Published in final edited form as:

Syst Pharmacol. 2013 ; 1: 1–9. doi:10.2478/sph-2012-0001.

Inhalation Anesthesia-Induced Neuronal Damage and Gene Expression Changes in Developing Rat Brain

Fang Liu^{1, #}, Lei Guo^{2, #}, Jie Zhang², Shuo W. Rainosek⁶, Leming Shi², Tucker A. Patterson¹, Quan-Zhen Li³, Natalya Sadovova⁴, Joseph P. Hanig⁵, Merle G. Paule¹, William Slikker Jr.¹, and Cheng Wang^{1, *}

¹Division of Neurotoxicology, National Center for Toxicological Research, U.S. Food & Drug Administration, 3900 NCTR Rd., Jefferson, AR 72079 USA

²Division of Systems Toxicology, National Center for Toxicological Research, U.S. Food & Drug Administration, 3900 NCTR Rd., Jefferson, AR 72079 USA

³Department of Immunology, Microarray Core Facility, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX 75390 USA

⁴Toxicologic Pathology Associates, 3900 NCTR Rd., Jefferson, AR 72079 USA

⁵Center for Drug Evaluation and Research, FDA, Silver Spring, MD 20933 USA

⁶Department of Anesthesiology, University of Arkansas for Medical Science

Abstract

Nitrous Oxide (N₂O), an N-methyl-D-aspartate (NMDA) receptor antagonist, and isoflurane (ISO), which acts on multiple receptors including postsynaptic gamma-aminobutyric acid (GABA) receptors, are frequently used inhalation anesthetics, alone or as a part of a balanced anesthetic regimen administered to pregnant women and to human neonates and infants requiring surgery. The current study investigated histological features and gene expression profiles in response to prolonged exposure to N₂O or ISO alone, and their combination in developing rat brains. Postnatal day 7 rats were exposed to clinically-relevant concentrations of N₂O (70%), ISO (1.0%) or N₂O plus ISO (N₂O + ISO) for 6 hours. The neurotoxic effects were evaluated and the brain tissues were harvested for RNA extraction 6 hours after anesthetic administration. The prolonged exposure to N₂O + ISO produced elevated neuronal cell death as indicated by an increased number of TUNEL-positive cells in frontal cortical levels compared with control. No significant neurotoxic effects were observed in animals exposed to N₂O or ISO alone. DNA microarray analysis revealed gene expression changes after N₂O, ISO or N₂O + ISO exposure. Differentially expressed genes (DEGs) from the N₂O + ISO group were significantly associated with 45 pathways directly related to brain functions. Although the gene expression profiles from animals exposed to N₂O or ISO alone were remarkably different from those of the control group, the pathways of these genes involved were not closely associated with neurons. These findings provide novel insights into the mechanisms by which N₂O + ISO cause neurotoxicity in the

* cheng.wang@fda.hhs.gov.

Denotes co-first authors

developing brain, suggesting multiple factors are involved in the neuronal cell death-inducing effects (cascades) of N₂O + ISO.

Keywords

Nitrous oxide; Isoflurane; DNA Microarray; Neurotoxicity; Development

1. Introduction

The anesthetic gas nitrous oxide (N₂O) and the volatile anesthetic isoflurane (ISO) are commonly used in surgical procedures for human infants and in veterinary and laboratory animal practice to produce loss of consciousness and analgesia. Mounting evidence indicates that ISO causes damage in various types of tissues and cells [1–4], including primary cortical and striatal neurons [2,5,6]. Developing brains are especially vulnerable to anesthesia-mediated neurotoxicity [7]. Pre-clinical studies in animal models showed that a 6-hour exposure to ISO alone, or in combination with N₂O, induced widespread neurodegeneration in postnatal day (PND) 7 rat brains, and was associated with subsequent memory and learning deficits [8]. Our recent work added more evidence that prolonged anesthetic exposure (N₂O + ISO) during a sensitive period of brain development causes an increased rate of cell death in nonhuman primates [9]. In addition, a previous behavioral study has shown that exposure of developing nonhuman primates to a widely-used general anesthetic (ketamine) resulted in long-lasting cognitive impairment [10]. Recent clinical studies suggest that some learning disabilities and behavioral disturbances in children correlate to anesthesia exposure during surgery before 4 years of age, especially children subjected to multiple surgeries that require anesthesia [11,12]. However, existing human data are insufficient to support or refute the possibility that the neurotoxic effects observed in animals, under certain conditions, could occur in human infants or children. Before any study approach is able to address this critical public health issue associated with the safe use of anesthetics and sedatives in young children, animal studies are still indispensable for exploring the effects of anesthetics on the developing brain; the mechanisms by which anesthetics cause detrimental changes in the brain as well as possible methods to prevent the neurotoxic effects of anesthetics on the central nervous system.

Recent genetic studies indicate the involvement of some anti- and pro-apoptotic genes in N₂O + ISO-induced neuronal cell death during development [13]. However, no literature has ever reported a complete gene expression analysis on brains exposed to N₂O and/or ISO. Microarray analysis, which simultaneously evaluates the changes in gene expression, is a powerful tool incorporated into toxicological research for identifying pathways through which any drug or chemical might mediate its actions. The present study assessed the gene expression profiles of rat brains using DNA microarray analysis combined with Terminal dUTP Nick-end Labeling (TUNEL)-Assay, in an attempt to explore the molecular pathogenesis and possible pathways through which N₂O and/or ISO cause neuronal cell death in developing rat brains.

2. Materials and Methods

2.1 Animals

A total of 24 PND 7 Sprague-Dawley rat pups (male and female) were used in the experiment. The animals were randomly divided into anesthetic-treated (n=6/group) and control (n=6) groups. The anesthetic-treated rat pups were exposed to either 70% N₂O and 1.0% ISO alone, or a combination of 70% N₂O plus 1.0% ISO for 6 hours. The control group received mock anesthesia (air was used as a substitute). Oxygen saturation was measured with a small clip that attached to an appendage (e.g. a foot) on the animal's body using MouseOx system (Starr Life Sciences Corp, Oakmont, PA, USA). Animal procedures were approved by the National Center for Toxicological Research's Institutional Animal Care and Use Committee and conducted in full accordance with the PHS Policy on Humane Care and Use of Laboratory Animals. Animal procedures were designed to minimize the number of animals required. Body temperatures were monitored using an anal thermal probe and kept normothermic throughout the experiments by a T/Pump Therapy System (Gaymar Industries, Orchard Park, NY).

N₂O and oxygen were blended and delivered by a calibrated anesthetic machine (Bird Corporation, Palm Springs, CA, USA). An agent-specific vaporizer (E-Z Anesthesia, Palmer, PA, USA) was used to deliver a specific percentage of ISO. The anesthetic-treated animals were kept in an anesthesia chamber made of clear acrylic plastic and sealed with a gasket to prevent gas/vapor leakage; the gas/vapor was exhausted through an outlet that was connected to a charcoal-filtered canister to ensure accurate concentrations of anesthetic exposure and avoid accumulation of carbon dioxide in the anesthesia chamber. In our previous study, the composition of the chamber gas was analyzed using a mass spectrometer [14]. A 6-hour withdrawal time was allowed before the animals were sacrificed. Brain tissues from frontal cortical regions of the rats were harvested for RNA isolation. The frontal cortical regions were defined by the Atlas of the Developing Rat Brain [15]. All rat pups in the histopathological studies (n=3 rats/group) were perfused with saline and then fixed by transaortic perfusion of ice-cold (4°C) paraformaldehyde (4%) in phosphate buffer (pH=7.2).

2.2 TUNEL-Assay

Condensed or fragmented DNA was assayed using TUNEL staining as previously described [16]. Deoxynucleotidyl transferase (TdT), a template-independent polymerase, was used to incorporate biotinylated nucleotides at sites of DNA breaks. The signal was then amplified using avidin-biotin peroxidase, enabling conventional histochemical identification by light microscopy. In brief, brain tissue was equilibrated with sucrose and embedded in optimum cutting temperature (OCT) tissue medium. The frontal cortex (corresponding to Figure 79 as defined by the Atlas of the Developing Rat Brain) [17] was selected for histological studies.

Coronal cryostat sections (10 µm) were treated with proteinase K to dissociate proteins from DNA and washed in PBS. The sections were then incubated in H₂O₂ to quench endogenous peroxidase, rinsed with PBS and incubated in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 90 min at room temperature followed by

incubation in TdT (0.3 U/ml; Roche, Indianapolis, IN) and biotinylated dUTP (0.2 nM/10 U TdT; Roche) in TdT buffer at 37°C for 60 min. The reaction was terminated by transferring the sections to buffer containing 300 mM NaCl and 30 mM sodium citrate for 15 min at room temperature, and rinsed with PBS. The sections were treated with 2% BSA for 10 min at room temperature and biotin-avidin (1:50 in PBS; Vectastain ABC kit; Vector, Burlingame, CA, USA) for 30 min at 37°C. The reaction product was visualized with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA).

Unbiased sampling of brain sections was performed by randomly selecting five fields (1.4 mm²/field) from five serial sections of each rat. The fields of view were selected as closely as possible from the same place in each animal's frontal cortex. These viewing fields (photographs) were counted by two examiners who were blind to the treatments. Quantitative analysis of TUNEL staining was presented as the mean values $\pm$ SD. The null hypothesis was rejected at a probability level of $p < 0.05$.

2.3 Brain tissue collection and RNA isolation

Total RNA from brain tissue (frontal cortex) was isolated using RNeasy® Lipid Tissue Mini Kits (Qiagen Inc., Valencia, CA, USA). The yielded RNA concentration was measured spectrophotometrically (NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA). The integrity of total RNA was evaluated using the RNA 6000 LabChip kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). High quality RNA with RNA integrity numbers (RINs) greater than 9.0 was used for the microarray experiments.

2.4 Microarray analysis

RNA samples from the rat brains were subjected to microarray analysis. Gene expression profiling was performed using the Illumina Rat Ref-12 Expression BeadChip platform containing 22,523 probes (Illumina Inc., San Diego, CA). Gene expression data from the Illumina Rat Ref-12 arrays were analyzed using ArrayTrack™, a software system developed at the NCTR/FDA for the management, analysis, visualization and interpretation of microarray data [18]. Correlation coefficients were calculated within JMP 7.0 (SAS Institute, Cary, NC). Microarray data from treated animals were compared to those from controls. Pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA) (<http://www.ingenuity.com/>).

3. Results

3.1 N₂O and ISO-induced neurodegeneration

In the present study, important physiological parameters including body temperature and percent oxygen saturation were monitored. The body temperatures were maintained in a normal range (36.5 $\pm$ 0.4°C) for all experimental rat pups and there were no significant group differences between control and anesthetic-treated animals (N₂O alone, ISO alone or their combination) over the duration of anesthesia. The percent oxygen saturation was within the normal range (averaged 96 $\pm$ 2%), and not significantly different between groups in anesthetic-exposed animals.

A combination of 70% N₂O and 1.0% ISO produced an elevated rate of apoptotic cell death (Figure 1B,C) as indicated by an increased number of TUNEL-positive cells exhibiting typical nuclear condensation and fragmentation in the frontal cortical levels as compared with the control group (Figure 1A). Coronal sections of the whole rat brain were also examined to screen vulnerable brain areas. N₂O + ISO-induced neuronal cell death in PND 7 rat brains was primarily restricted to the neocortical brain regions, especially in layers II and III of the frontal cortex; no significant increase in the number of TUNEL-positive cells was observed in other brain regions such as striatum, hippocampus, thalamus, and amygdala. Although a few TUNEL-positive cells were observed in the brain, N₂O or ISO alone did not significantly increase neuronal cell death (data not shown) compared with the corresponding controls.

3.2 Evaluation of DNA microarray data quality

To assess the overall quality and reproducibility of the DNA microarray data used in the present study, the Pearson's correlation coefficients for pair-wise log₂ intensity correlations between each biological replicate within each experimental group was calculated. Pearson's correlation matrix of 12 arrays was calculated based on all data points (22,523 probes), no filtering applied for evaluation of microarray quality. These analyses indicated that there were high correlations for samples within the treated and control groups: the treated groups (N₂O + ISO, N₂O alone, and ISO alone) showed an average correlation coefficient of 0.987, 0.986 and 0.987, respectively. The correlation coefficient of the control group was 0.990. These results demonstrated the high degree of gene expression similarity for samples within the same group. Figure 2 presents representative scatter plots from the N₂O-treated group, which had the lowest correlation coefficient of 0.986.

Hierarchical Cluster Analysis (HCA) (Figure 3) was conducted to determine the gene expression profiles for control, N₂O + ISO, N₂O, and ISO-treated samples. Four apparent clusters were revealed in HCA with the samples separating well into control, N₂O + ISO, N₂O, and ISO-treated groups. The N₂O + ISO-treated group showed the greatest difference from the controls among the 3 treatment groups, while both the N₂O- and ISO-treated groups were closer to controls, suggesting less treatment effects on neuronal cell gene expression, as compared to the N₂O + ISO treatment.

3.3 Differentially expressed genes (DEGs)

DEGs were selected using criteria of a *p*-value less than 0.05 and a fold change greater than 1.4 (up or down). This straightforward gene selection approach of combining a *p*-value cutoff (*e.g.* 0.05) and a fold-change ranking has generated highly reproducible gene lists [19,20]. The criterion of a *p*<0.05 reduces the chance of false discovery (*i.e.* a gene is selected as differentially expressed when it is not), whereas ranking the genes that meet the *p*<0.05 by fold-change criterion allows genes with a larger magnitude of differential expression to be reproducibly identified as differentially expressed, thus balancing the sensitivity, specificity, and reproducibility of DEGs from a microarray gene expression study [21]. Based on these two criteria, 378 (172 up-regulated genes, 206 down-regulated genes) from the N₂O + ISO-exposed group; 174 (94 up-regulated genes, 80 down-regulated genes) from the N₂O alone group; and 145 genes (73 up-regulated genes, 72 down-regulated genes)

from the ISO alone group, respectively, satisfied the requirements and were identified as DEGs. These genes were selected for further bioinformatics analysis. Among the three groups there were 17 overlapping DEGs between N₂O alone and ISO alone groups, 26 overlapping DEGs between ISO alone and N₂O + ISO-exposed groups, 65 overlapping DEGs between N₂O alone and N₂O + ISO-exposed groups, and 9 overlapping DEGs among the three treatment groups (Figure 4). Our previous work demonstrated that male and female perinatal animals display similar gene expression profiles in response to anesthetic exposure [22,23]. Therefore, male and female rat pups were randomly assigned to different groups in the current study.

3.4 Pathway analysis of the DEGs

Ingenuity Pathway Analysis (IPA) software was used to determine the most relevant biological pathways affected by DEGs.

3.4.1 Pathways involved in the N₂O + ISO-treated group—335 out of 378 DEGs in the N₂O + ISO-treated group were identified by IPA and overlaid onto a global molecular network developed from information contained in the database. Forty-five canonical pathways were found to be significantly altered (Table 1) with the $-\log(p\text{-value})$ greater than 1.4 (equivalent to $p\text{-value} < 0.05$, which describes the significance in the case that the annotation does not occur by chance). Each pathway was associated with specific genes and involved different functions. The first pathway, G-protein coupled receptor signaling, containing the largest number of genes, was related to cell death and cellular function and maintenance. In addition, serotonin receptor signaling, calcium signaling, CREB signaling in neurons, TNFR1 signaling, and endoplasmic reticulum stress pathway were significantly altered by the ISO + N₂O treatment and contributed to the histological feature of the ISO + N₂O-treated brain. The top biological functions these DEGs involved in are closely related to neurological diseases, behavior, cognition, learning, development of brain, synaptic transmission of neurons, and apoptosis of neurons (Table 2).

3.4.2 Pathways involved in the N₂O alone group—144 of 174 DEGs from the N₂O alone group were identified by IPA. Five canonical pathways, including RAN signaling, cell cycle control and chromosomal replication, androgen and estrogen metabolism, aminoacyl tRNA biosynthesis, glycosaminoglycan degradation and keratin sulfate biosynthesis, were significantly altered. The IPA database did not provide direct evidence that these pathways were closely associated with neuronal damage, supporting the histological findings that N₂O-treatment did not cause significant neuronal cell death.

3.4.3 Pathways involved in ISO alone group—113 of 145 genes from ISO alone group were identified by IPA. Five canonical pathways, such as AMPK signaling, angiopoietin signaling, serotonin receptor signaling, cAMP-mediated signaling, and relaxin signaling were significantly altered. Similarly, these pathways were not directly associated with neuronal damage.

4. Discussion

Anesthetic-induced immobilization and reduced metabolic rate can cause hypothermia of experimental animals [24,25]. In the present study body temperatures were effectively controlled with external heat sources during the experiment, so the enhanced neurotoxicity observed could not be attributed to low body temperatures during anesthesia. In addition, no evidence of abnormal oxygen saturation was observed in anesthetic-treated animals, excluding the possibility of cerebral hypoperfusion and hypoxia-related cell death resulting from prolonged respiratory or cardiac compromise during anesthesia. It should be mentioned that the inhaled anesthetic combination of 70% N₂O + 1% ISO produced and maintained a steady light surgical plane of anesthesia in the rat pups, as evidenced by lack of voluntary movement, decreased muscle tone and minimal reaction to physical stimulation with maintenance of an intact palpebral reflex; whereas the 70% N₂O alone or 1% ISO alone failed to maintain a steady state of anesthesia. Consistent with our previous study [9], no significant neurotoxic effects were observed from the animals exposed to N₂O alone or ISO alone. Although a number of DEGs were identified in N₂O or ISO-treated groups, these DEGs involved networks were not directly relevant to neuronal cell death and neuron functions, except the serotonin receptor signaling altered by ISO treatment. This may explain why there were identified DEGs, but no obvious apoptotic neurons were found in N₂O or ISO-alone treated brains. Importantly, N₂O + ISO caused much more changes (the most changes) in gene expression than that in the N₂O or ISO-alone groups and enhanced apoptotic cell death. Since the underlying mechanisms by which N₂O + ISO exposure causes the neurotoxic effects have been poorly characterized, the novel aspect of the present study lies in elucidating the possible molecular pathways leading to neuronal cell damage. The basic findings of this microarray study are: 1) N₂O + ISO significantly altered several networks that are closely associated with neuronal cell death; 2) N₂O + ISO caused calcium dysregulation or disturbed calcium homeostasis; and 3) dysfunction of the GABA receptor system in the brain, which is a critical factor in causing neuronal cell death.

There were 378 DEGs recognized in N₂O + ISO-treated brains, with a high percentage of overlapping genes with N₂O (65 overlapping genes) and ISO (26 overlapping genes), suggesting that the N₂O and ISO work in concert to induce the changes in gene expression, which resulted in enhanced neuronal cell death as indicated by increased number of TUNEL-positive cells (Figure 1). Analysis by IPA indicated the majority of the top 50 biological functions the 378 DEGs involved, are directly related to brain functions (Table 2). Among them, a number of molecules are associated with neurological diseases and development of the central nervous system. About 20 molecules directly contribute to the neuronal apoptosis. It should be mentioned that caspase 3, the executioner of apoptosis, was not affected by either N₂O or ISO, whereas N₂O + ISO exposure significantly up-regulated caspase 3 expression, supporting the findings from the TUNEL assay that N₂O + ISO exposure induced enhanced neuronal apoptosis.

Glutathione peroxidase 1, encoded by GPX1, is one of the most important antioxidant enzymes [26,27]. Here, the higher level expression of GPX1 could indicate the neuronal reaction to oxidative stress is induced by prolonged N₂O + ISO exposure. Meanwhile, GLRX which participates in glutathione metabolism (encoding protein-disulfide reductase)

[28], was also highly expressed in the N₂O + ISO-treated group, further implying an imbalance between the production of reactive oxygen species and the antioxidant system.

Calcium plays a central role in signal transduction in cells, thereby affecting nervous system development and function, even apoptosis [29]. Prolonged N₂O + ISO exposure caused the alteration of calcium signaling, resulting in the disturbance of intracellular cations and/or anions, which could further induce mitochondrial dysfunction. Moreover, the expression of CACNA1A (encoding voltage-dependent calcium channel) was significantly elevated in the N₂O + ISO-exposed group. This kind of alteration could contribute to the intracellular calcium changes and guide neurons to death. In addition, canonical pathway analysis by IPA indicated that the top functions of several pathways, such as G-protein coupled receptor signaling, Huntington's Disease signaling, protein kinase A signaling, and the endoplasmic reticulum stress pathway, are closely related to the apoptotic cell death cascades. Thus, the development of the central nervous system could be significantly affected by prolonged N₂O + ISO exposure and a long lasting brain function deficit might subsequently occur.

Co-administration of N₂O and ISO is a frequently-used inhaled anesthetic regimen during surgery in the neonate. Previous studies [8,9] have demonstrated that a striking enhancement in brain damage was noted when N₂O was combined with ISO, and maximal neurodegeneration was observed after 6 hours. However, only a small amount of damage (not significant) was detected at 4 hours, and no significant effects at 2 hours. These data provide evidence that anesthetic exposure duration is an important factor in anesthetic-induced neurotoxic effects when similar anesthetic depth was achieved [30]. Work from others [31,32] has also demonstrated a significant increase of neuronal cell death when ISO was administered alone at a concentration of 1.5% for 5 hours, suggesting the neurotoxic effects of a higher dose of ISO in the developing brain and deeper anesthesia induced by higher concentrations of ISO is critical in triggering abnormal neuronal apoptosis.

It has been suggested that the prolonged exposure of the developing brain to NMDA antagonists (with continued blockade of the NMDA receptor), such as occurs with ketamine, causes neuronal cell death by a mechanism that involves a compensatory up-regulation of NMDA receptor subunits [22,23,33,34]. This leads to abnormal elevation of intracellular calcium and generation of reactive oxygen species (ROS) originating from mitochondria [16,35], subsequently activating caspases and causing apoptosis. It was also proposed that immature GABA receptors are excitatory early in development and convert to an inhibitory role when neurons mature [36]. Thus, prolonged supraphysiological stimulation of immature neurons by GABA agonists may contribute to increased excitability during early development. This increase of excitability along with NMDA antagonist-induced alteration of NMDA receptors could lead to neuronal cell death. Although no significant changes of NMDA receptor subunits were observed in N₂O + ISO-exposed group, changes in GABA receptor signaling was identified by IPA. In the current study, the GABA_A receptor, with the most relevant biological functions, was significantly down-regulated. However, the GABA_A receptor was not significantly affected by either N₂O or ISO alone exposed animals. Thus, a more complicated situation was introduced when neurons were simultaneously exposed to both an NMDA receptor antagonist and a GABA_A receptor agonist, and the final expression profile of the GABA_A receptor is a consequence of the balanced effects of N₂O and ISO.

Taken together, the present DNA microarray and morphological data indicate that the frontal cortex is the brain region most vulnerable to inhalation anesthetic-induced neurotoxicity. A complete gene expression profile was described after exposure to a clinically-relevant combination of ISO and N₂O during development. The fate of neurons depends mostly on the interactions of involved networks, the dose of anesthetic, the duration of exposure, the route of administration, the receptor subtype activated, and the stage of development or maturity at the time of exposure. Since the majority of analysis is at the transcriptional level, the limitation of the study is obvious. To verify the relationship between each gene, further follow-up studies at the protein level are necessary, using both *in vivo* and *in vitro* models.

Acknowledgments

This work was supported by the National Center for Toxicological Research (NCTR)/U.S. Food and Drug Administration (FDA), the Center for Drug Evaluation and Research (CDER)/FDA, and the National Institute of Child Health and Human Development (NICHD).

Disclaimer: This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

References

1. Wise-Faberowski L, et al. Isoflurane-induced neuronal degeneration: an evaluation in organotypic hippocampal slice cultures. *Anesth Analg*. 2005; 101(3):651–7. table of contents. [PubMed: 16115969]
2. Wei H, et al. The common inhalational anesthetic isoflurane induces apoptosis via activation of inositol 1,4,5-trisphosphate receptors. *Anesthesiology*. 2008; 108(2):251–60. [PubMed: 18212570]
3. Yang H, et al. Inhalational anesthetics induce cell damage by disruption of intracellular calcium homeostasis with different potencies. *Anesthesiology*. 2008; 109(2):243–50. [PubMed: 18648233]
4. Xie Z, et al. The inhalation anesthetic isoflurane induces a vicious cycle of apoptosis and amyloid beta-protein accumulation. *J Neurosci*. 2007; 27(6):1247–54. [PubMed: 17287498]
5. Wei H, et al. Isoflurane and sevoflurane affect cell survival and BCL-2/BAX ratio differently. *Brain Res*. 2005; 1037(1–2):139–47. [PubMed: 15777762]
6. Liang G, et al. A presenilin-1 mutation renders neurons vulnerable to isoflurane toxicity. *Anesth Analg*. 2008; 106(2):492–500. table of contents. [PubMed: 18227305]
7. Loepke AW, Soriano SG. An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. *Anesth Analg*. 2008; 106(6):1681–707. [PubMed: 18499597]
8. Jevtovic-Todorovic V, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci*. 2003; 23(3):876–82. [PubMed: 12574416]
9. Zou X, Liu F, Zhang X, Patterson T, Hanig Joseph, Paule Merle, Slikker William, Wang C. Inhalation anesthetic induced neurodegeneration in developing rhesus monkeys. *Neurotoxicology and Teratology*. 2011
10. Paule MG, Li Mi, Allen RR, Liu F, Zou X, Hotchkiss C, Hanig JP, Patterson TA, Slikker W Jr, Wang C. Ketamine Anesthesia during the First Week of Life can Cause Long-Lasting Cognitive Deficits in Rhesus Monkeys. *Neurotoxicology and teratology*. 2011
11. Wilder RT, et al. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology*. 2009; 110(4):796–804. [PubMed: 19293700]
12. Kalkman CJ, et al. Behavior and development in children and age at the time of first anesthetic exposure. *Anesthesiology*. 2009; 110(4):805–12. [PubMed: 19293699]
13. Wang C, et al. L-carnitine protects neurons from 1-methyl-4-phenylpyridinium-induced neuronal apoptosis in rat forebrain culture. *Neuroscience*. 2007; 144(1):46–55. [PubMed: 17084538]

14. Zou X, et al. The effects of L-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex. *Neuroscience*. 2008; 151(4):1053–65. [PubMed: 18201836]
15. Paxinos, G., Tork, I., Tecott, LH., Valentino, K. Atlas of The Developing Rat Brain. Academic Press, Inc. Harcourt Brace Jovanovich; 1991.
16. Johnson KM, et al. Chronic phencyclidine induces behavioral sensitization and apoptotic cell death in the olfactory and piriform cortex. *J Neurosci Res*. 1998; 52(6):709–22. [PubMed: 9669320]
17. Paxinos, GTI., Tecott, LH., Balentino, KL., Fritchle, ALR. Atlas of the Developing Rat Brain. Academic Press, Inc; 1991.
18. Tong W, et al. ArrayTrack—supporting toxicogenomic research at the U.S. Food and Drug Administration National Center for Toxicological Research. *Environ Health Perspect*. 2003; 111(15):1819–26. [PubMed: 14630514]
19. Guo L, et al. Rat toxicogenomic study reveals analytical consistency across microarray platforms. *Nat Biotechnol*. 2006; 24(9):1162–9. [PubMed: 17061323]
20. Shi L, et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol*. 2006; 24(9):1151–61. [PubMed: 16964229]
21. Shi L, et al. The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies. *BMC Bioinformatics*. 2008; 9(Suppl 9):S10.
22. Shi Q, et al. Gene expression profiling in the developing rat brain exposed to ketamine. *Neuroscience*. 2010; 166(3):852–63. [PubMed: 20080153]
23. Liu F, et al. Changes in gene expression after phencyclidine administration in developing rats: a potential animal model for schizophrenia. *Int J Dev Neurosci*. 2011; 29(3):351–8. [PubMed: 20691775]
24. Lee JI, et al. Immobilization with ketamine HCl and tiletamine-zolazepam in cynomolgus monkeys. *J Vet Sci*. 2003; 4(2):187–91. [PubMed: 14610374]
25. Young SS, et al. Short duration anaesthesia with medetomidine and ketamine in cynomolgus monkeys. *Lab Anim*. 1999; 33(2):162–8. [PubMed: 10780820]
26. Crack PJ, et al. Lack of glutathione peroxidase-1 exacerbates Abeta-mediated neurotoxicity in cortical neurons. *J Neural Transm*. 2006; 113(5):645–57. [PubMed: 16252075]
27. McLean CW, et al. Overexpression of glutathione peroxidase protects immature murine neurons from oxidative stress. *Dev Neurosci*. 2005; 27(2–4):169–75. [PubMed: 16046851]
28. Maher P. Redox control of neural function: background, mechanisms, and significance. *Antioxid Redox Signal*. 2006; 8(11–12):1941–70. [PubMed: 17034341]
29. Szalai G, Krishnamurthy R, Hajnoczky G. Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J*. 1999; 18(22):6349–61. [PubMed: 10562547]
30. Zou X, et al. Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. *Int J Dev Neurosci*. 2009; 27(7):727–31. [PubMed: 19580862]
31. Brambrink AM, et al. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. *Anesthesiology*. 2010; 112(4):834–41. [PubMed: 20234312]
32. Olney JW, Brambrink AM, Avidan MS, Farber NB, Back SA. Isoflurane-induced oligoapoptosis in neonatal rhesus macaque brain. 2010
33. Wang C, et al. The role of the N-methyl-D-aspartate receptor in ketamine-induced apoptosis in rat forebrain culture. *Neuroscience*. 2005; 132(4):967–77. [PubMed: 15857702]
34. Wang C, et al. Blockade of N-methyl-D-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. *Toxicol Sci*. 2006; 91(1):192–201. [PubMed: 16500925]
35. Slikker W Jr, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci*. 2007; 98(1):145–58. [PubMed: 17426105]
36. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci*. 2002; 3(9):728–39. [PubMed: 12209121]

Figure 1.

N₂O + ISO - induced neuronal cell death in PND 7 rats assessed by TUNEL assay.

Representative photographs indicate that numerous darkly stained TUNEL-positive cells were observed in layers II and III of the frontal cortex in the brain exposed to N₂O + ISO (B). Only a few TUNEL-positive cells were observed in the control rat brain (A). For each group, three animals were randomly assigned (n=3/group). Quantitative data for the four groups are presented as means $\pm$ S.E.M. A probability of * $p < 0.05$ was considered significant (C). Scale bar = 60 μ m.

Figure 2.

The log₂ raw intensity data of the three biological replicates in N₂O-treated groups were plotted against each other. As shown in the scatter plot, for most spots, the intensity values from the replicate microarrays accumulated along the diagonal axis with a correlation coefficient of 0.986.

Figure 3. Hierarchical Cluster Analysis (HCA) of gene expression profiles for N₂O + ISO, N₂O alone, ISO alone-treated groups and the control group. Each column represents the results from an individual animal. Each row represents for one particular gene.

Figure 4.

Venn Diagram shows that there are 17 overlapping DEGs between N₂O alone and ISO alone groups, 26 overlapping DEGs between ISO alone and N₂O + ISO-exposed groups, 65 overlapping DEGs between N₂O alone and N₂O + ISO-exposed groups and 9 overlapping DEGs among the three treated groups.

Table 1

Significant Canonical Pathways Altered by ISO+N2O identified by Ingenuity Pathways Analysis Software.

Ingenuity Canonical Pathways	−log(p-value)	Ratio
G-Protein Coupled Receptor Signaling	3.56E+00	3.77E−02
Neuroprotective Role of THOP1 in Alzheimer's Disease	3.35E00	9.26E−02
Ephrin Receptor Signaling	3.28E00	5E−02
cAMP-mediated signaling	3.14E00	5.02E−02
Role of NFAT in Cardiac Hypertrophy	3.07E00	4.74E−02
Cellular Effects of Sildenafil (Viagra)	2.79E00	5.16E−02
Serotonin Receptor Signaling	2.72E00	8.7E−02
Calcium Signaling	2.67E00	4.35E−02
Cholecystokinin/Gastrin-mediated Signaling	2.22E00	5.66E−02
Relaxin Signaling	2.21E00	4.43E−02
Melatonin Signaling	2.21E00	6.25E−02
RAR Activation	2.17E00	4.28E−02
GABA Receptor Signaling	2.13E00	7.02E−02
Cardiac Hypertrophy Signaling	1.97E00	3.67E−02
Axonal Guidance Signaling	1.93E00	3E−02
CXCR4 Signaling	1.91E00	4.14E−02
Protein Kinase A Signaling	1.89E00	3.32E−02
Circadian Rhythm Signaling	1.87E00	8.57E−02
GNRH Signaling	1.81E00	4.11E−02
Non-Small Cell Lung Cancer Signaling	1.68E00	5.06E−02
PPARα/RXRα Activation	1.67E00	3.72E−02
CREB Signaling in Neurons	1.66E00	3.47E−02
Amyotrophic Lateral Sclerosis Signaling	1.65E00	4.2E−02
PI3K Signaling in B Lymphocytes	1.65E00	4.08E−02
Role of NFAT in Regulation of the Immune Response	1.64E00	3.5E−02
Aryl Hydrocarbon Receptor Signaling	1.62E00	3.77E−02
Chemokine Signaling	1.59E00	5.48E−02
Huntington's Disease Signaling	1.59E00	3.36E−02
Renal Cell Carcinoma Signaling	1.57E00	5.41E−02
Neuropathic Pain Signaling In Dorsal Horn Neurons	1.57E00	4.46E−02
Taurine and Hypotaurine Metabolism	1.57E00	4.17E−02
Renin-Angiotensin Signaling	1.49E00	3.97E−02
Endoplasmic Reticulum Stress Pathway	1.47E00	1.11E−01
Corticotropin Releasing Hormone Signaling	1.46E00	3.68E−02
Inositol Phosphate Metabolism	1.44E00	3.09E−02
EGF Signaling	1.42E00	5.77E−02
β-alanine Metabolism	1.39E00	3.23E−02

Ingenuity Canonical Pathways	−log(p-value)	Ratio
Colorectal Cancer Metastasis Signaling	1.39E00	3.1E−02
TNFR1 Signaling	1.37E00	5.66E−02
Aldosterone Signaling in Epithelial Cells	1.37E00	3.45E−02
Biosynthesis of Steroids	1.35E00	1.65E−02
TGF- β Signaling	1.34E00	4.49E−02
P2Y Purigenic Receptor Signaling Pathway	1.33E00	3.57E−02
CD27 Signaling in Lymphocytes	1.31E00	5.26E−02
Gap Junction Signaling	1.3E00	3.26E−02

Table 2

Top Biological Functions of the DEGs from ISO+N2O-Treated Brain Identified by Ingenuity Pathways Analysis Software.

Category	Functions Annotation	p-Value	# Molecules
Behavior	behavior	6.71E-17	42
Neurological Disease	dyskinesia	2.67E-14	46
Neurological Disease	Huntington's disease	8.38E-14	44
Genetic Disorder	Huntington's disease	8.38E-14	44
Skeletal and Muscular Disorders	Huntington's disease	8.38E-14	44
Neurological Disease	movement disorder	4.97E-13	63
Behavior	locomotion	1.59E-12	26
Neurological Disease	encephalopathy	1.73E-10	55
Tissue Development	tissue development	2.51E-10	86
Neurological Disease	neuromuscular disease	1.04E-09	60
Skeletal and Muscular Disorders	neuromuscular disease	1.04E-09	60
Behavior	feeding	2.43E-09	15
Digestive System Development and Function	feeding	2.43E-09	15
Metabolic Disease	glucose metabolism disorder	4.31E-09	87
Tissue Development	development of nervous tissue	7.08E-09	32
Cell Signaling	neuropeptide signaling pathway	7.25E-09	9
Cellular Growth and Proliferation	proliferation of cells	1.92E-08	84
Behavior	learning	2.24E-08	16
Gastrointestinal Disease	disorder of pancreas	2.32E-08	87
Nervous System Development and Function	development of central nervous system	3.24E-08	29
Behavior	cognition	3.56E-08	17
Tissue Development	development of brain	3.85E-08	24
Nervous System Development and Function	development of brain	3.85E-08	24
Embryonic Development	development of brain	3.85E-08	24
Organ Development	development of brain	3.85E-08	24
Organismal Development	development of brain	3.85E-08	24
Amino Acid Metabolism	quantity of GABA	7.54E-08	6
Molecular Transport	quantity of GABA	7.54E-08	6
Small Molecule Biochemistry	quantity of GABA	7.54E-08	6
Psychological Disorders	substance-related disorder	8.44E-08	14
Nervous System Development and Function	depolarization of neurons	1.01E-07	7
Cell Morphology	depolarization of neurons	1.01E-07	7
Cell-To-Cell Signaling and Interaction	depolarization of neurons	1.01E-07	7
Cellular Function and Maintenance	depolarization of neurons	1.01E-07	7
Nervous System Development and Function	neurotransmission of neurons	1.40E-07	17
Cell-To-Cell Signaling and Interaction	neurotransmission of neurons	1.40E-07	17

Category	Functions Annotation	p-Value	# Molecules
Neurological Disease	schizophrenia	1.79E-07	30
Genetic Disorder	schizophrenia	1.79E-07	30
Psychological Disorders	schizophrenia	1.79E-07	30
Cellular Assembly and Organization	development of cellular protrusions	2.00E-07	24
Cell Morphology	depolarization of cells	2.56E-07	8
Cellular Function and Maintenance	depolarization of cells	2.56E-07	8
Genetic Disorder	obesity	5.65E-07	22
Nutritional Disease	obesity	5.65E-07	22
Molecular Transport	quantity of metal	5.73E-07	25
Nervous System Development and Function	synaptic transmission of neurons	5.84E-07	16
Cell-To-Cell Signaling and Interaction	synaptic transmission of neurons	5.84E-07	16
Cellular Movement	cell movement of carcinoma cell lines	6.42E-07	9
Cardiovascular Disease	heart disease	7.30E-07	57
Cell Death	apoptosis of neurons	7.43E-07	20