Hubei University of Medicine and Affiliated Taihe Hospital of Hubei University of Medicine, China (2013-07).

Isolation, proliferation, and differentiation of NSCs

NSCs were harvested from rats following a previously described protocol (Lu et al., 2012a, b; Li et al., 2014). The original NSC culture medium was proliferation medium plus 50 IU/mL penicillin and 50 μg/mL streptomycin. Pure proliferation of NSCs was induced by 10 ng/mL basic fibroblast growth factor (Promega, Madison, WI, USA) and 10 ng/mL epidermal growth factor (Promega) in basic culture medium, which contained Dulbecco's Modified Eagle Media: Nutrient Mixture F12 (DMEM/F12, 1:1, v/v, Gibco, Grand Island, NY, USA), 2% B27 (Gibco), 1% N2 (Gibco) supplement, 0.5 mM L-glutamine, and 0.5 mM non-essential amino acid (Gibco). Pure differentiation culture medium mainly contained 1% fetal bovine serum (Gibco) and 1% serum replacement (Gibco) in basic culture medium without basic fibroblast growth factor and epidermal growth factor (Vescovi and Snyder, 1999; Wen et al., 2002, 2007; Yu et al., 2007; Lu et al., 2012a, b; Li et al., 2014; SanMartin et al., 2014). In each treatment group, different concentrations of GM1 and/or propofol combined with remifentanil were added into pure proliferation or differentiation culture medium at the beginning, and co-cultured during the entire proliferation or differentiation process.

Identification of NSCs

NSCs were identified by immunocytochemistry and reverse transcription polymerase chain reaction (RT-PCR) following a previously described protocol, with identification results also shown in our previous studies (Lu et al., 2012a, b; Li et al., 2014).

Drug exposure

After culture in proliferation culture medium or differentiation culture medium for 8 hours, NSCs were exposed at the same time to propofol (AstraZeneca, London, UK) and remifentanil (Yichang Humanwell Pharmaceutical Co., Ltd., Yichang, Hubei Province, China), and/or GM1 (TRB Pharmaceutical Co., Buenos Aires, Argentina) for 30 minutes. Low, moderate, and high doses of propofol combined with remifentanil were 1, 2–2.5, and 4–5 times respectively, the effective clinical drug blood concentration (Van't Veer et al., 2009). There were 11 experimental groups (*n* = 5 wells in each treatment), see **Table 1** for details.

NSC proliferation assay

Proliferation of NSCs was assessed by immunocytochemistry using our previously described protocol (Lu et al., 2012a, b; Li et al., 2014). From 6 to 8 hours after drug exposure,

Table 1 Drug exposure paradigm

Group	Remifentanil (ng/mL)	Propofol (μg/mL)	Monosialoganglioside 1 (μg/mL)	Intralipid (%)
Control (blank control)				
Intralipid (Huarui Pharmacy Ltd., Wuxi, Jiangsu Province, China)				20
Low concentration				
$GM1_1$			12.5	
R_1+P_1	5	1.0		
$R_1+P_1+GM1_1$	5	1.0	12.5	
Moderate concentration				
$GM1_2$			25	
R_2+P_2	10	2.5		
$R_2+P_2+GM1_2$	10	2.5	25	
High concentration				
$GM1_3$			50	
R_3+P_3	20	5.0		
$R_3+P_3+GM1_3$	20	5.0	50	

GM1: Monosialoganglioside 1; R: remifentanil; P: propofol.

Figure 1 Isolation, proliferation, and differentiation of NSCs (× 200).
(A) Original NSCs and tissue isolated from hippocampi of neonatal rats (postnatal day 1). (B) Single NSCs and tissue formed small neurospheres on day 1. (C) Large neurospheres were formed after 7 days of culture. (D) NSCs were split and re-suspended (into almost single cells) and re-cultured in differentiation culture medium. (E, F) Differentiation of NSCs to astroglia (marked with GFAP) and neurons (marked with β-tubulin) on days 4 and 12 after culture. (A–D) Original (general) light observations using an inverted microscope. (E, F) Immunofluorescence staining by confocal microscopy. Red: TRITC, β-tubulin(+); green: FITC, GFAP(+); blue: DAPI, nuclei. NSC: Neural stem cells; GFAP: glial fibrillary acidic protein; FITC: fluorescein isothiocyanate; DAPI: 4′,6-diamidino-2-phenylindole.
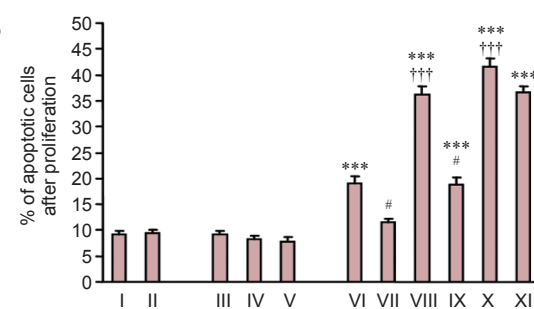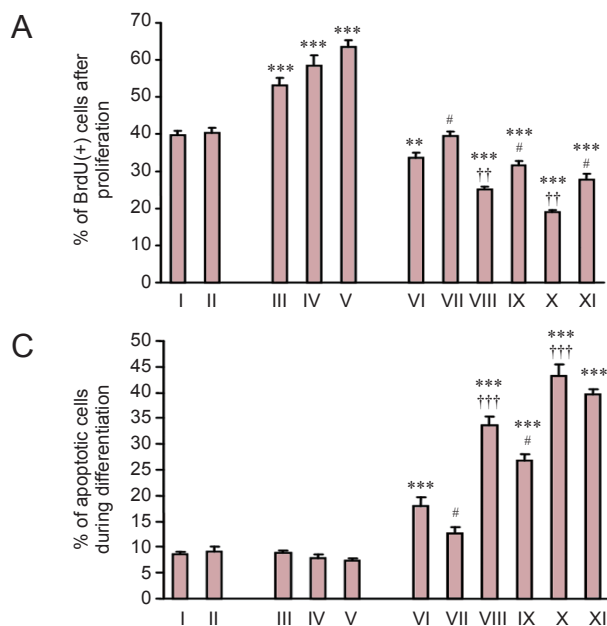

Figure 2 Effect of propofol, remifentanil, and/or GM1 on NSCs: BrdU(+) and apoptosis during proliferation and differentiation.
Data are expressed as the mean ± SEM and were analyzed by one-way analysis of variance and Bonferroni *post hoc* tests. **$P < 0.01$, ***$P < 0.001$, *vs.* control group; #$P < 0.05$, *vs.* group with the same dosage of propofol and/or remifentanil without GM1 treatment; ††$P < 0.01$, †††$P < 0.001$, *vs.* $R_1 + P_1$ group. I: Control; II: Intralipid; III: $GM1_1$; IV: $GM1_2$; V: $GM1_3$; VI: $R_1 + P_1$; VII: $R_1 + P_1 + GM1_1$; VIII: $R_2 + P_2$; IX: $R_2 + P_2 + GM1_2$; X: $R_3 + P_3$; XI: $R_3 + P_3 + GM1_3$. GM1: Monosialoganglioside 1; P: propofol; R: remifentanil; BrdU: 5-bromodeoxyuridine.

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NSCs were incubated with 5-bromodeoxyuridine (BrdU) (5 μM; Sigma, St. Louis, MO, USA) for 20 minutes.

NSC apoptosis assay

The protocol was described in our previous studies (Lu et al., 2012a, b; Li et al., 2014). At 48 hours after drug exposure, NSCs were washed with phosphate buffered saline (PBS) and digested. NSCs were gently dissociated using a pipette and centrifuged at 2,000 r/min for 5 minutes. NSCs were then re-suspended in 400 μL binding buffer at a density of 1 × 10^6 cells/mL, and treated with 5 μL Annexin V-FITC and propidium iodide at 2–8°C in the dark. Stained cells were evaluated by flow cytometry (Beckman-Coulter, Miami, FL, USA) at a wavelength of 488 nm. Fluorescein isothiocyanate (FITC) and propidium iodide emissions were measured. Apoptotic analysis was represented by the ratio of apoptotic cells to total cells.

NSC differentiation assay

The effects of the drugs examined on differentiation of NSCs were examined by immunocytochemistry at 72 hours after drug exposure. NSCs were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. After washing with PBS, NSCs were blocked with PBS and 10% goat serum for 30 minutes at room temperature. NSCs were then incubated in PBS supplemented with 0.4% Triton-X-100 (Sigma) and 0.1% bovine serum albumin. NSCs were re-washed and treated with mouse anti-rat β-tubulin monoclonal primary antibody (1:400; Millipore, Billerica, MA, USA) and rabbit anti-rat glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma) overnight at 4°C. After washing with PBS, NSCs were incubated with FITC goat anti-rabbit IgG (1:100; Thermo Fisher Scientific Inc., Rockford, IL, USA) and TRITC goat anti-mouse IgG (1:100; Thermo Fisher Scientific Inc.) for 2 hours at room temperature in a wet box. Next, NSCs were incubated with 4′,6-diamidino-2-phenylindole (DAPI) staining solution. Fluorescence signal was observed using a confocal microscope (Carl Zeiss GmbH, Jena, Germany) and the percentage of differentiated cells was determined by flow cytometry (Beckman-Coulter), in accordance with our previously described protocol (Lu et al., 2012a, b; Li et al., 2014).

Determination of intracellular calcium ($[Ca^{2+}]_i$) concentration in NSCs

The protocol was described in our previous study (Li et al., 2014). Following exposure, cells were washed and digested, triturated into suspension, and re-washed. Cells were then re-suspended and Fluo 3-AM (AAT Bioquest, Inc., Sunnyvale, CA, USA) added. Next, cells were incubated for 1 hour in the dark, washed, and re-suspended in Ca^{2+}-free PBS. Intracellular calcium concentration was measured at a wavelength absorbance of 526 nm.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance and Bonferroni *post hoc* tests were used. A value of $P < 0.05$ was considered statistically significant.

Results

Morphology and identification of NSCs

Hippocampal cells were isolated from neonatal rats (postnatal day 1) and cultured in NSC culture medium (**Figure 1A**). Suspended single NSCs formed small neurospheres (composed of 10–20 cells) on day 1 (**Figure 1B**), and large neurospheres (composed of 50–100 cells) on day 7 after culture (**Figure 1C**). NSCs were split from large neurospheres into suspended NSCs (almost single cells), and cultured in differentiation culture medium (**Figure 1D**). Differentiation of NSCs to astroglia (marked with GFAP) and neurons (marked with β-tubulin) (Lu et al., 2012a, b; Li et al., 2014) was observed on days 4 and 12 after culture.

Effect of propofol, remifentanil, and GM1 on NSC proliferation

The effect of the drugs on NSC proliferation was examined by measuring the percentage of BrdU(+) cells. As a vehicle, intralipids did not affect BrdU(+) cell percentage. GM1 increased BrdU(+) cell percentage in a dose-dependent manner ($P < 0.001$). Moreover, combined propofol (1 μg/mL) and remifentanil (5 ng/mL) decreased BrdU(+) cell percentage compared with control groups ($P < 0.01$). Further, propofol and remifentanil co-treatment decreased BrdU(+) cell percentage in a dose-dependent manner ($P < 0.01$) (**Figure 2A**). In contrast, BrdU(+) cell percentage increased when GM1 was added to propofol and remifentanil compared with propofol and remifentanil alone at the same dosage ($P < 0.05$) (**Figure 2A**).

Effect of propofol, remifentanil, and GM1 on NSC apoptosis during proliferation and differentiation

The effect of propofol, remifentanil, and GM1 on NSC apoptosis was examined during both proliferation and differentiation. In proliferation culture medium, intralipids and GM1 did not affect the apoptotic percentage of NSCs compared with the control group ($P > 0.05$; **Figure 2B**). Combined treatment of propofol (1 μg/mL) and remifentanil (5 ng/mL) increased the percentage of apoptotic cells compared with the control group ($P < 0.001$). Treatment with both propofol and remifentanil induced NSC apoptosis in a dose-dependent manner (**Figure 2B**). Co-treatment of GM1 with propofol and remifentanil decreased the percentage of apoptotic cells compared with propofol and remifentanil at low and moderate doses ($P < 0.05$; **Figure 2B**). However, co-treatment of GM1 (at 50 μg/mL) with propofol and remifentanil did not significantly decrease the percentage of apoptotic NSCs compared with propofol (5.0 μg/mL) and remifentanil (20 ng/mL) at high doses ($P > 0.05$; **Figure 2B**).

In differentiation culture medium, intralipids and GM1 did not affect the percentage of apoptotic cells ($P > 0.05$). Combined propofol and remifentanil induced NSC apoptosis in a dose-dependent manner ($P < 0.001$). Co-treatment of GM1 (at 12.5 μg/mL or 25 μg/mL) with propofol and remifentanil decreased the percentage of apoptotic NSCs

compared with propofol and remifentanil at the same dose ($P < 0.05$; **Figure 2C**). Nevertheless, co-treatment of GM1 (at 50 μg/mL) with propofol and remifentanil did not significantly decrease the percentage of apoptotic NSCs compared with propofol (5.0 μg/mL) and remifentanil (20 ng/mL) at high doses ($P > 0.05$; **Figure 2C**).

Effect of propofol, remifentanil, and/or GM1 on NSC differentiation
The effect of drug treatment on NSC differentiation was examined by measuring the percentage of cells labeled with GFAP or β-tubulin. Intralipids and GM1 did not affect GFAP or β-tubulin cell percentage ($P > 0.05$). Compared with the control group, combined propofol (5 μg/mL) and remifentanil (20 ng/mL) decreased β-tubulin(+) cell percentage ($P < 0.05$) and increased GFAP(+) cell percentage ($P < 0.05$). Moreover, the other groups showed no significant changes in β-tubulin(+) or GFAP(+) cell percentage (**Figures 3A**, **B**, **and 4**).

Effect of propofol, remifentanil, and/or GM1 on intracellular Ca^{2+} ([Ca^{2+}]$_i$) concentration of NSCs
Incubation of intralipids and GM1 did not affect [Ca^{2+}]$_i$ of NSCs. Moreover, combined propofol (1 μg/mL) and remifentanil (5 ng/mL) decreased [Ca^{2+}]$_i$ in NSCs compared with propofol (1 μg/mL) ($P < 0.05$). Co-treatment of propofol and remifentanil decreased NSC [Ca^{2+}]$_i$ levels in a dose-dependent manner ($P < 0.01$ or 0.001). Co-treatment of GM1 with propofol and remifentanil increased [Ca^{2+}]$_i$ levels compared with propofol and remifentanil at the same dosage ($P < 0.05$; **Figure 3C**).

Discussion

Major hippocampal functions in the brain include memory formation and navigation. Proliferation and differentiation of these NSCs and hippocampal neural progenitor cells are associated with behavioral and cognitive states, including memory and learning (Deng et al., 2010; Shetty, 2014; Blaya et al., 2015; Gu et al., 2015; Iggena et al., 2017). Therefore, NSCs and hippocampal neural progenitor cells are widely used as *in vitro* models in anesthesia-induced neurotoxicity studies. Previously, our research and other studies have shown that general anesthesia exerts damaging effects on the immature central nervous system, especially at moderate and high concentrations (Davidson, 2011; Braz et al., 2012; Creeley et al., 2013; Li et al., 2014; Van Biesen et al., 2015; Satomoto and Makita, 2016; Ward et al., 2016; Earley et al., 2017; Jevtovic-Todorovic et al., 2017; McCann and de Graaff, 2017; Vutskits and Davidson, 2017; Walters and Paule, 2017; Zanghi and Jevtovic-Todorovic, 2017). In rats at postnatal day 14, but not adult (postnatal day 60), repeated isoflurane infusion causes impairment of reversal learning and object recognition, a decrease in the hippocampal stem cell pool, and neurogenesis reduction *in vivo*. Furthermore, such deficits and impairments induced by isoflurane exposure become worse when affected rats get older (Zhu et al., 2010; Cheng

and Levy, 2014; Schenning et al., 2017). Consequently, we used an *in vitro* model of NSCs derived from neonatal rat (postnatal day 1) in this study.

GM1 is the main constituent of lipid rafts in the outer plasma membrane layer of neurons and is distributed in various brain areas (Skaper et al., 1991; Baek et al., 2010; Colsch et al., 2011; Ji et al., 2015; Dalton et al., 2017). In the immature hippocampus, GM1 signaling plays important roles in neural activities, such as axonal growth and neuronal differentiation. Plasma membrane ganglioside sialidase is essential for synthesis of endogenous GM1 and is expressed at high abundance in the developing hippocampus (Rodriguez et al., 2001; Iwamori et al., 2005; Schneider et al., 2015). Neurons overexpressing plasma membrane ganglioside sialidase show enhanced axon growth and accelerated polarization *in vitro* (Rodriguez et al., 2001; Iwamori et al., 2005; Schneider et al., 2015). Moreover, GM1 has neuroprotective properties in the central nervous system under various pathological conditions. Exogenous GM1 prevents loss of neurogenesis induced by D-galactose in the mouse hippocampus (Zhang et al., 2005). Transgenic mice without GM1 synthesis show impaired neuronal activity, including dysfunction of calcium regulation, loss of neuroprotection, and enhanced susceptibility to kainate-induced neuronal apoptosis (Wu et al., 2005; Huang et al., 2007; Wu et al., 2016; Zanghi and Jevtovic-Todorovic, 2017). Recently, studies also found a potential association between GM1 signaling and anesthesia-induced neurotoxicity. Accordingly, in the current study, we first investigated the neuroprotective effects of GM1 on NSCs against neurotoxicity induced by propofol combined with remifentanil.

The calcium signaling pathway plays modulative roles in neurogenesis and differentiation of NSCs derived from the immature brain, while dysregulation of intracellular calcium is associated with developmental disorders (Slusarski and Pelegri, 2007; U et al., 2014; Deng et al., 2015; Wang et al., 2016). Here, we found that propofol combined with remifentanil decreased [Ca^{2+}]$_i$ levels in NSCs in a dose-dependent manner. Direct evidence for a modulative role of GM1 in neuronal calcium signaling has not yet been found. The present results suggest that neuronal calcium signaling may account for combined propofol and remifentanil-induced neurotoxicity, as well as GM1 induced neuroprotection of NSCs.

GM1 appears to alleviate combined propofol and remifentanil-induced neurotoxicity, especially at moderate and high concentrations, in NSCs *in vitro*. Neuronal calcium signaling may account for the combined propofol and remifentanil-induced neurotoxicity and neuroprotective effects of GM1. Our findings raise concerns about the potential central nervous system adverse effects of propofol and remifentanil in neonatal anesthesia. The protective role of GM1 in the immature central nervous system warrants further investigation.
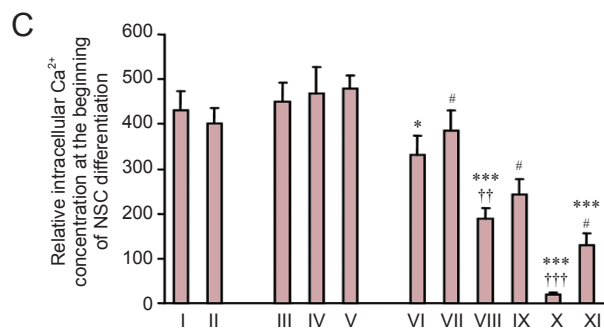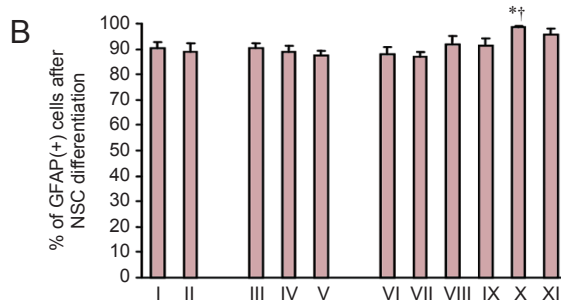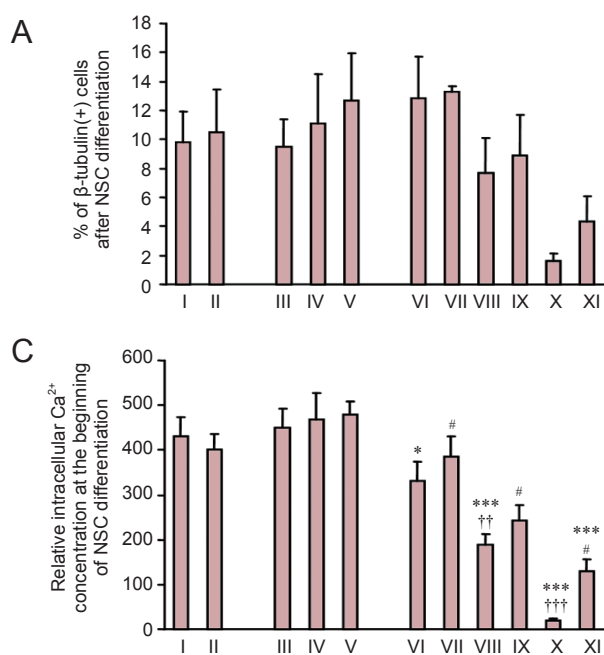
Figure 3 Effect of propofol, remifentanil, and/or GM1 on neural stem cell (NSC) differentiation.

Percentage of β-tubulin(+) cells (A) and GFAP(+) cells (B), and levels of intracellular Ca^{2+} (C) are summarized. Data are expressed as the mean ± SEM and were analyzed by one-way analysis of variance and Bonferroni *post hoc* tests. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* group with the same dosage of propofol and/or remifentanil treatment and without GM1 treatment; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, *vs.* $R_1 + P_1$ group. I: Control; II: Intralipid; III: $GM1_1$; IV: $R_1 + P_1$; V: $R_1 + P_1 + GM1_1$; VI: $GM1_2$; VII: $R_2 + P_2$; VIII: $R_2 + P_2 + GM1_2$; IX: $GM1_3$; X: $R_3 + P_3$; XI: $R_3 + P_3 + GM1_3$. GM1: Monosialoganglioside 1; P: propofol; R: remifentanil; GFAP: glial fibrillary acidic protein.
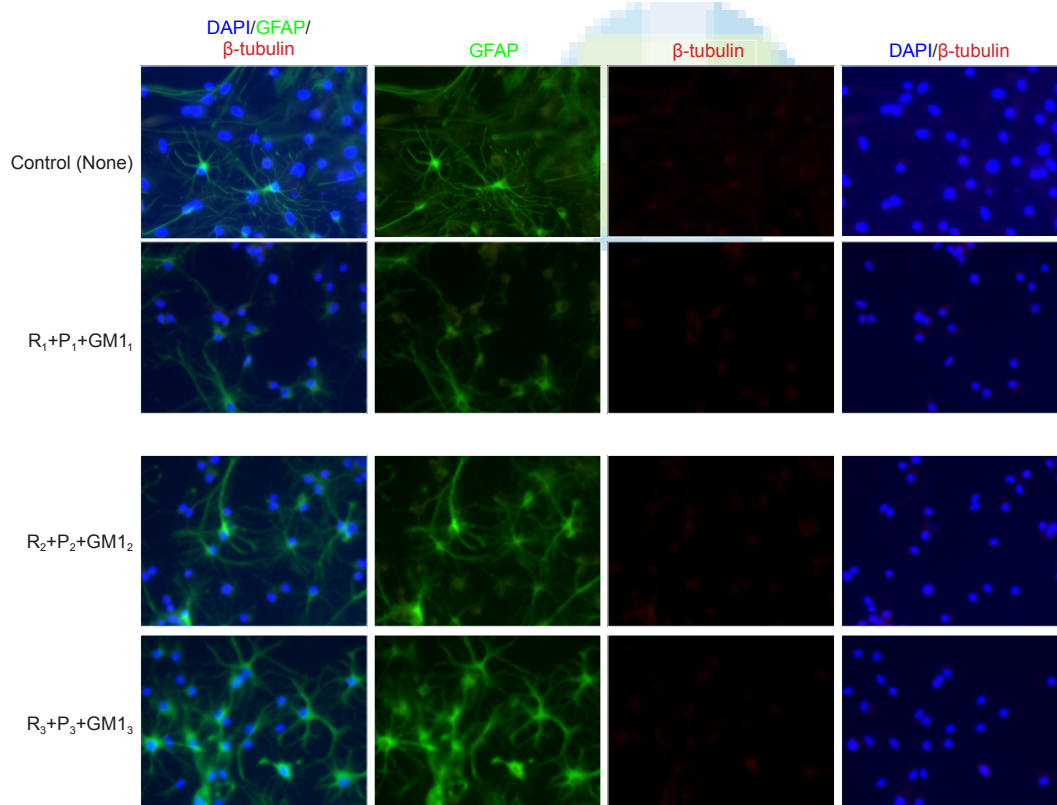

Figure 4 Effects of different concentrations of propofol combined with remifentanil and monosialoganglioside 1 (GM1) on neural stem cell (NSC) morphology (immunofluorescence staining, × 400).

NSCs were split and re-suspended (into almost single cells) and re-cultured in each differentiation culture medium. NSCs differentiation was detected after 12 days in each group. Green: Astrocyte-specific glial fibrillary acidic protein (GFAP; FITC staining); red: neuron-specific β-tubulin (TRITC staining); blue: nuclei (DAPI staining). P_1: 1.0 μg/mL propofol; P_2: 2.5 μg/mL propofol; P_3: 5.0 μg/mL propofol; R_1: 5 ng/mL remifentanil; R_2: 10 ng/mL remifentanil; R_3: 20 ng/mL remifentanil; $GM1_1$: 12.5 μg/mL GM1; $GM1_2$: 25 μg/mL GM1; $GM1_3$: 50 μg/mL GM1; $R_1 + P_1 + GM1_1$: 5 ng/mL remifentanil + 1.0 μg/mL propofol + 12.5 μg/mL GM1; $R_2 + P_2 + GM1_2$: 10 ng/mL remifentanil + 2.5 μg/mL propofol + 25 μg/mL GM1; $R_3 + P_3 + GM1_3$: 20 ng/mL remifentanil + 5.0 μg/mL propofol + 50 μg/mL GM1. NSCs: Neural stem cells; GM1: Monosialoganglioside 1; GFAP: glial fibrillary acidic protein; FITC: fluorescein isothiocyanate; DAPI: 4′,6-diamidino-2-phenylindole.

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