Developmental Toxicology: Putting the Puzzle Together

Joseph Warkany Lecture

Teratology Society – June 28, 2015
Montreal, Canada
Josef Warkany (1902–1992): an Austrian American pediatrician known as the "father of teratology".

Warkany was born in Vienna and this is where he completed his medical studies. By 1932, he had published over 23 articles, before moving to Cincinnati, Ohio, in 1932, where he remained for the rest of his life.

Two genetic syndromes are named for him: Warkany syndrome 1 and Warkany syndrome 2.
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Disclosure Slide

• DISCLAIMER: The views expressed in this presentation are those of the presenter and do not necessarily reflect the views or policies of the U.S. Food and Drug Administration.

• No conflicts of interest to disclose.
Neonatal anesthesia and sedation

• Our increasing ability to keep premature infants and compromised neonates alive is resulting in an ever-increasing population in our nation’s neonatal intensive care units.

• Part of this success lies in the increased number of complicated surgical and other interventions that are brought to bear in this already-at-risk population.

• Many of these procedures are carried out under various forms of anesthesia and/or sedation, often in combination with other therapeutics.

• Concerns over the potential adverse effects of these kinds of exposures have prompted the need for studies to address this issue.
Neonatal drug exposure and NMDA receptors

• Interest piqued by the findings that the blockade of NMDA receptors by ketamine causes robust increases in apoptotic cell death in the rat during the brain growth spurt (PND7) (Ikonomidou et al., 1999).

• These findings were subsequently replicated and extended in our own laboratories (Scallet et al., 2004).

• Subsequent studies in nonhuman primates confirmed ketamine-induced selective brain cell death in a developmental stage dependent and duration of exposure dependent manner (Slikker et al., 2007).
Impact of anesthetic exposure during early life in nonhuman primates and children

• Following a single bout of ketamine-induced anesthesia during the neonatal period, long-lasting cognitive deficits were observed for at least the first 3 years of life in nonhuman primates (Paule et al., 2011).

• Exposure to multiple, but not single, episodes of anesthetic/surgery significantly increased the risk of developing learning disabilities (hazard ratio: 2.12 [95% confidence interval: 1.26-3.54]), even when accounting for health status (Flick et al., 2011).

• Children exposed to anesthesia before age 3 had an increased long-term risk of clinical deficit in receptive and expressive language and abstract reasoning even after a single exposure in this birth cohort study (Ing, et al., 2012).
Role of NMDA and GABA receptors in development

• Amino acid neurotransmitters play an important role by regulating neuronal survival, axonal and dendritic structure, and synaptogenesis and plasticity.

• There has been speculation that the infant brain may be more responsive to agents that affect NMDA and GABA receptor function than are adult brains.
Representative Anesthetic Agents
(alone or in combination)

1) NMDA Antagonists:
   Ketamine
   Nitrous oxide

2) GABA Agonists:
   Propofol

3) Inhalation agents: alone or in combination
   Isoflurane and Nitrous Oxide
   Sevoflurane
The NMDA Receptor

Channel Blockers
- Mg²⁺
- Memantine

Agonists
- Glutamate
- NMDA

Coagonists
- Glycine
- D-serine

Antagonists
- APV
- Mrz 2/576
- Ifenprodil (2B)
- Zn²⁺

Modulator
- Polyamines

Alternative splicing
Glycosylation site
Subunits of the NMDA Receptor

NR1
- Essential for the function of the receptor

HOMOMERIC
\[ NR1 - NR1 \]

HETEROMERIC
\[ NR1 - NR2A \]
\[ NR1 - NR2B \]
\[ NR1 - NR2C \]
\[ NR1 - NR2D \]
**Ketamine Dose Response & Time Course**

**Frontal Cortex**

![Graph showing Fluoro-Jade C-positive neuronal profiles across different ketamine doses and injection numbers.](image)

**Number of Neurodegenerative Profiles in Several Rat Brain Regions (PND 7)**

(20 mg/kg; 6 injections)

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>Ketamine</th>
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<tr>
<td>Frontal Cortex</td>
<td>4 ± 0.8</td>
<td>42 ± 3.2*</td>
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<tr>
<td>Striatum</td>
<td>4 ± 1.2</td>
<td>14 ± 3.2*</td>
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<tr>
<td>Hippocampus</td>
<td>7 ± 2.8</td>
<td>16 ± 4.1*</td>
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<tr>
<td>Thalamus</td>
<td>4 ± 0.7</td>
<td>10 ± 1.0</td>
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<tr>
<td>Amygdala</td>
<td>2 ± 1.1</td>
<td>7 ± 0.8*</td>
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*Zou et al., 2009*
Frontal Cortex (*PND-7 Rat Pups*)

*In Vivo* Exposure to Ketamine

Electron Microscopy

20 mg/kg × 6 (2 hr interval)

A: **Control** (saline): normal neuron with intact cytoplasm and nuclear membrane.

B-1: **Ketamine**: apoptotic neuron with DNA (nucleus) fragmentation.

B-2: **Ketamine**: apoptotic neurons with typical nuclear condensation.
Distribution of Ketamine in the 7 day old rat pup plasma and brain

Determination of brain cell death with ELISA
Ketamine Effects on NMDA Receptor Expression

A: Control (Saline × 6)
B: Ketamine (20 mg/kg × 6)

NR1 mRNA Signaling

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<th>Control</th>
<th>Ketamine</th>
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<tr>
<td>NR1 mRNA Signaling</td>
<td>10 ± 2</td>
<td>25 ± 3</td>
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* Indicates significant difference from control.
Ketamine Effects on NMDA Receptor Expression

Selective validation of the microarray results by Q-PCR

<table>
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<tr>
<th>Gene symbols</th>
<th>Fold-change (Q-PCR)</th>
<th>Fold-change (microarray)</th>
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<tr>
<td>Grin1 (NR1)</td>
<td>1.8*</td>
<td>1.5*</td>
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<tr>
<td>Grin2a (NR2A)</td>
<td>1.5*</td>
<td>1.2</td>
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<tr>
<td>Grin2b (NR2B)</td>
<td>1.0</td>
<td>0.9</td>
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<td>Grin2c (NR2C)</td>
<td>1.7*</td>
<td>1.5*</td>
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<tr>
<td>Grin2d (NR2D)</td>
<td>1.2</td>
<td>1.1</td>
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* P<0.05, as compared to the control

Shi, Q. et al., 2010
1. Normal voltage-dependent activation of the NMDA receptor by glutamate (Glu) opens Ca\(^{2+}\) channels.

2. Non-competitive inhibition of the NMDA receptor by ketamine (Ke) blocks the channel, preventing Ca\(^{2+}\) entry into the cell.

3. Compensatory upregulation of the NMDA receptor allows for the accumulation of toxic levels of intracellular Ca\(^{2+}\) under normal physiological conditions. Cell death via apoptosis or necrosis occurs.

Slikker et al., 2005
Serial images recorded with a Gatan 3View Serial Block Face Apparatus mounted on a Zeiss Merlin FEG-SEM, set to 1.4 kV, 40 pA, 5.4 nm/pixel and a slice thickness of 50 nm

Control: Cell from the frontal cortex of a Non-treated Postnatal Day 7 rat pup, Yellow scale bar length= 5 µm, Mitochondria: multi-colored

Treated: Cell from the frontal cortex of a Postnatal Day 7 rat pup treated with ketamine Hydrochloride: 6 subcutaneous injections at 20 mg/kg and 2-h intervals, Yellow scale bar length= 5 µm, Mitochondria: multi-colored

Manuscript: Using two- and three-dimensional electron microscopy techniques to quantify mitochondria defects in the developing Rat brain following Ketamine treatment (2014), Trisha Eustaquio, Angel Paredes, Christopher Dugard, Nysia George, Fang Liu, William Slikker, Merle Paule, Paul Howard, and Cheng Wang
A Neural Stem Cell is a subclass of precursors that:

1. is **self-renewing**: capable of making additional copies of itself by division.
   a. symmetric - both daughters are stem
   b. asymmetric - one daughter is stem cell

2. is **multipotent**: capable of making daughters other than itself.
   a. committed progenitors
   b. neurons, astrocytes, oligodendrocytes
   c. non-neural tissues (plasticity)?

3. **can generate all or part of neural tissue**
   a. normal development
   b. functional reconstitution
Rat Embryonic Neural Stem Cells

A  DIV 2  DIV 4  DIV 6  DIV 8

B  Nestin  Nestin + EdU

C
Anesthetics Used in Children

Ketamine, a non-competitive NMDA receptor antagonist, has been used as a general pediatric anesthetic for surgical procedures in infants.

Propofol (marketed as Diprivan) is a short-acting, general anesthetic agent.

Propofol is a GABA receptor agonist.

Animal model studies suggest that exposure to anesthetics during certain periods of development has long-term deleterious effects including deficits in cognitive function.

At the cellular level, there is evidence that anesthetic agents induce cell death, cause synaptic remodeling and alter morphology of the developing brain.
Embryonic Neural Stem Cell Culture

Embryonic Day 16

1x10^6/ml, Growth Medium (PDGF, NT-3, EGF, bFGF)

Day In Vitro (DIV) 8

Medium changed every 3 days

Exposure to anesthetics

Experiments (LDH, MTT, TUNEL, EdU, DCF and 8-oxo-guanine assays, cell death ELISA, etc.)
Effect of propofol on undifferentiated stems (24 hr. exposure)

A

B

C

D

Control

Propofol (50 µM)
EdU-DAPI Staining

Control

Propofol (50 µM; 24 hours)

A

EdU

B

C

DAPI

D

E

EdU-DAPI

Neural Stem Cell Proliferation

(Propofol; 24-hour Exposure)

G

% of EdU-positive Nuclei

100

80

60

40

C

P

C+L-Ca

P+L-Ca

C = Control; P = Propofol (50µM); L-Ca = Acetyl-L-Carnitine (10 µM)
Neural Stem Cell Differentiation Flow Chart

Embryonic Day 16

1x10^6/ml, Growth Medium (PDGF, NT-3, EGF, bFGF)

Day In Vitro (DIV) 6

Medium changed every 3 days

Transition Medium
EGF, bFGF, & 3% FCS

2 days

Differentiation Medium
GNF, BDNF & 10% FCS

1 day

24h exposure to anesthetics
NMDA Receptor NR1(Subunit)-labeled Neurons

(A) Control
(B) Ketamine (10 μM)

C

Protein Expression (Ratio of NR1/actin)

Control Ketamine

D

NMDA Receptor NR1

(Fang Liu et al., 2013)
Changes in $[Ca^{2+}]i$ in Fura-2-Loaded Neurons

A representative control neuron

A representative ketamine-treated neuron

(Fang Liu et al., 2013)
Western Blotting Analysis

\[ C = \text{Control}; \ P = \text{Propofol}; \ K = \text{Ketamine} \]

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<th>Growth Medium</th>
<th>Differentiation Medium</th>
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<tr>
<td></td>
<td>C</td>
<td>P</td>
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<tr>
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<td>β-Actin</td>
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Conclusions

• Stems cells can be used to understand the effects of anesthetics on developing systems
• Knowledge of the stage of development of the stem cell is critical to the interpretation of the toxicity data
• Under well controlled conditions, stem cell data may be predictive of in vivo derived data
Time Windows of Vulnerability to the Neurotoxic Effects of NMDA Receptor Antagonists for Rat

(Postulated for Monkey and Human)

Wright et al., 2007

![Diagram showing time windows of vulnerability for rats, rhesus monkeys, and humans.]

- **Rats**
  - Conception  
  - Birth  
  - 14 d  
  - 1.5 mo

- **Rhesus Monkeys**
  - Conception  
  - Birth  
  - 2 mo  
  - 3 yr

- **Humans**
  - Conception  
  - Birth  
  - 3 yr  
  - 11 yr

- Red: Apoptotic Neurodegeneration
- Green: Excitotoxic Neurodegeneration
- Purple: No Neurodegeneration
Experimental Design

PHASE 1
Determine Sensitive Stage

PHASE 2
Define Duration Response

PHASE 3
Determine Reversibility

PHASE 4
Bio-imaging and Omics
In Vivo Monitoring

Pulse oximetry:
- Heart rate
- Oxygen saturation

Body temperature
- Blood pressure
- Blood glucose
- Hematocrit

Capnography:
- Respiratory rate
- Expired CO$_2$

Blood gas values

Plasma Ketamine Concentrations
(Samples taken at 2-4 hr intervals)
Ketamine plasma levels in the monkey

Slikker et al., 2007
The percentage of hemoglobin (Hb) which is saturated with oxygen is not affected by ketamine in pregnant and in infants monkeys.
Ketamine effects in the 5 day old monkey.

Note cell death as shown by silver stain (dark cells).

Slikker et al., 2007
Effects of ketamine-induced anesthesia on the frontal cortex of the developing monkey

Slikker et al., 2007
National Center for Toxicological Research (NCTR)
Operant Test Battery (OTB) Assessments

• Learning
• Motivation
• Color and Position Discrimination
• Short-term Memory
Early postnatal ketamine anesthesia and long lasting cognitive deficits in rhesus monkeys

- 24-hr iv ketamine anesthesia on PND 5 or 6.
- Wean at 6 months of age.
- Begin OTB behavioral assessments at 7 months of age: daily 50 min sessions (M-F).
- Monitor for at least two years (currently at >1500 sessions, >300 weeks/80 months (>6 years) of testing; animals now >7 years old).
National Center for Toxicological Research (NCTR) Operant Test Battery (OTB) Assessments

- Learning [Incremental Repeated Acquisition (IRA) Task]
- Motivation [Progressive Ratio (PR) Task]
- Color and Position Discrimination [Conditioned Position Responding (CPR) Task]
- Short-term Memory [Delayed Matching-To-Sample (DMTS) Task]
Learning Task
Oxidative Mechanisms of Neurotoxicity: Modes of Neuroprotection

- **Antioxidants—*In Vitro***
  - Superoxide Dismutase mimetic, M40403 (Wang et al. 2003)
  - 7-Nitroindazole, NOS inhibitor (Wang et al. 2008)

- **Antioxidants—*In Vivo***
  - Melatonin (Jevtovic-Todorovic and Reiter, 2004)
  - Pramipexole (restores mitochondrial integrity) (Boscolo et al. 2012)
  - L-Carnitine (mitochondrial protection)
Preventative/ameliorative agents/strategies

- L-carnitine
- Nicotinamide
- Melatonin
- Beta-estradiol
- Clonidine
- H₂ gas
- Erythropoietin
- Vitamins C/D₃
- Preconditioning
- Hypothermia
- Env. Enrichment
- 7-nitroindazole
- Lithium
- Dexmedetomidine
- Pramipexole
- Xenon
- Cannabinoid1R
- Roscovitine
- Ca channel blockers
Innovative Science to Improve Public Health

Developmental *In Vivo* Rat Model Postnatal Day 7

- **Inhaled Anesthetic Study**
  - Isoflurane (ISO): 0.55%
  - Nitrous Oxide (N₂O): 75%
  - Combination
    - without L-Carnitine
    - with L-Carnitine
Nitrous Oxide (N\textsubscript{2}O) and Isoflurane (ISO) anesthesia in the 7 day old rat

Zou et al. Neuroscience, 2008
The number of Caspase-3 neuronal profiles

Zou et al. Neuroscience, 2008
The number of silver grains

Control 2h 4h 6h 8h

The Number of Silver Grains

Zou et al. Neuroscience, 2008
A

Control

B

N₂O+ISO(6h)

C

N₂O+ISO(6h)+L-Cn

Caspase-3 Positive Neuronal Profiles

L-Carnitine 0 to 500 mg/kg, sc

Zou et al. Neuroscience, 2008
Bio-Imaging at NCTR/FDA

MicroPET
23 cm bore

Biospec MRI
7 Tesla, 30 cm bore
Developmental exposure to ketamine in the rat

- PND 7: Single episode of anesthesia with ketamine, rat pups in the experimental group were exposed to 6 subcutaneous injections of ketamine (20 mg/kg) and control rat pups received 6 injections of saline.

- MicroPET scan with
  - $[^{18}\text{F}]$-Annexin V: (Zhang et al., Tox Sci, 2009)
microPET images from a ketamine-treated rat using the specific tracer $^{18}$F-Annexin V

$[^{18}F]$-Annexin V

Apoptosis

Externalization of phosphatidylserine
MicroPET Images of Rat Brain after $[^{18}\text{F}]$ Annexin V administration

- **Control**
- **Ketamine**

Images at:
- 5 min
- 10 min
- 15 min
- 20 min
- 25 min
- 30 min
- 35 min
- 40 min
Dynamic Uptake of [18F]-AnnexinV

SUV Ratio vs Time (m)

Control
Ketamine

Evidence of neuroapoptosis in vivo without neurohistology
Developmental exposure to ketamine in the rat

- PND 7: Single episode of anesthesia with ketamine, rat pups in the experimental group were exposed to 6 subcutaneous injections of ketamine (20 mg/kg) and control rat pups received 6 injections of saline.

- microPET scan with
  - $[^{18}F]$-Annexin V on PND 35: Zhang et al., Tox Sci, 2009

  - $[^{18}F]$-FEPPA: time course study (ongoing experiment).
    - PND 14: n=4
    - PND 21: n=4
    - PND 28: n=4
    - PND 35: n=7
FEPPA interacts with peripheral benzodiazepine receptors and used as a marker of glial activation in response to neuronal damage and inflammation.
microPET images from a ISO/N2O NHP using the specific tracer $^{18}$F-FEPPA

**Astrocyte**

**Resting Microglia**

**Mitochondria**

$^{18}$F-FEPPA

Translocator protein/ Peripheral Benzodiazepine Receptor (PBR)

Outer Mitochondrial Membrane

Inner Mitochondrial Membrane
Dynamic Uptake of 18F-FEPPA

PND 14

7 days after exposure to ketamine
Dynamic Uptake of 18F-FEPPA

14 days after exposure to ketamine

PND 21
Dynamic Uptake of 18F-FEPPA

PND 28
21 days after exposure to ketamine

SUV

Time (min)

0.07
0.06
0.05
0.04
0.03
0.02
0.01

0 30 40 50 60 70 80 90 100 110 120

Contral
Ketamine
Dynamic Uptake of 18F-FEPPA

28 days after exposure to ketamine

PND 35

Time course data from the same animals

Innovative Science to Improve Public Health
Physiological Parameters for Infant Monkeys Exposed to Inhaled Gaseous Anesthetics: 1% Isoflurane (ISO) and/or 70% Nitrous Oxide (N$_2$O) for 8 hrs.

<table>
<thead>
<tr>
<th>PND 5/6 Monkeys</th>
<th>Control</th>
<th>ISO</th>
<th>N$_2$O</th>
<th>ISO+N$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory rate (breaths/min)</td>
<td>63±4.8</td>
<td>66±3.1</td>
<td>73±5.5</td>
<td>60±12.5</td>
</tr>
<tr>
<td>Heart rate (beats per min)</td>
<td>219±24.1</td>
<td>186±44.6</td>
<td>213±24.7</td>
<td>188±28.5</td>
</tr>
<tr>
<td>O$_2$ saturation (%)</td>
<td>95±2.5</td>
<td>91±1.8</td>
<td>95±3.7</td>
<td>94±0.9</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.6±0.5</td>
<td>34.8±1.4</td>
<td>36.3±0.6</td>
<td>34.5±1.8</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>77±9.5</td>
<td>75±4.5</td>
<td>79±11.7</td>
<td>86±12.1</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>49±4.4</td>
<td>43±3.6</td>
<td>58±10.5</td>
<td>59±13.9</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>68±13.5</td>
<td>72±13.7</td>
<td>85±17.4</td>
<td>80±10.1</td>
</tr>
<tr>
<td>Venous pCO$_2$</td>
<td>45±9.2</td>
<td>60±1.0</td>
<td>49±6.9</td>
<td>54±10.6</td>
</tr>
<tr>
<td>Venous pO$_2$</td>
<td>26±15.0</td>
<td>28±4.2</td>
<td>27±5.5</td>
<td>28±4.7</td>
</tr>
<tr>
<td>Venous pH</td>
<td>7.3±0.1</td>
<td>7.3±0.02</td>
<td>7.2±0.04</td>
<td>7.3±0.08</td>
</tr>
</tbody>
</table>

Zou and Liu et al, Neurotox Teratol, 2011
Caspase 3 Immuno-staining (Frontal Cortex, Monkey)
Fluoro-Jade C Staining
(Frontal Cortex)
Effects of ISO + N₂O - induced anesthesia (1% Isoflurane (ISO) and 70% Nitrous Oxide (N₂O) for 8 hrs.) in the 5/6 day old monkey
Dynamic Uptake of $[^{18}\text{F}]-$FEPPA (PND 6 Monkeys)

A

Dorsal
Ventral
Control

B

Dorsal
Ventral
$\text{N}_2\text{O} (70\%) + \text{ISO (1.0\%)}$

C

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>SUV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>20</td>
<td>0.06</td>
</tr>
<tr>
<td>40</td>
<td>0.08</td>
</tr>
<tr>
<td>60</td>
<td>0.10</td>
</tr>
<tr>
<td>80</td>
<td>0.10</td>
</tr>
<tr>
<td>100</td>
<td>0.10</td>
</tr>
<tr>
<td>120</td>
<td>0.10</td>
</tr>
<tr>
<td>140</td>
<td>0.10</td>
</tr>
</tbody>
</table>

- **Control**
- **Treated**
Dynamic Uptake of $[^{18}\text{F}]$-FEPPA on PND 6 (Temporal Lobe)

Evidence of neuroprotection
Advantages of \textit{In vivo} Imaging in Safety Assessment

- Noninvasive/reduction in animal number
- Development – aging in same animal
- Animal serves as its own control
- Multiple studies per day in the same animal
- Anatomical and functional assessments in parallel
Disruption of membrane phospholipid integrity
~ 70 studies in rats

Primary endpoints:

Apoptosis
Long-term potentiation
Pre-pulse inhibition
Maze behaviors
Reactive oxygen species
Dendritic spine morphology and density
Social behavior
Neurogenesis
Reflex development
Mitochondrial integrity and density number and
~ 15 studies in nonhuman primates

Primary endpoints:

- Apoptosis
- Social behaviors
- Cognition/Executive function
- Glial activation/neuroinflammation
Conclusions

- The phenomenon has been observed in all species studied from round worms to zebrafish to rodents, pigs and nonhuman primates.

- There seems to be a clear dose/exposure duration response.

- All general anesthetics tested (NMDA antagonists and GABA agonists, including ethanol) with the exception of xenon—cause the effect.

- The most sensitive periods are those during rapid synaptogenesis.
Conclusions (continued)

- Resulting functional effects depend upon brain areas affected: timing of exposure dictates this.
- The functional effects observed typically occur in important cognitive domains relevant to executive function and intelligence and social behavior.
- Noted functional effects are very long-lasting if not permanent: effects seem larger as animals get older.
- Several approaches have proven ameliorative: provide mechanistic information and avenues for intervention.
Help ensure the safety of anesthetics used in infants and young children, donate today

www.SmartTots.org
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NICHDI NTP, CDER, and NCTR
The Annual Teratology Society Volleyball Games, 1982-2015
33 Years of NETworking!
Thank you for this honor and your attention.