Developmental Toxicology: Putting the Puzzle Together

Joseph Warkany Lecture

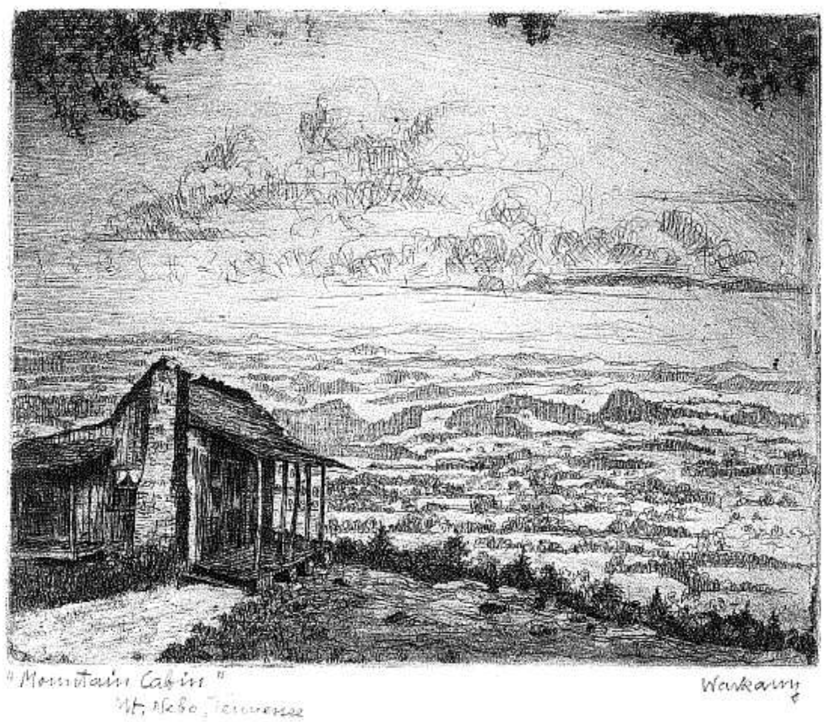
Teratology Society – June 28, 2015
Montreal, Canada

National Center for Toxicological Research/ FDA
Jefferson, Arkansas

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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Portsmouth, NH- 1980
Harriman, NY 1964
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Cedar, MI 1979
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French Lick, IN 1982
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Boca Raton, FL 1984, 1991, 1992
Puerto Rico 1994, 2009

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.

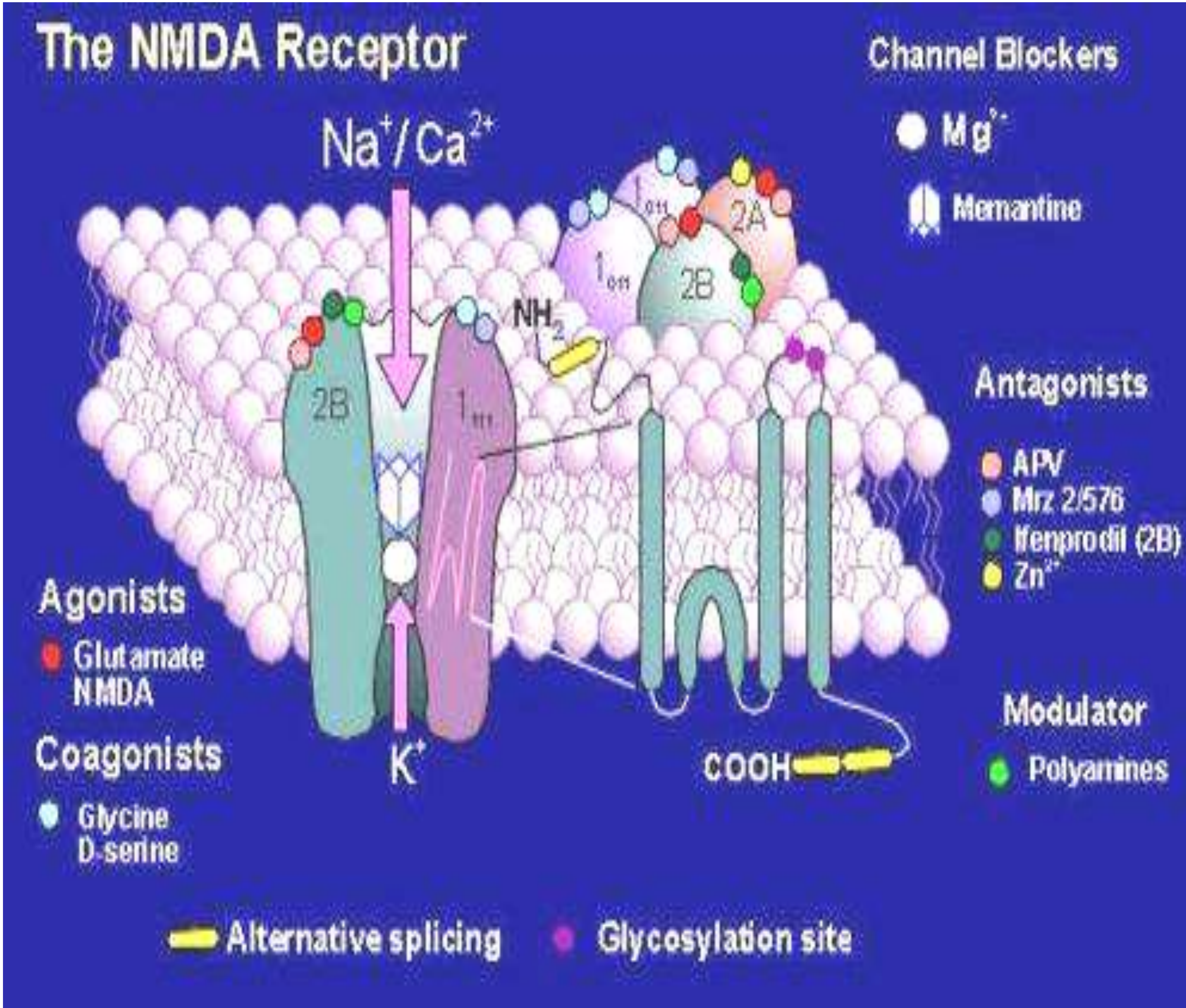
- There has been speculation that the infant brain may be more responsive to agents that affect NMDA and GABA receptor function than are adult brains.

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
Na+/Ca2+
1
2B
2A
NH2
2B
1
Antagonists
APV
Mrz 2/576
Ifenprodil (2B)
Zn2+
Agonists
Glutamate
NMDA
Coagonists
Glycine
D-serine
K+
COOH
Modulator
Polyamines
Alternative splicing
Glycosylation site

Subunits of the NMDA Receptor

NR1

- **Essential for the function of the receptor**

NR2A
NR2B
NR2C
NR2D

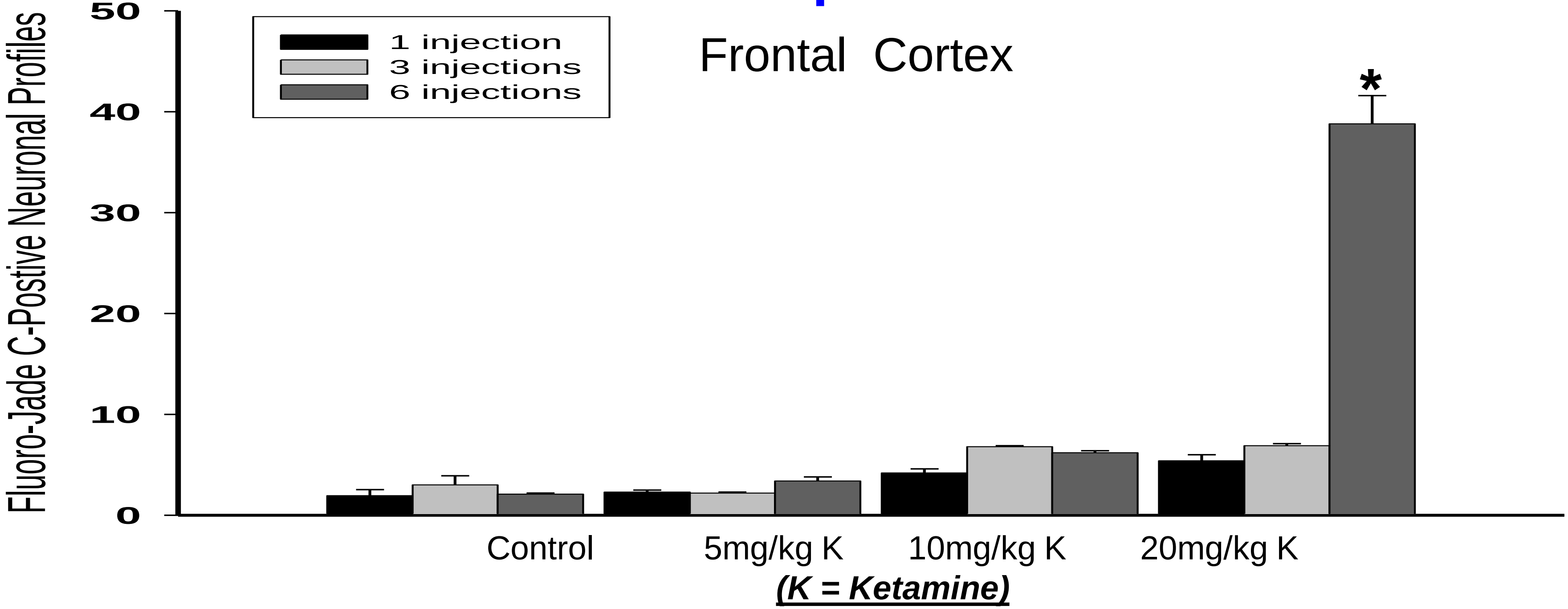
HOMOMERIC

NR1 – NR1

HETEROMERIC

NR1 – NR2A
NR1 – NR2B
NR1 – NR2C
NR1 – NR2D

Ketamine Dose Response & Time Course



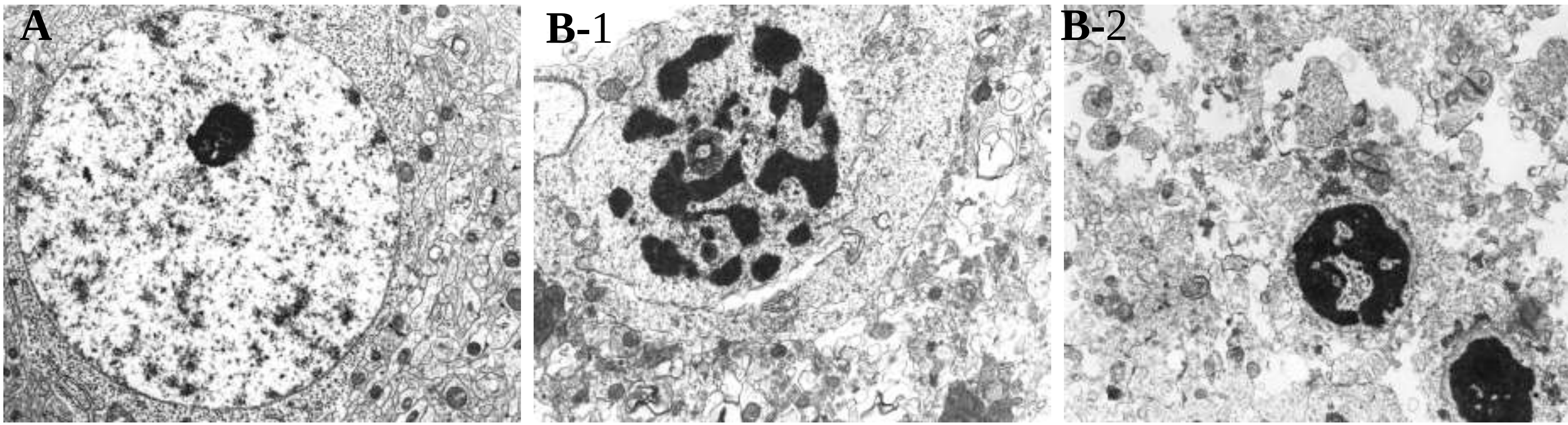
Number of Neurodegenerative Profiles in Several Rat Brain Regions (PND 7)
(20 mg/kg; 6 injections)

	Frontal Cortex	Striatum	Hippocampus	Thalamus	Amygdala
Control	4 ± 0.8	4 ± 1.2	7 ± 2.8	4 ± 0.7	2 ± 1.1
Ketamine	42 ± 3.2*	14 ± 3.2*	16 ± 4.1*	10 ± 1.0	7 ± 0.8*

Zou et al., 2009

Frontal Cortex *(PND-7 Rat Pups)*
In Vivo Exposure to Ketamine
Electron Microscopy

20 mg/kg × 6 (2 hr interval)

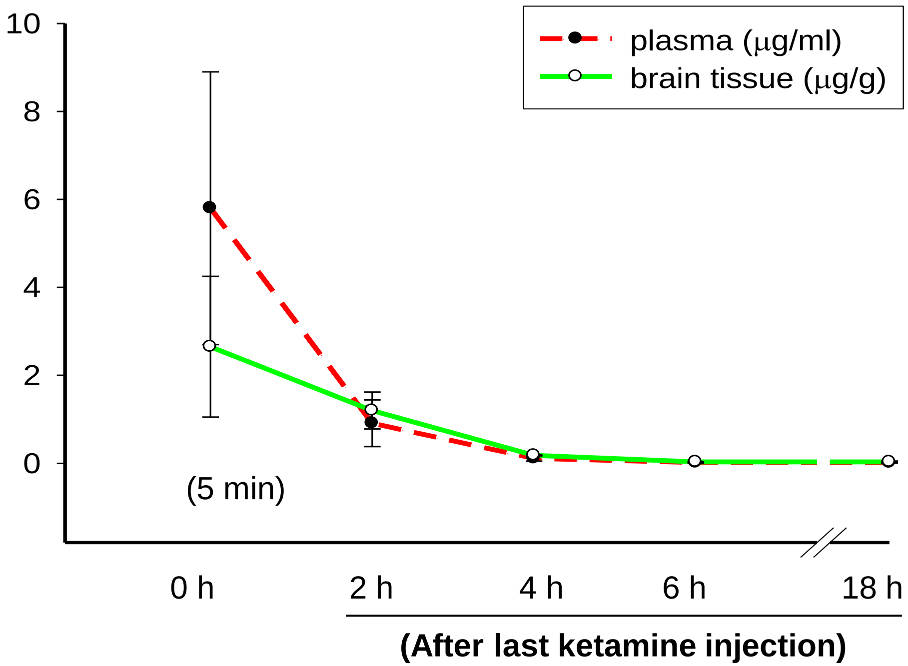


A: Control (saline): normal neuron with intact cytoplasm and nuclear membrane.

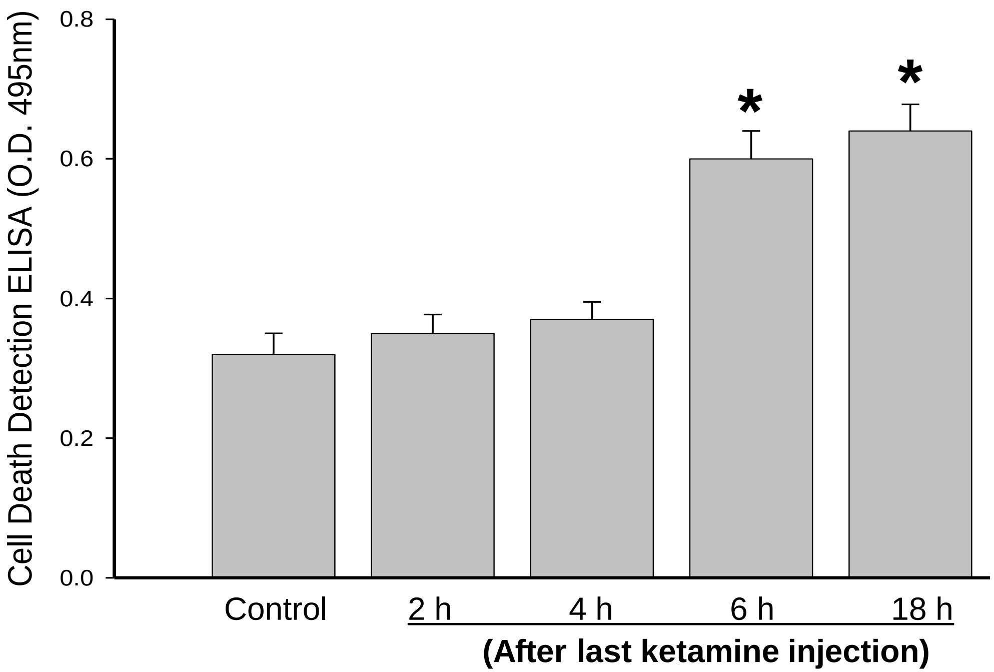
B-1**: Ketamine:** apoptotic neuron with DNA (nucleus) fragmentation.

B-2**: Ketamine:** apoptotic neurons with typical nuclear condensation.

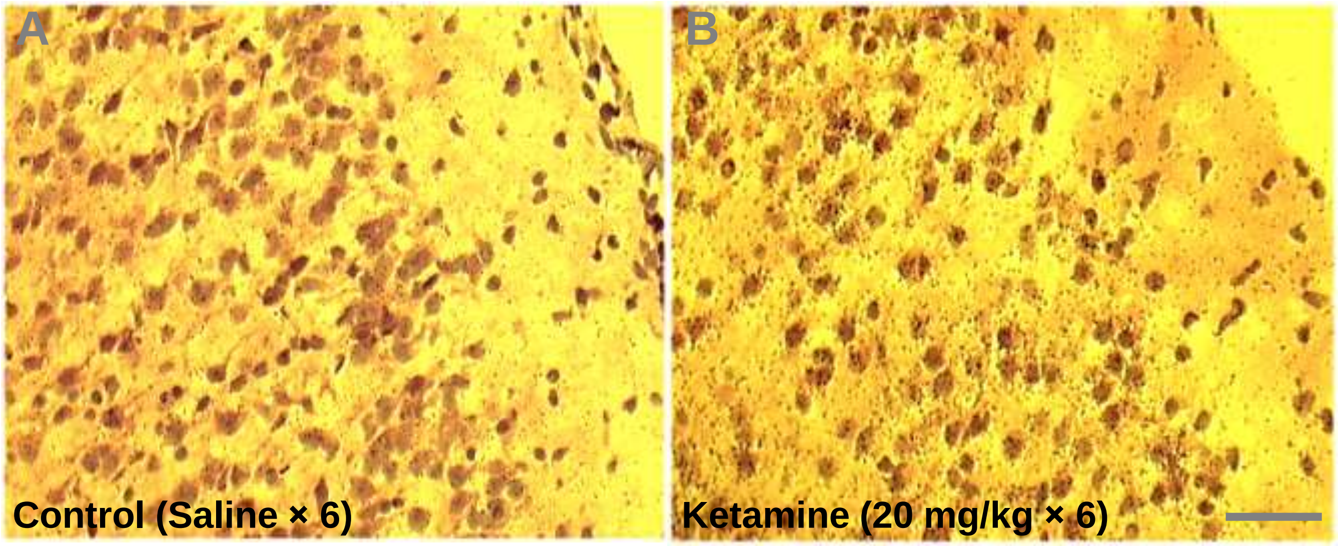
Distribution of Ketamine in the 7 day old rat pup plasma and brain

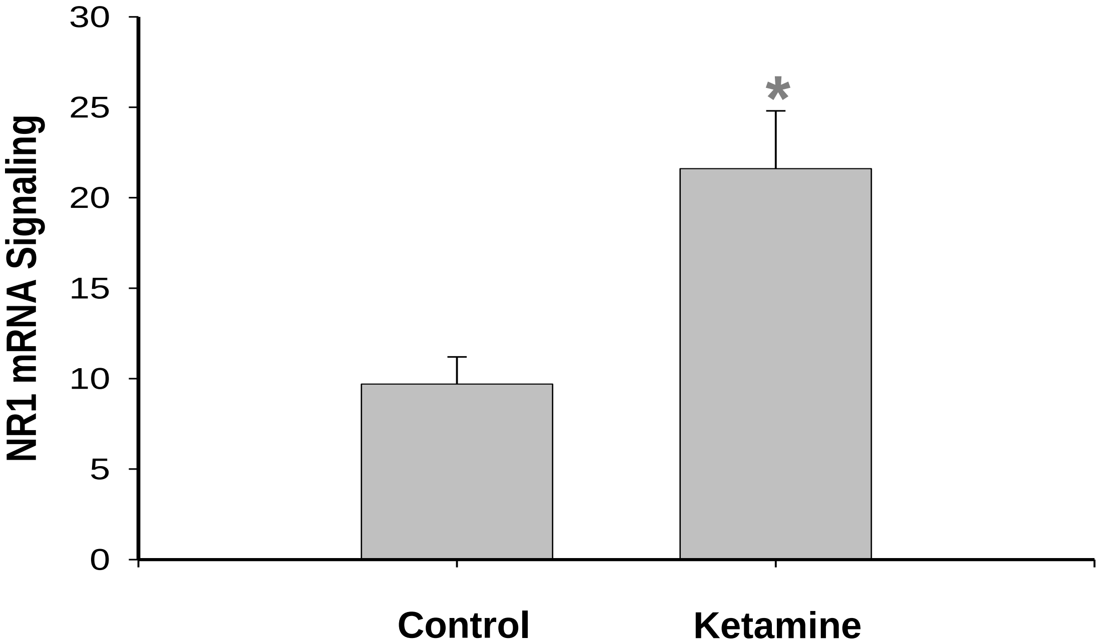


Determination of brain cell death with ELISA



Ketamine Effects on NMDA Receptor Expression





Ketamine Effects on NMDA Receptor Expression

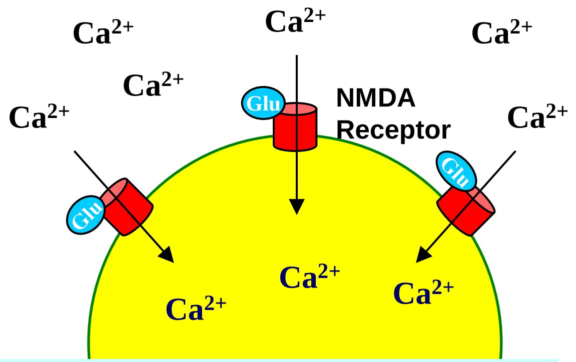
Selective validation of the microarray results by Q-PCR

Gene symbols	Fold-change (Q-PCR)	Fold-change (microarray)
Grin1 (NR1)	1.8*	1.5*
Grin2a (NR2A)	1.5*	1.2
Grin2b (NR2B)	1.0	0.9
Grin2c (NR2C)	1.7*	1.5*
Grin2d (NR2D)	1.2	1.1

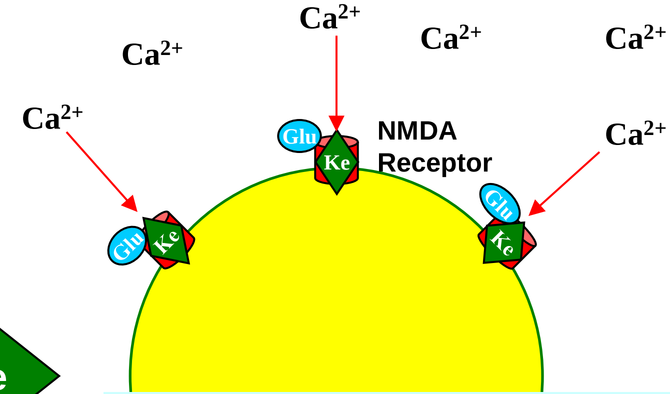
** P<0.05, as compared to the control*

Shi, Q. et al., 2010

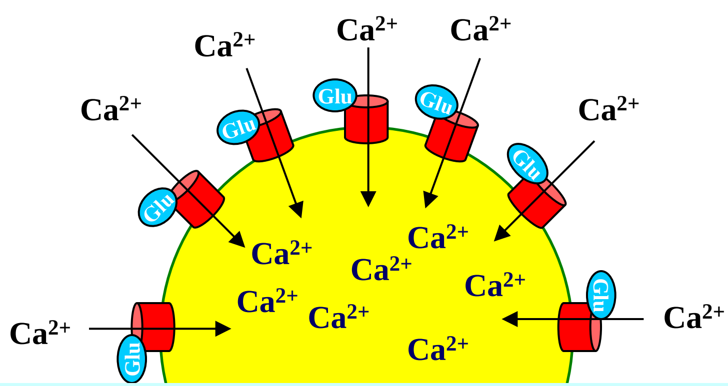
Working Model of the Effects of Ketamine Exposure on NMDA Neurons



1. Normal voltage-dependent activation of the NMDA receptor by glutamate (Glu) opens Ca^{2+} channels.



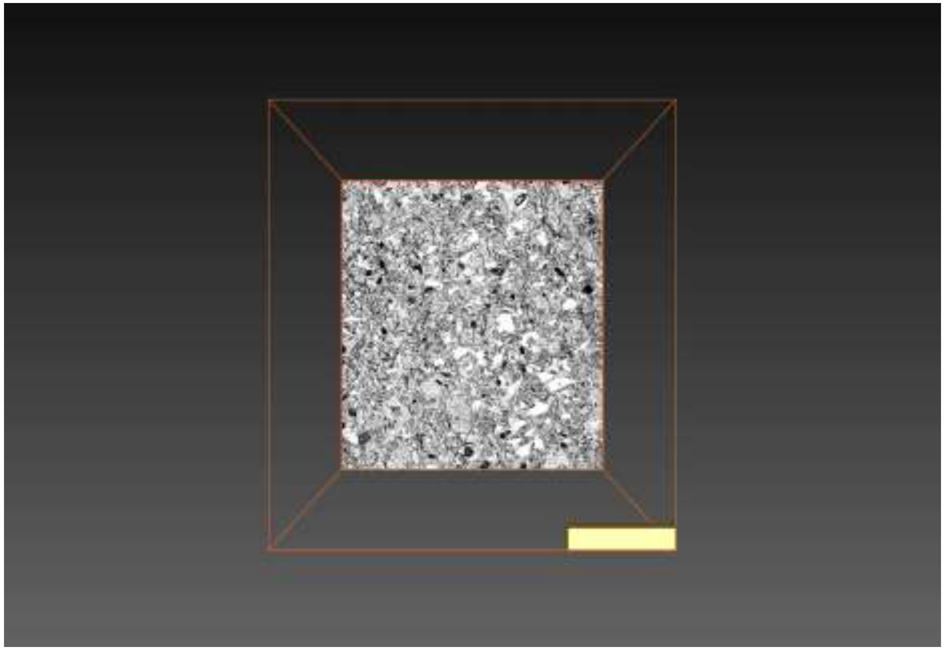
2. Non-competitive inhibition of the NMDA receptor by ketamine (Ke) blocks the channel, preventing Ca^{2+} entry into the cell.

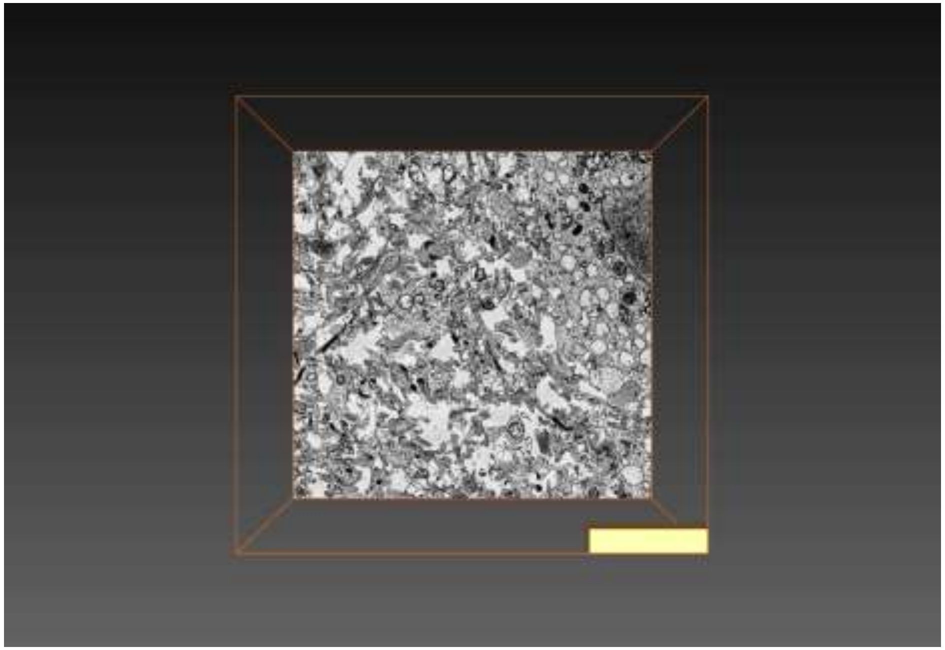


3. Compensatory upregulation of the NMDA receptor allows for the accumulation of toxic levels of intracellular Ca^{2+} under normal physiological conditions. Cell death via apoptosis or necrosis occurs.

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

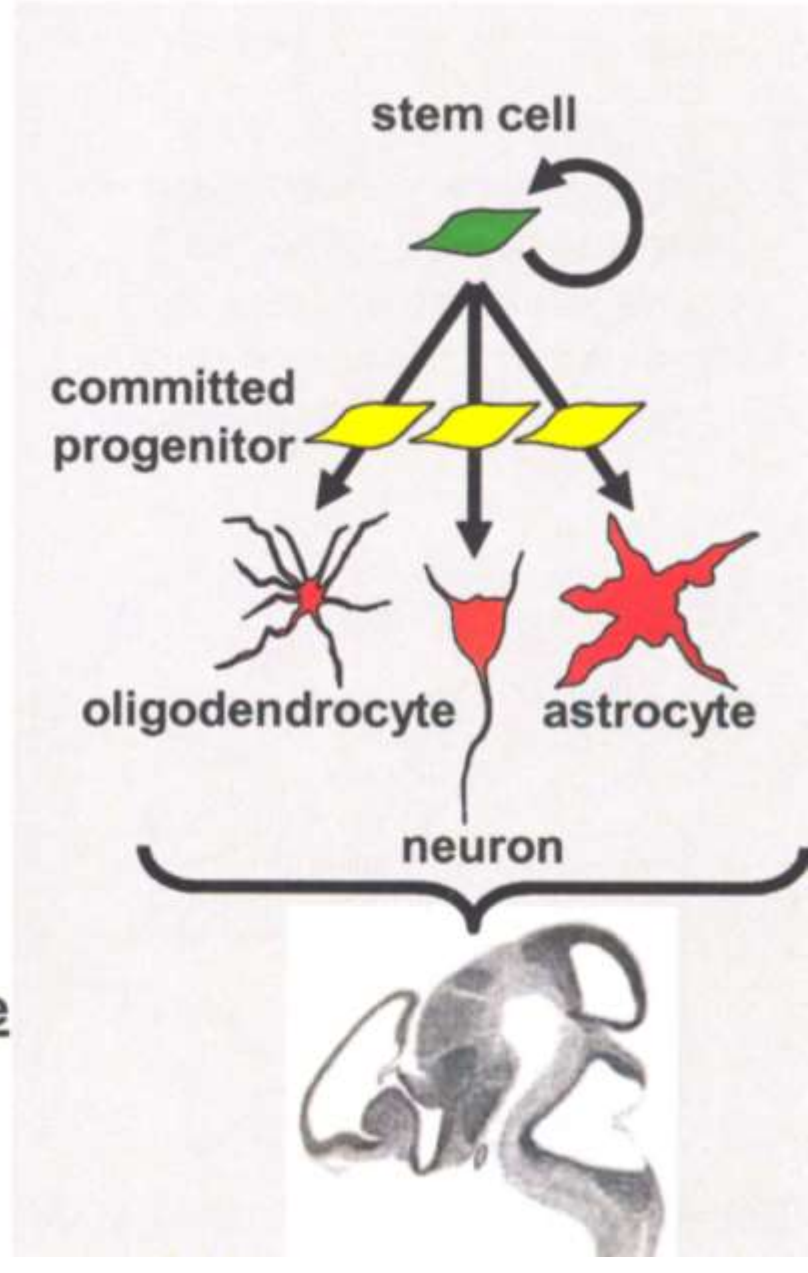
Manuscript: Using two- and three-dimensional electron microscopy techniques to quantify mitochondria defects in the developing Rat brain following Ketamine treatment (2014), *Trisha Eustaquio, Angel Paredes, Christopher Dugard, Nysia George, Fang Liu, William Slikker, Merle Paule, Paul Howard, and Cheng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

A Neural **Stem Cell** is a subclass of precursors that:

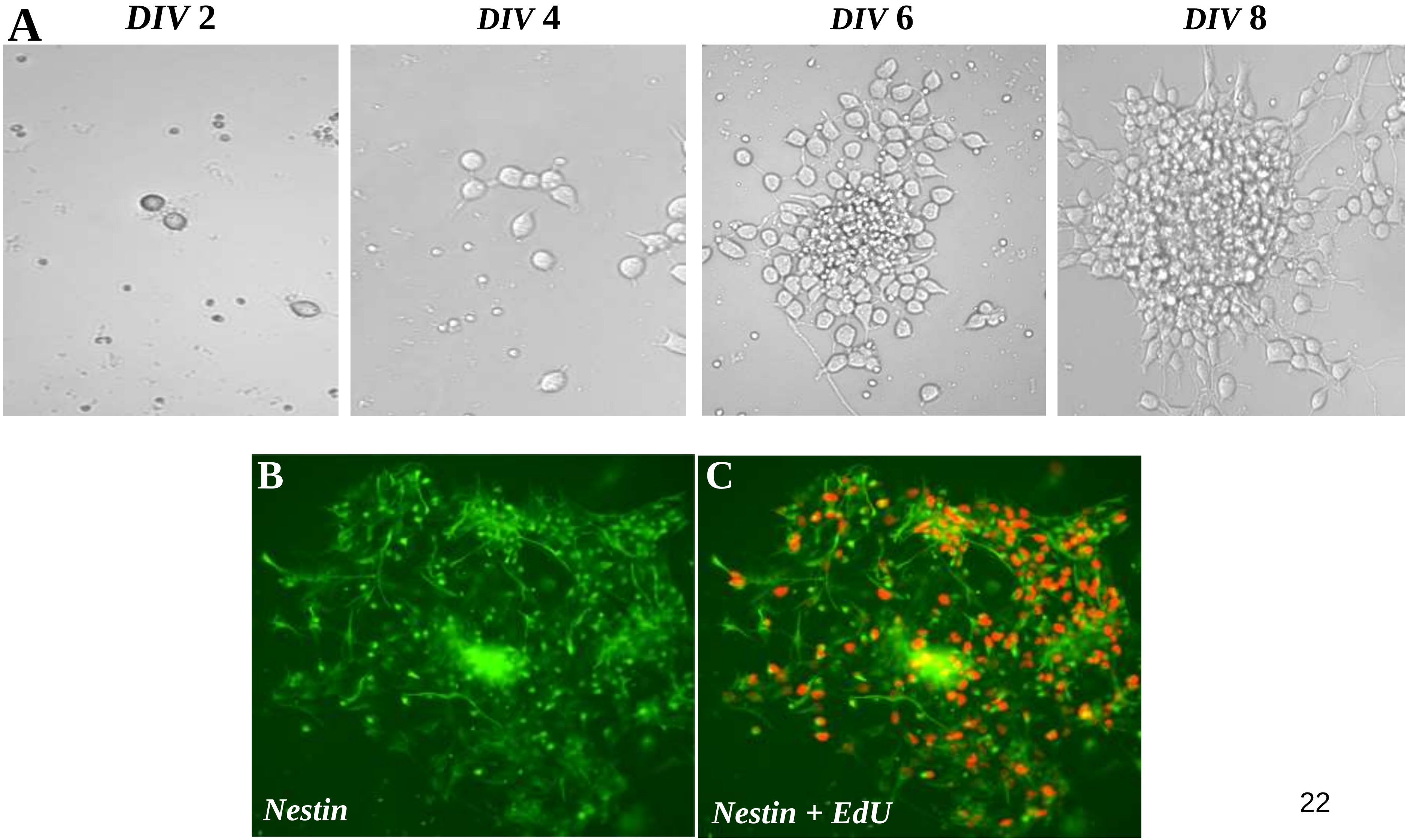
1. is **self-renewing**: capable of making additional copies of itself by division.
   a. symmetric - both daughters are stem
   b. asymmetric - one daughter is stem cell

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

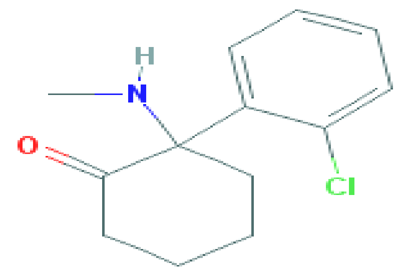


Rat Embryonic Neural Stem Cells



Anesthetics Used in Children

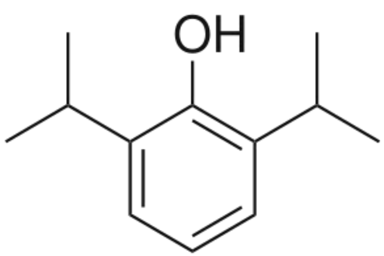
Ketamine, a non-competitive NMDA receptor antagonist, has been used as a general pediatric anesthetic for surgical procedures in infants.



Ketamine

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

Propofol is a GABA receptor agonist.

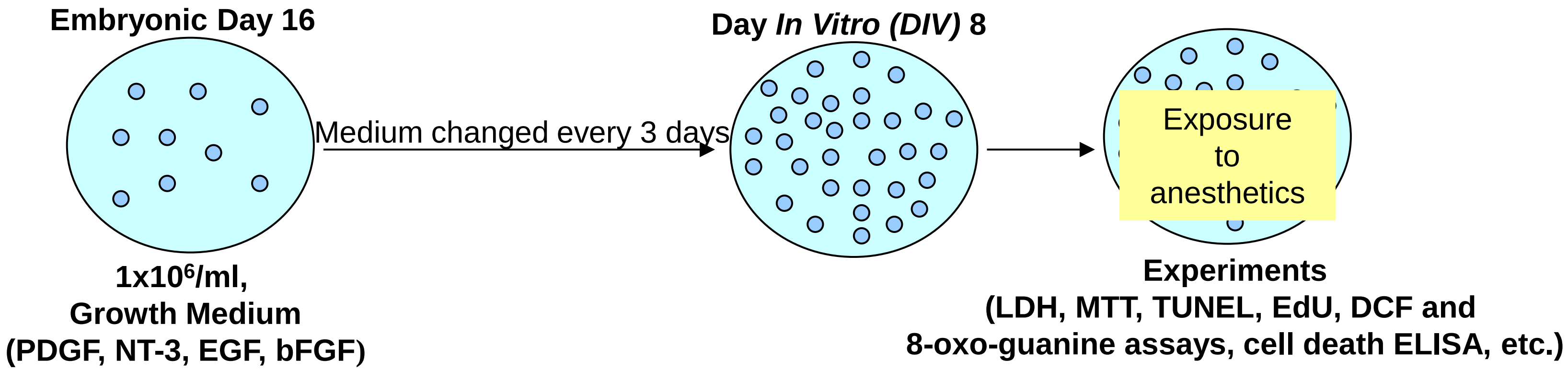


2,6-diisopropylphenol (propofol)

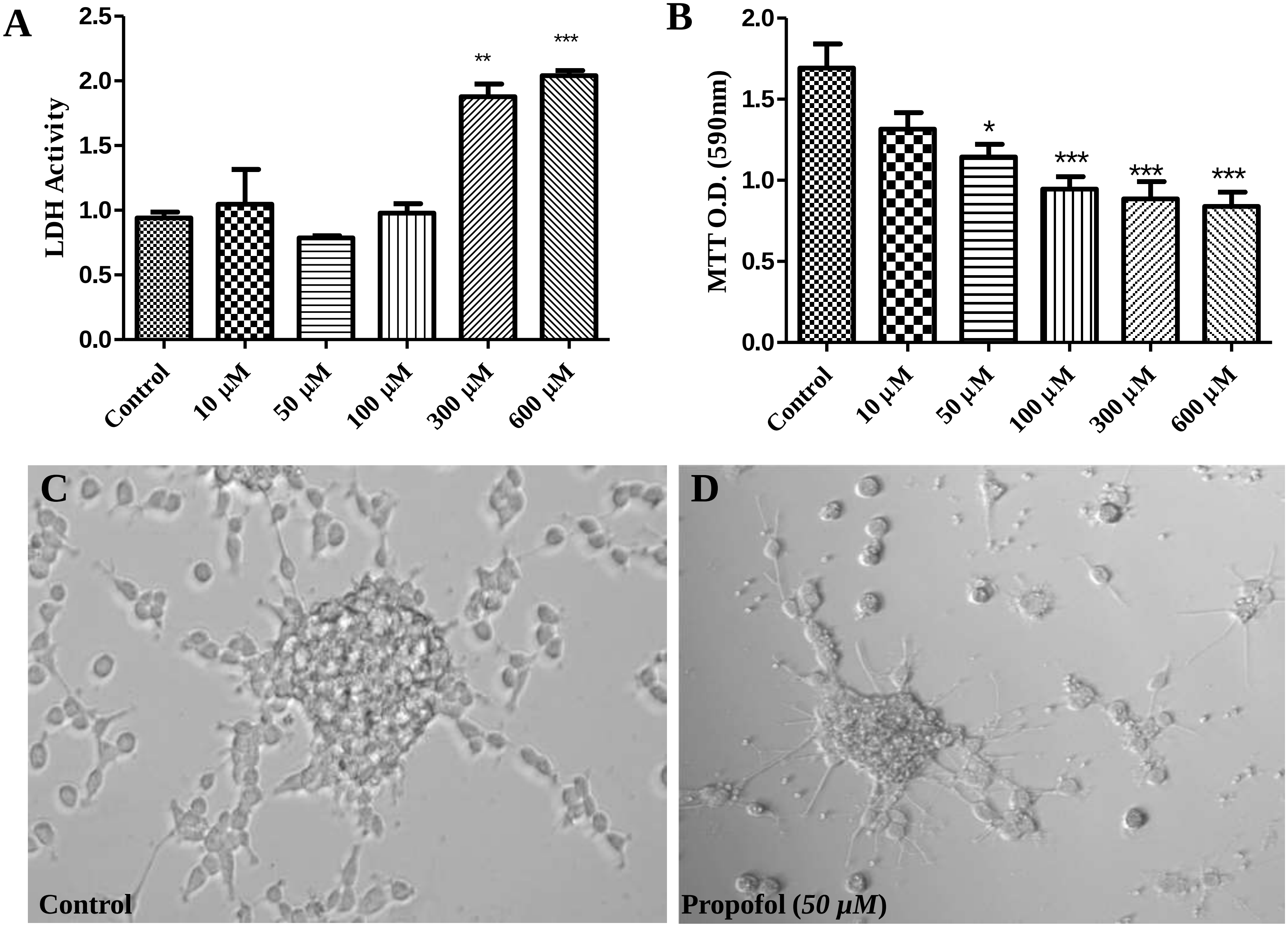
Animal model studies suggest that exposure to anesthetics during certain periods of development has long-term deleterious effects including deficits in cognitive function.

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

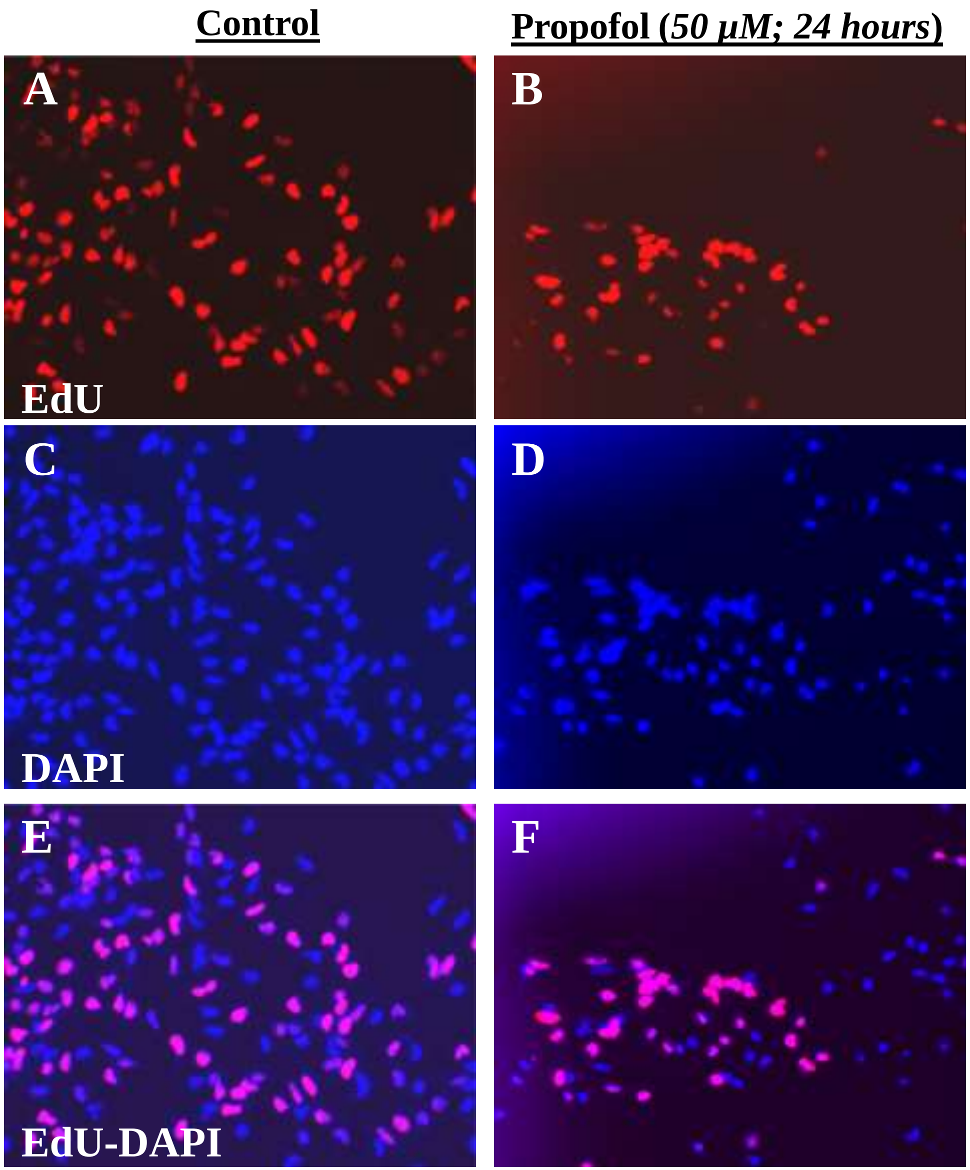
Embryonic Neural Stem Cell Culture



Effect of propofol on undifferentiated stems (24 hr. exposure)

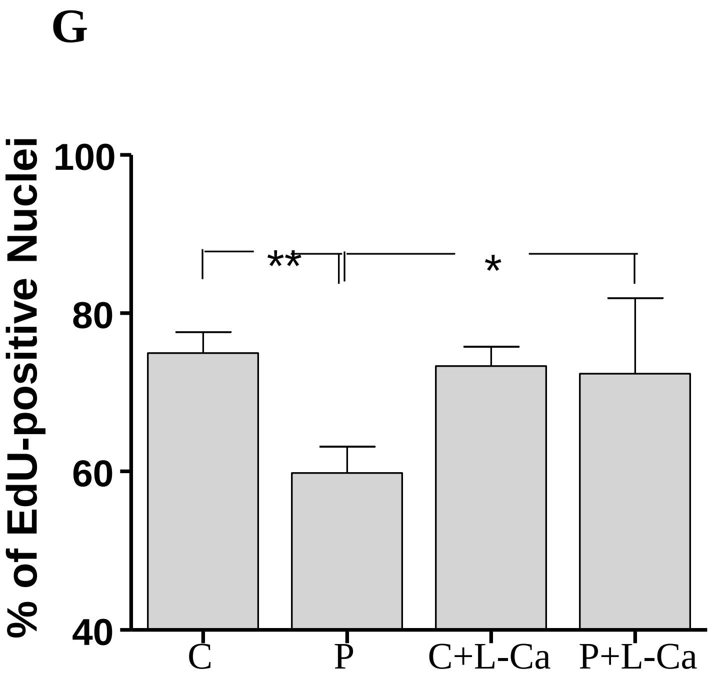


EdU-DAPI Staining



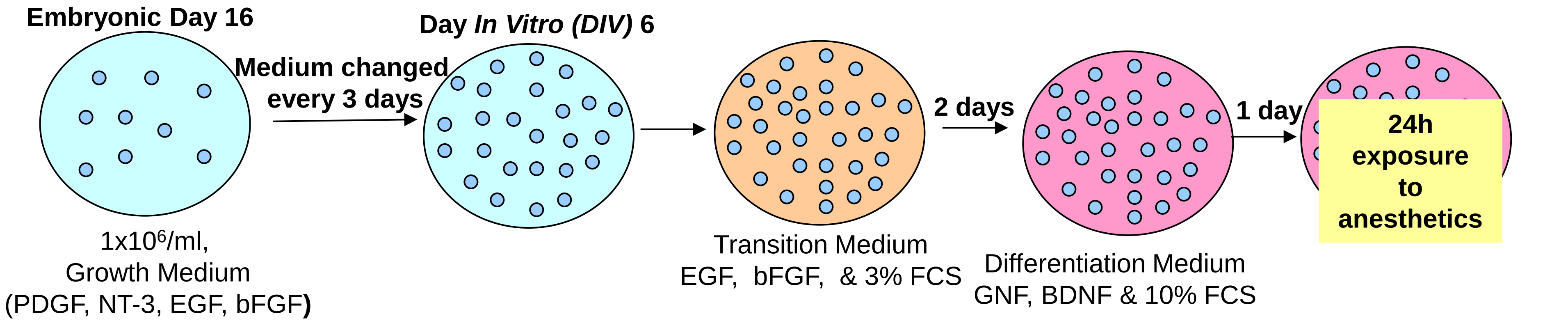
Neural Stem Cell Proliferation

(*Propofol; 24-hour Exposure*)

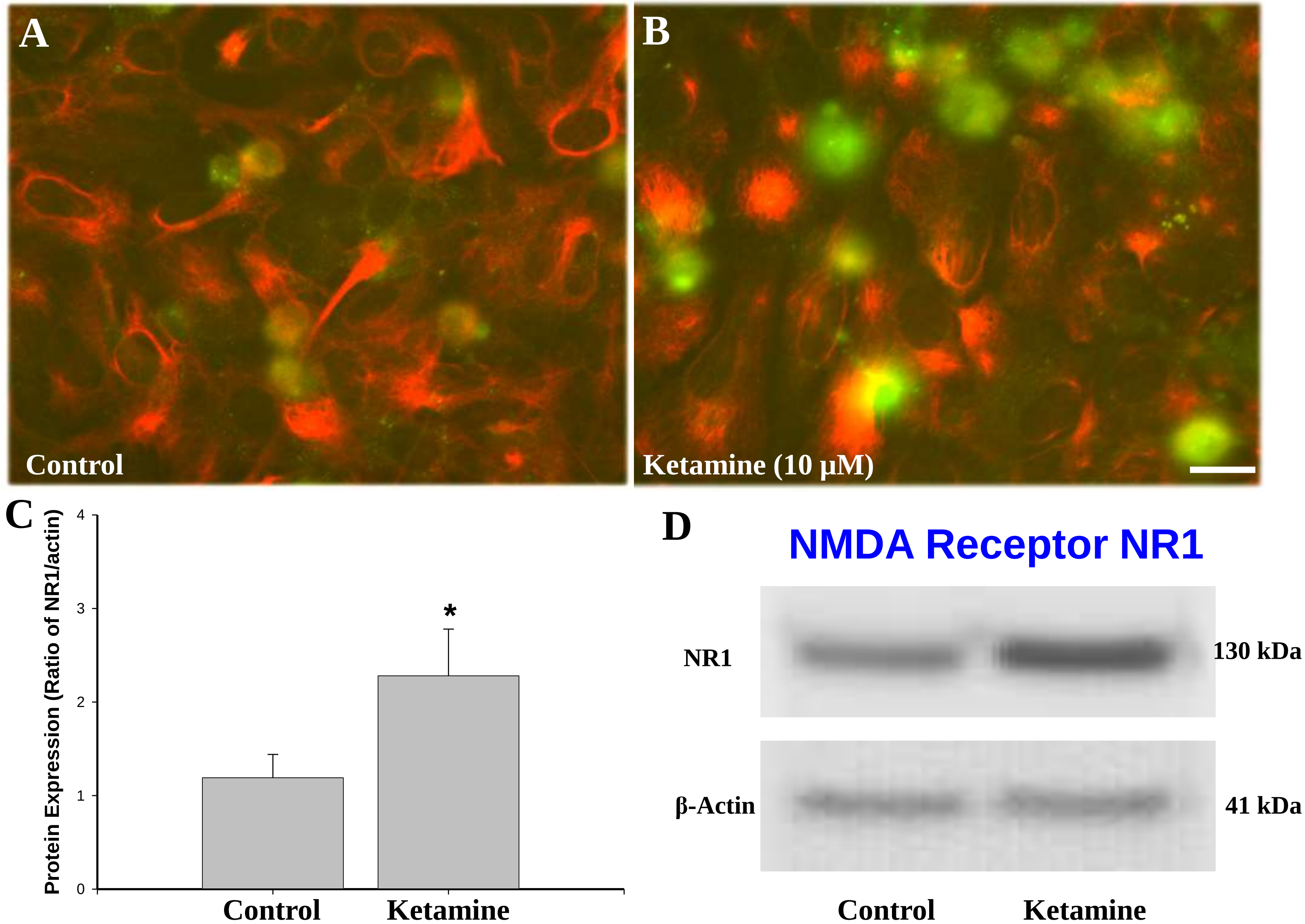


C = Control; P = Propofol (*50μM*);
L-Ca = Acetyl-L-Carnitine (*10 μM*)

Neural Stem Cell Differentiation Flow Chart



NMDA Receptor NR1(Subunit)-labeled Neurons

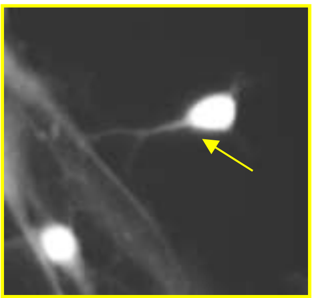


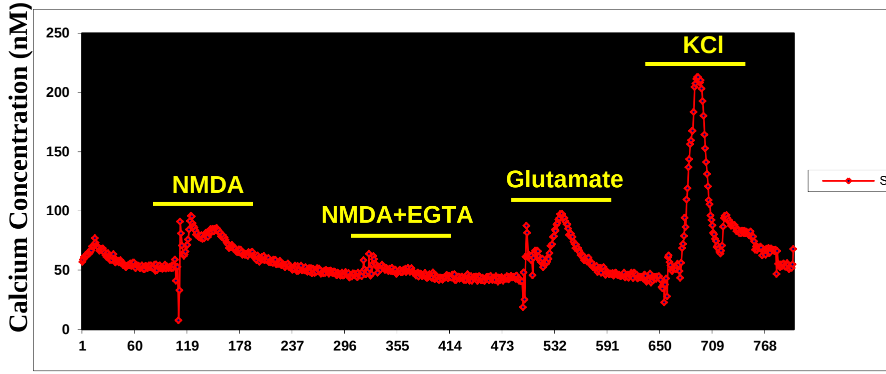
(Fang Liu et al., 2013)

Changes in *$[Ca^{2+}]i$* in Fura-2-Loaded Neurons

A representative control neuron

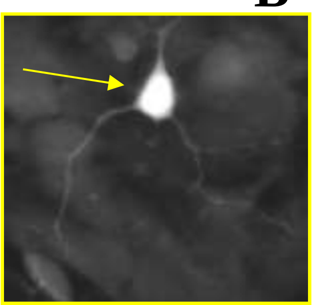
A

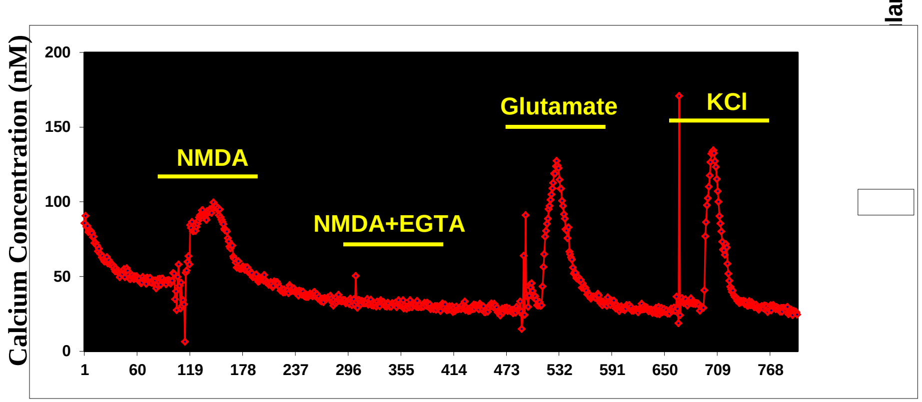




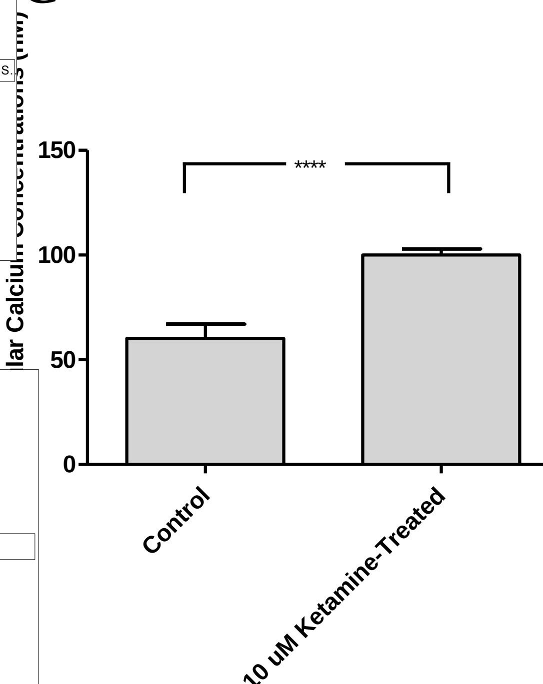
A representative ketamine-treated neuron

B





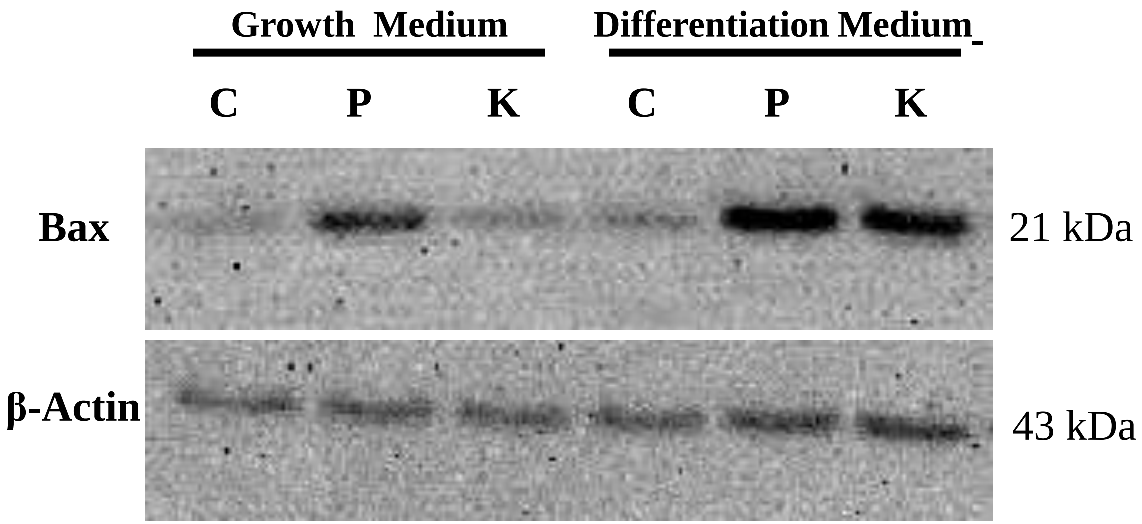
C



(Fang Liu et al., 2013)

Western Blotting Analysis

C = Control; P = Propofol; K = Ketamine



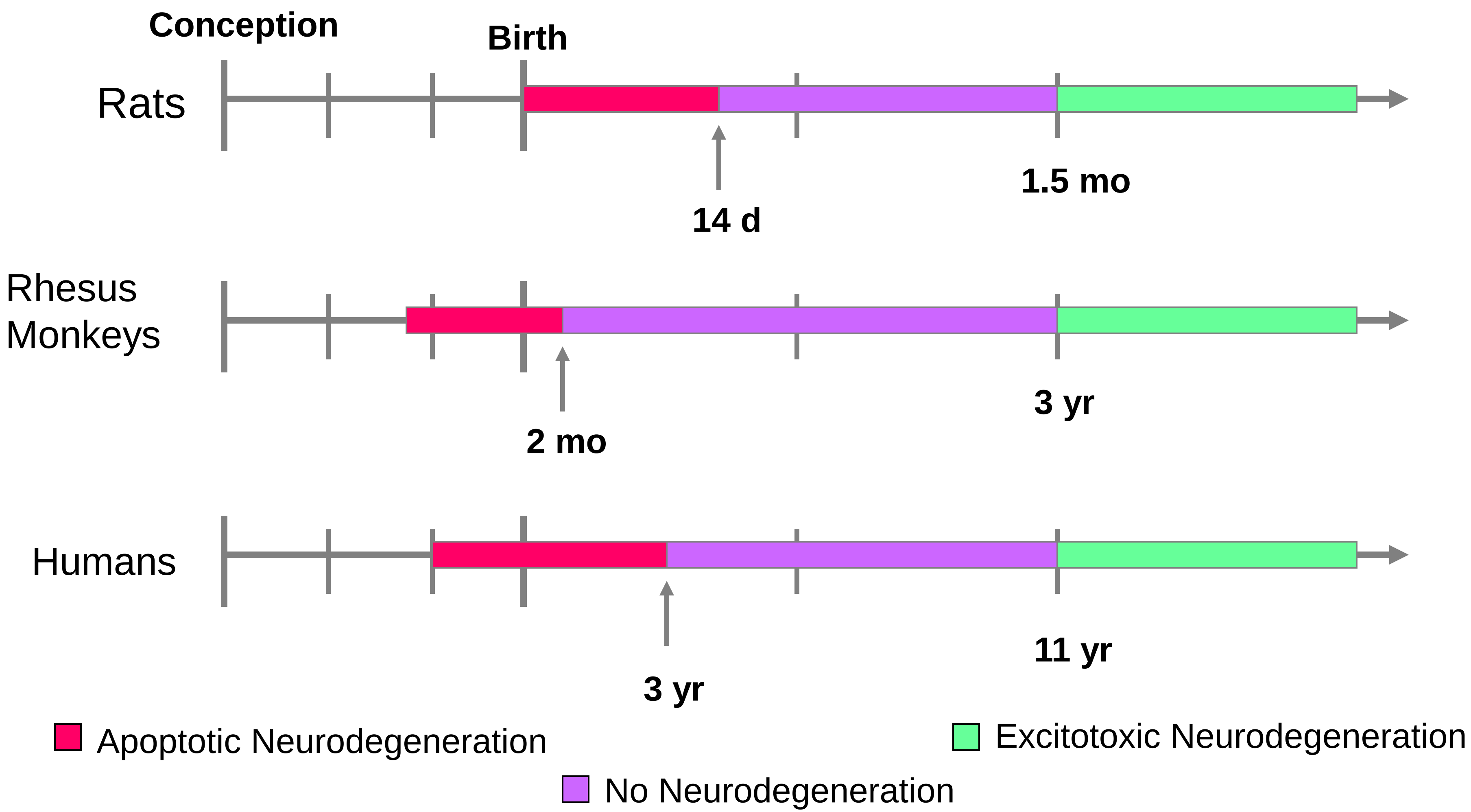
Conclusions

- 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



Experimental Design

Preliminary Experiment

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

Blood gas values

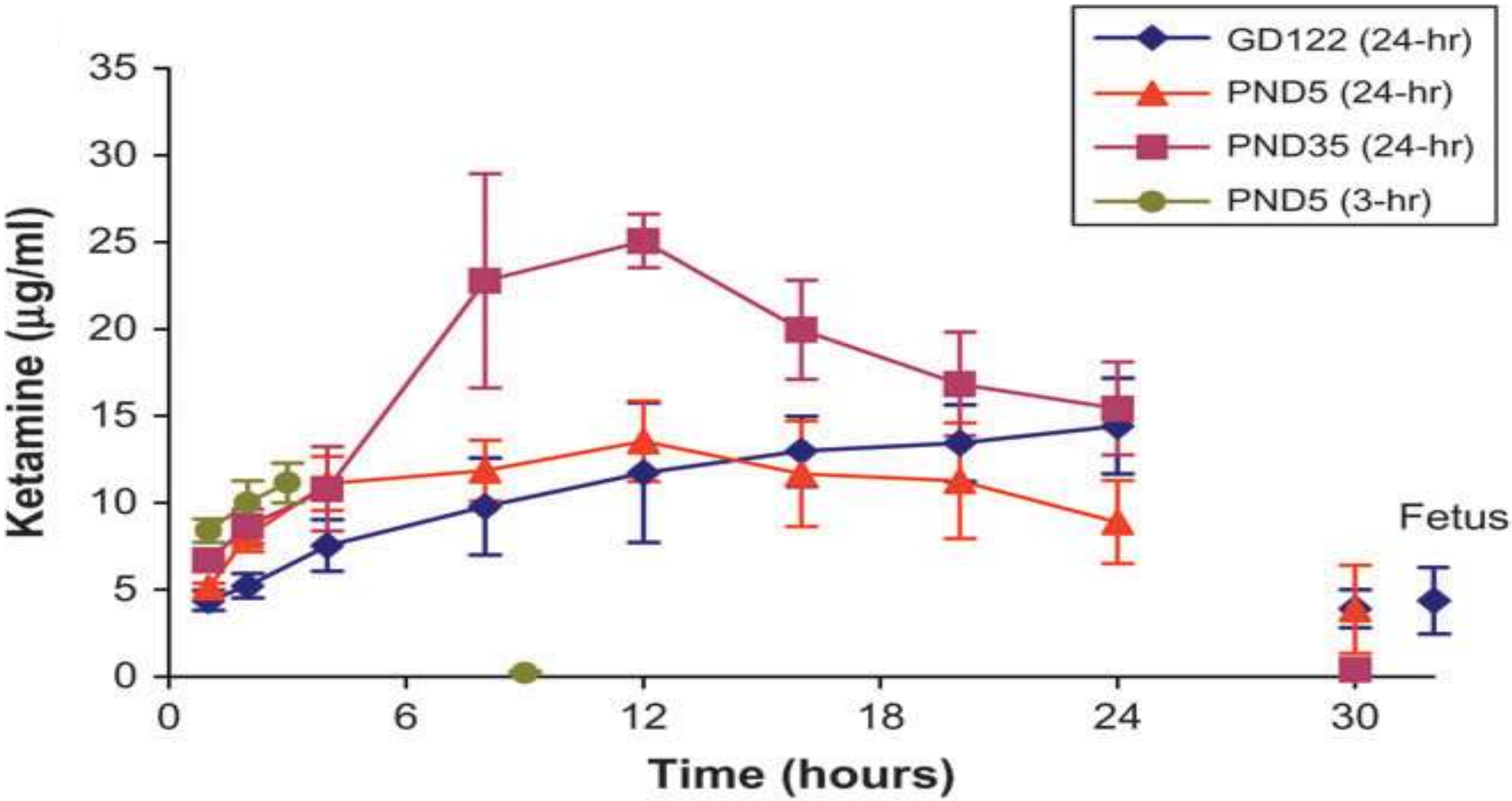
Capnography:
Respiratory rate
Expired CO_2

Plasma Ketamine Concentrations

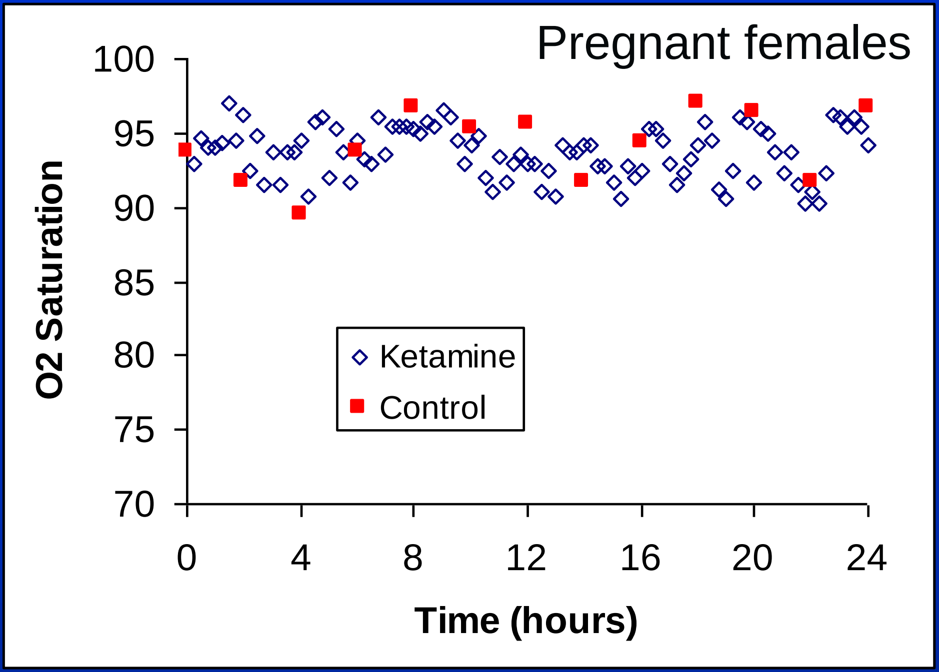
(Samples taken at 2-4 hr intervals)

Body temperature
Blood pressure
Blood glucose
Hematocrit

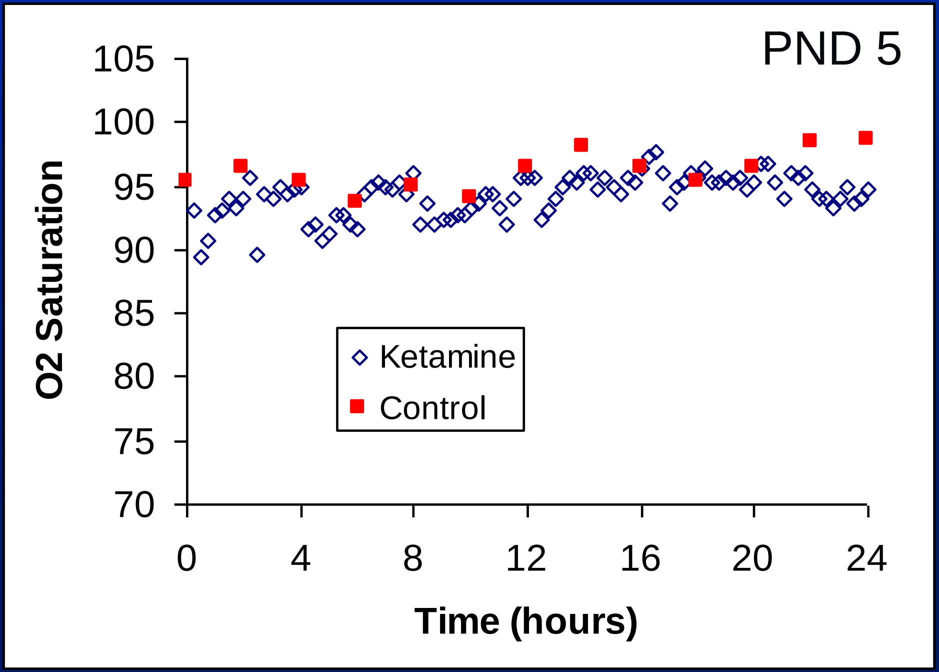
Ketamine plasma levels in the monkey

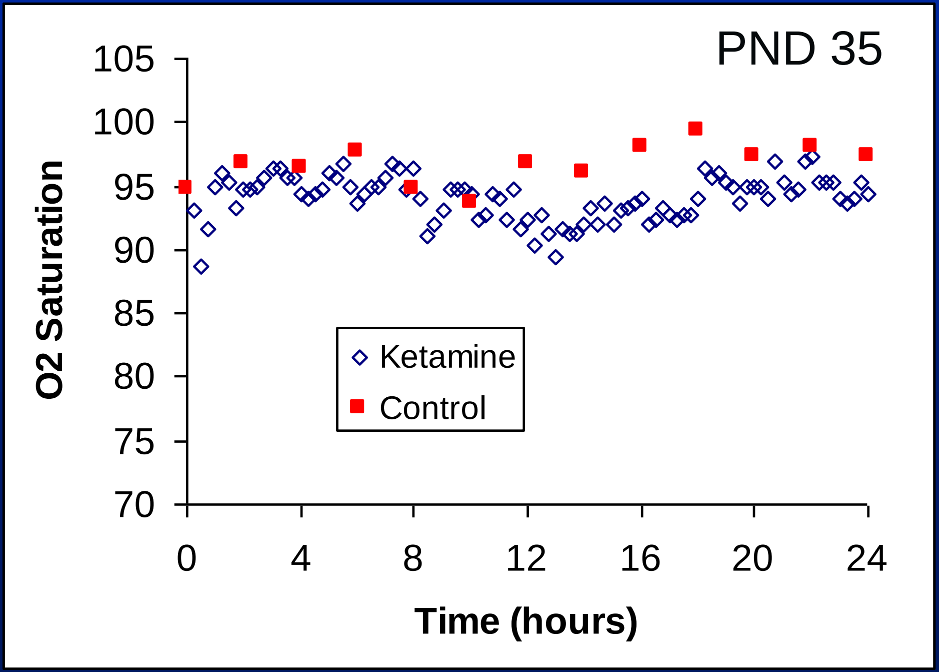


Slikker et al., 2007



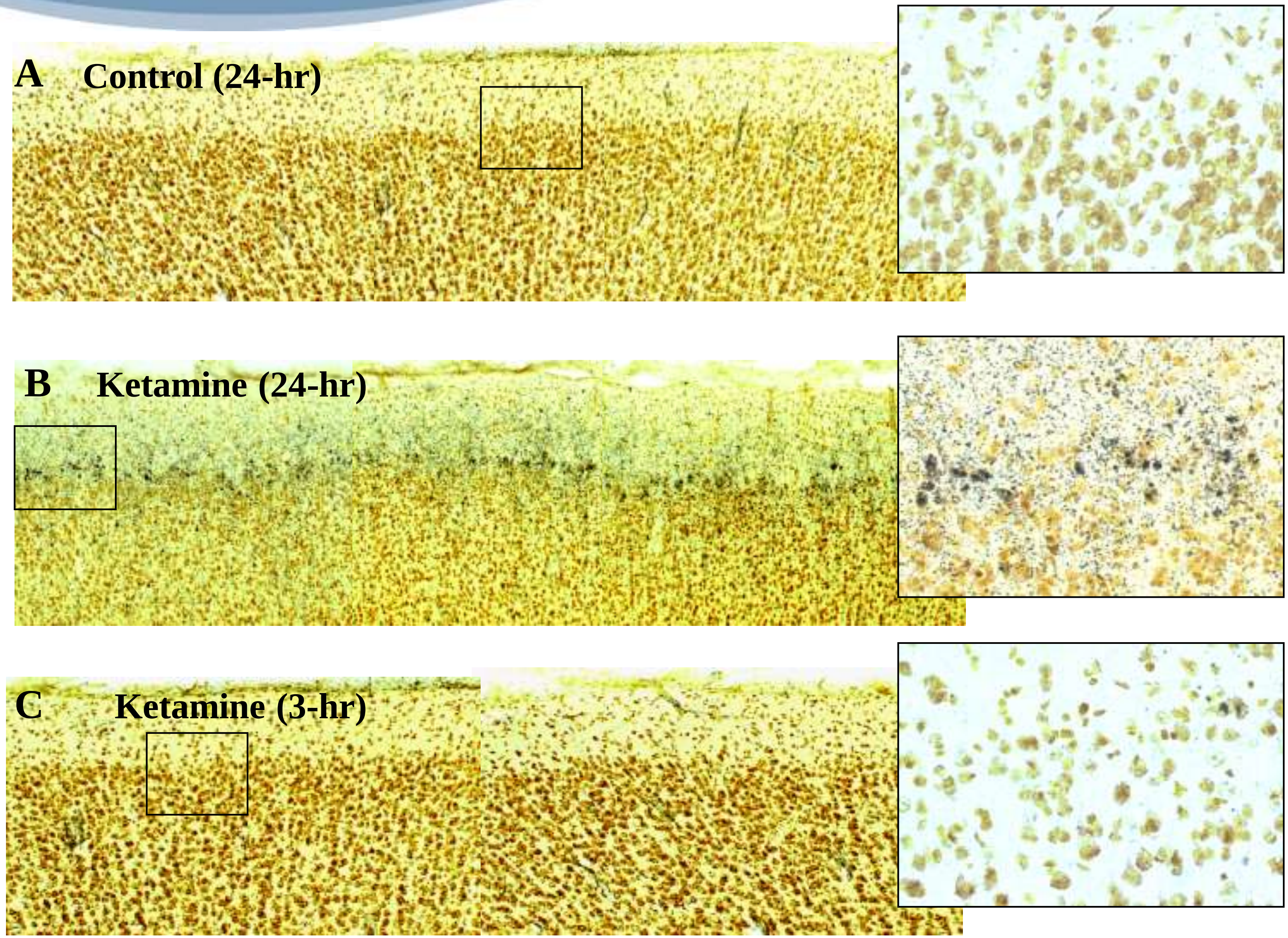
The percentage of hemoglobin (Hb) which is saturated with oxygen is not affected by ketamine in pregnant and in infants monkeys





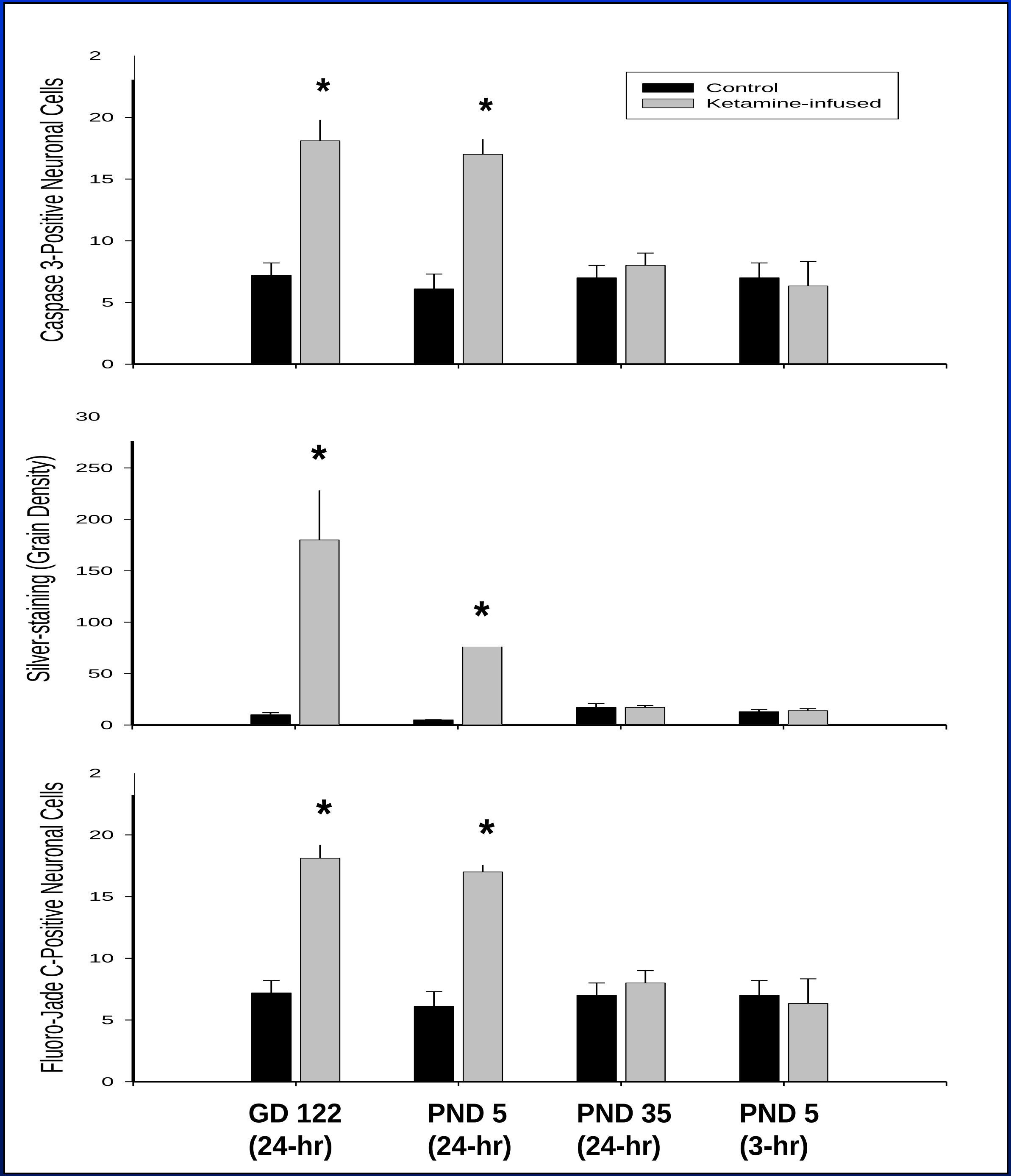
Ketamine effects in the 5 day old monkey.

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

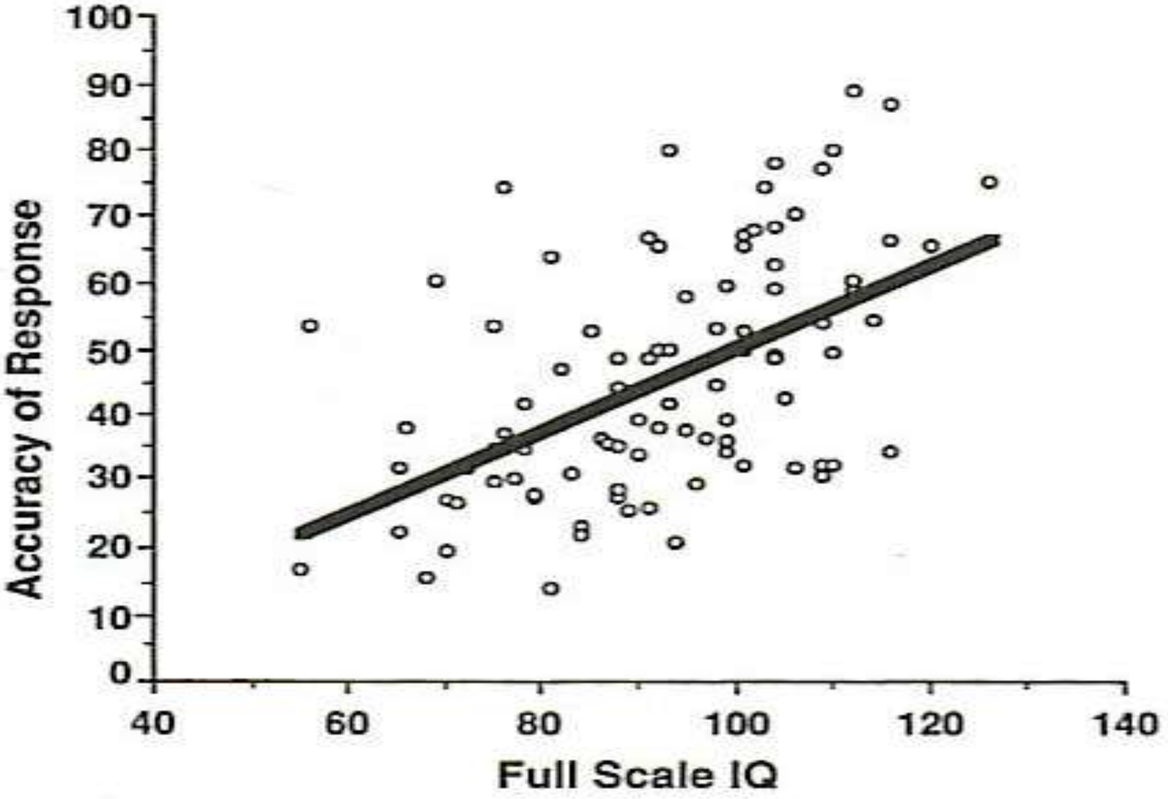
Early postnatal ketamine anesthesia and long lasting cognitive deficits in rhesus monkeys

- 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).

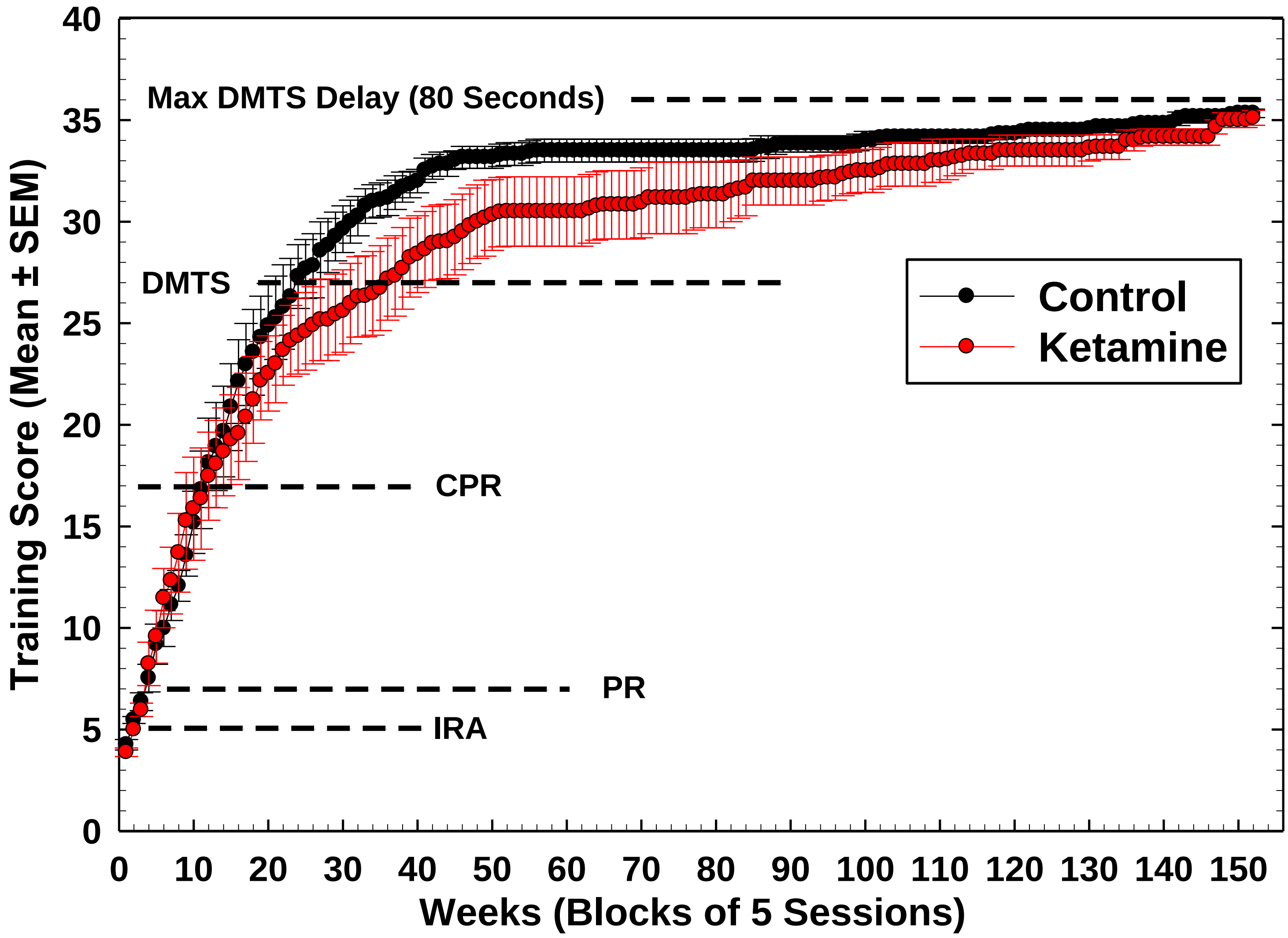
National Center for Toxicological Research (NCTR) Operant Test Battery (OTB) Assessments

- 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]

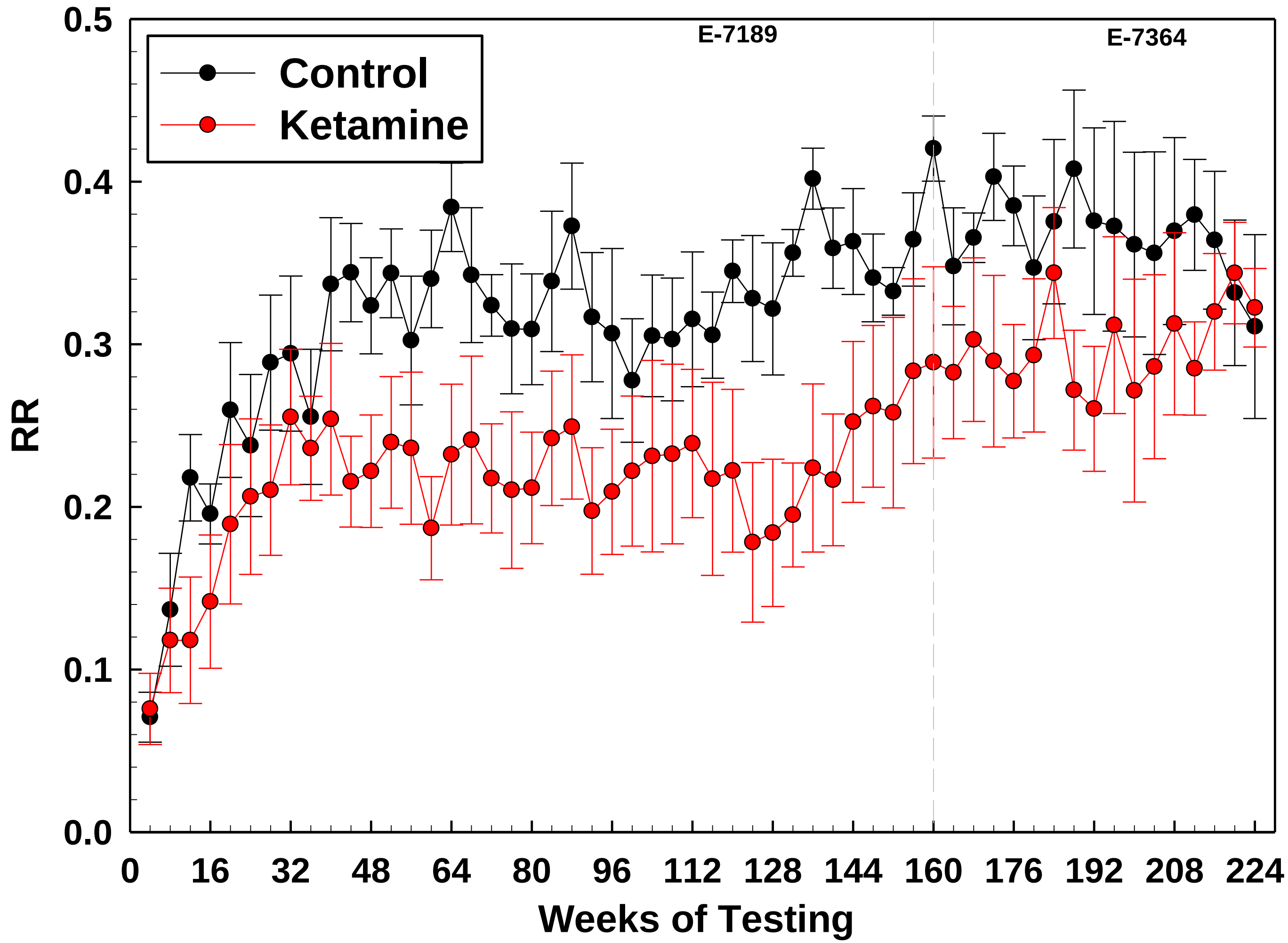
Learning Task



OTB Training Data



IRA Response Rate



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)

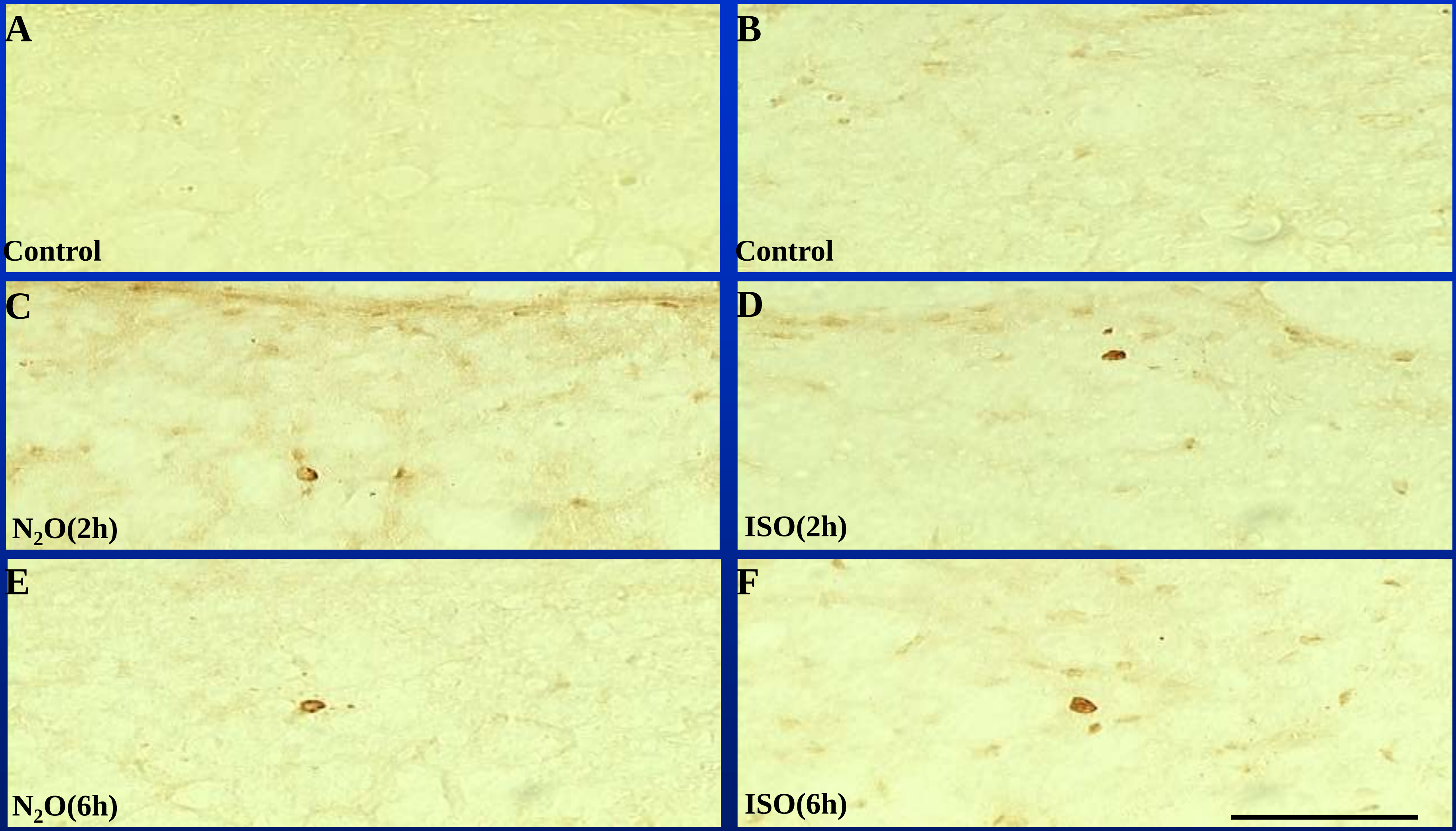
Preventative/ameliorative agents/strategies

L-carnitine	Erythropoietin	Lithium
Nicotinamide	Vitamins C/D_3	Dexmedetomidine
Melatonin	Preconditioning	Pramipexole
Beta-estradiol	Hypothermia	Xenon
Clonidine	Env. Enrichment	Cannabinoid1R
H_2 gas	7-nitroindazole	Roscovitine
	Ca channel blockers	

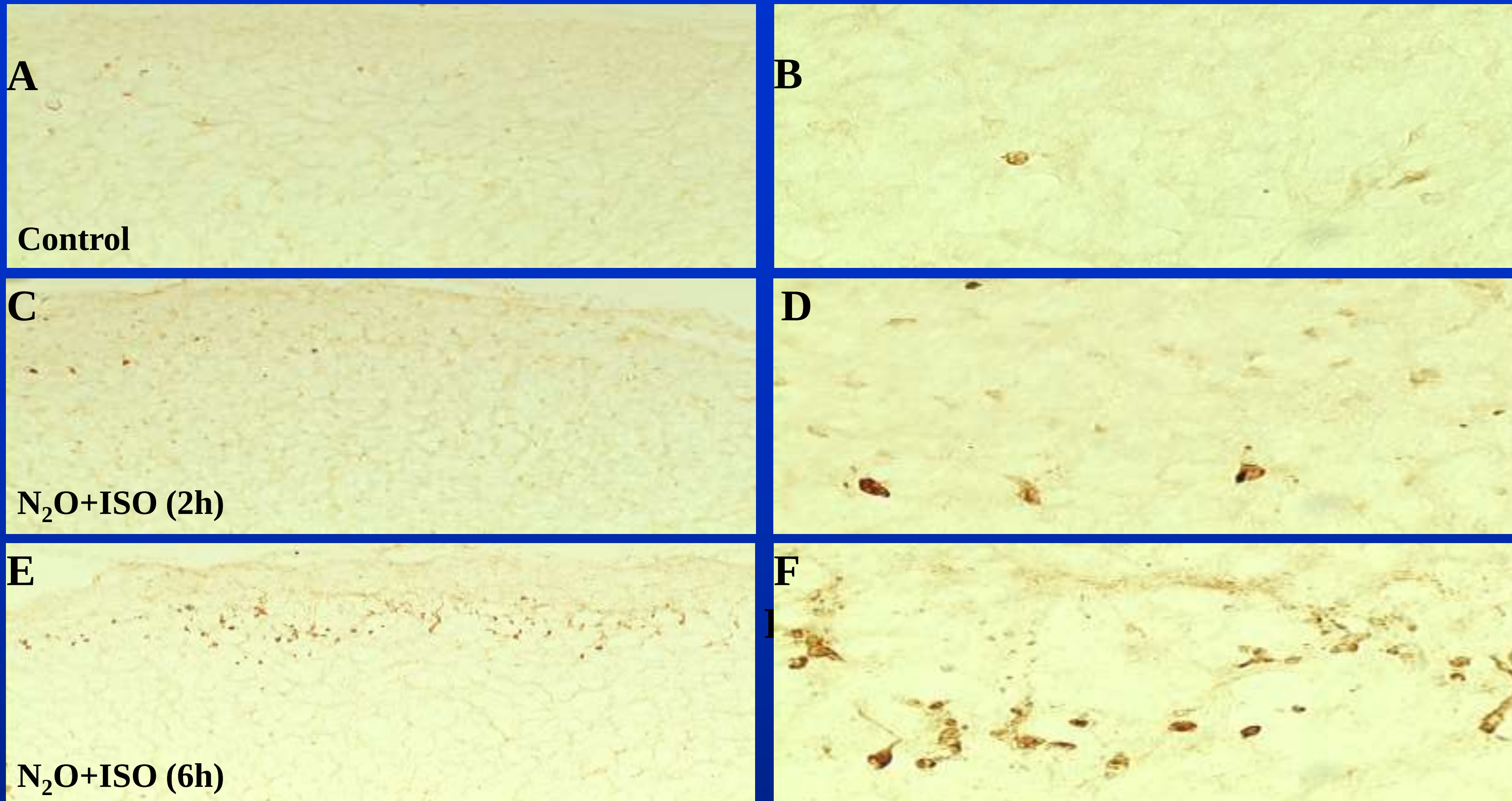
Developmental *In Vivo* Rat Model
Postnatal Day 7

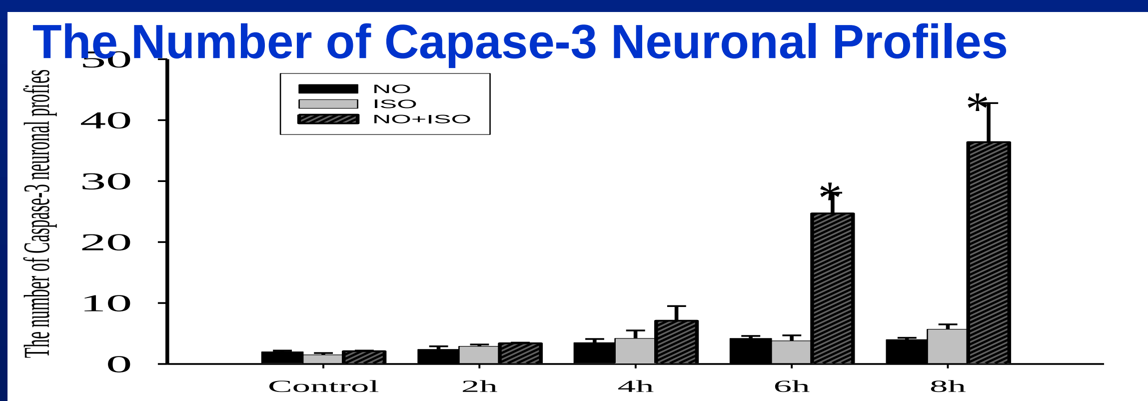
- **Inhaled Anesthetic Study**
  - Isoflurane (ISO): 0.55%
  - Nitrous Oxide (N_2O): 75%
  - Combination
    - without L-Carnitine
    - with L-Carnitine

Nitrous Oxide (N_2O) and Isoflurane (ISO) anesthesia in the 7 day old rat

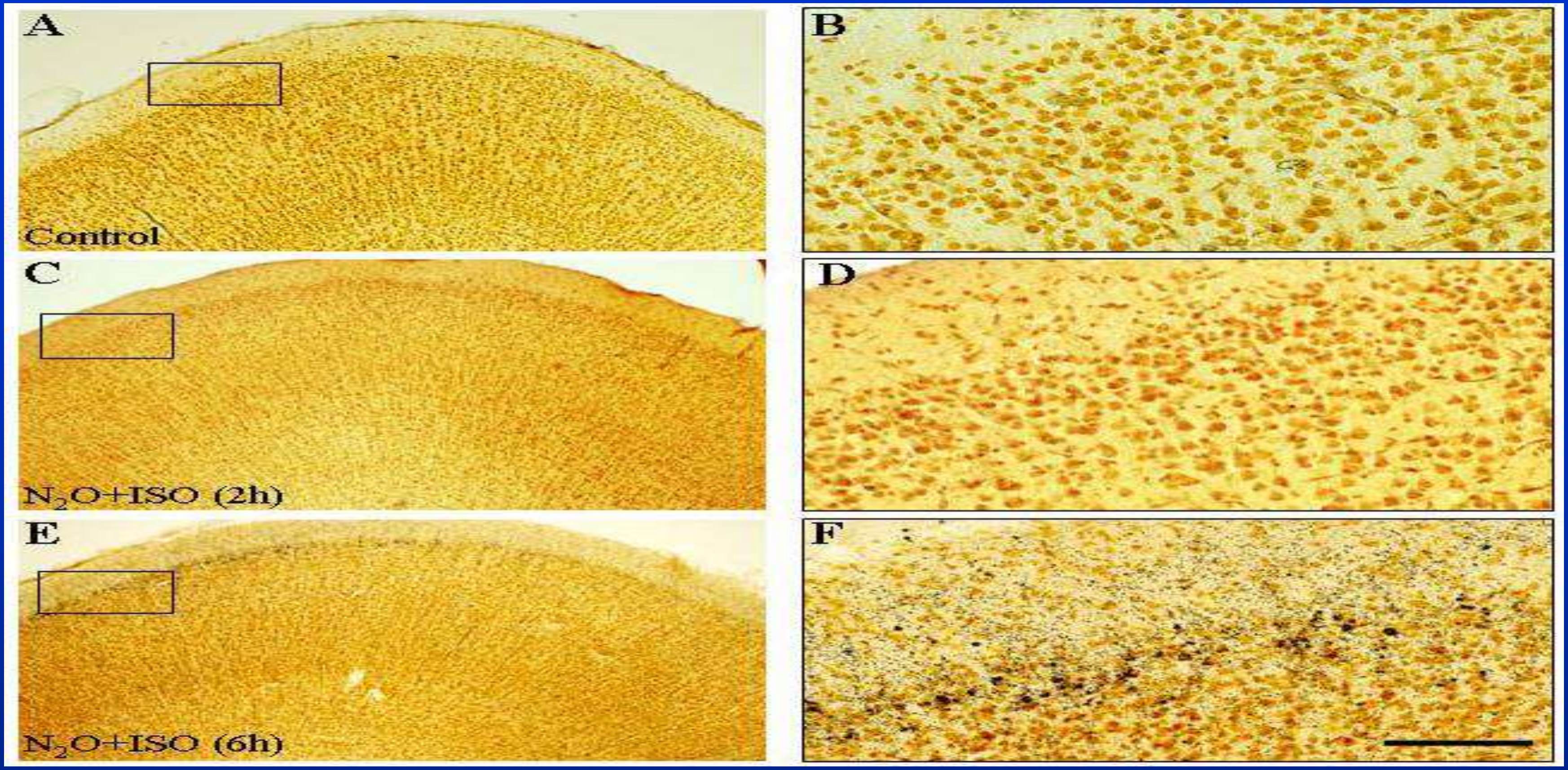


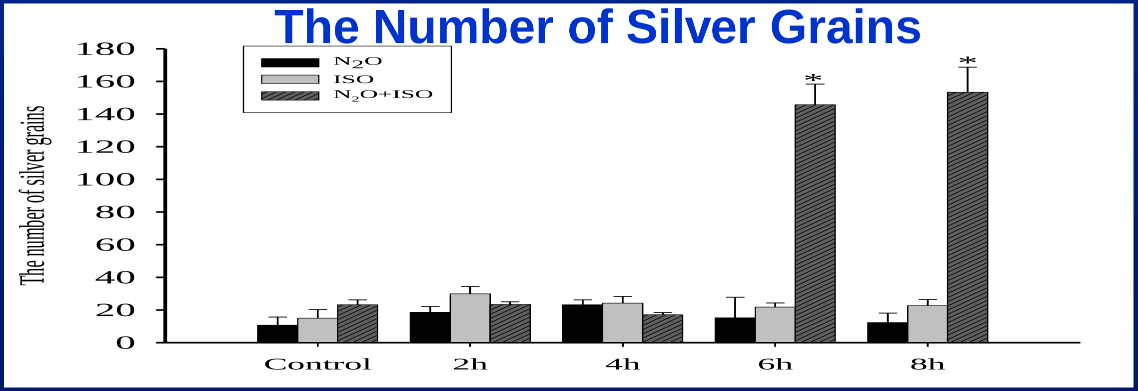
Zou et al. Neuroscience, 2008



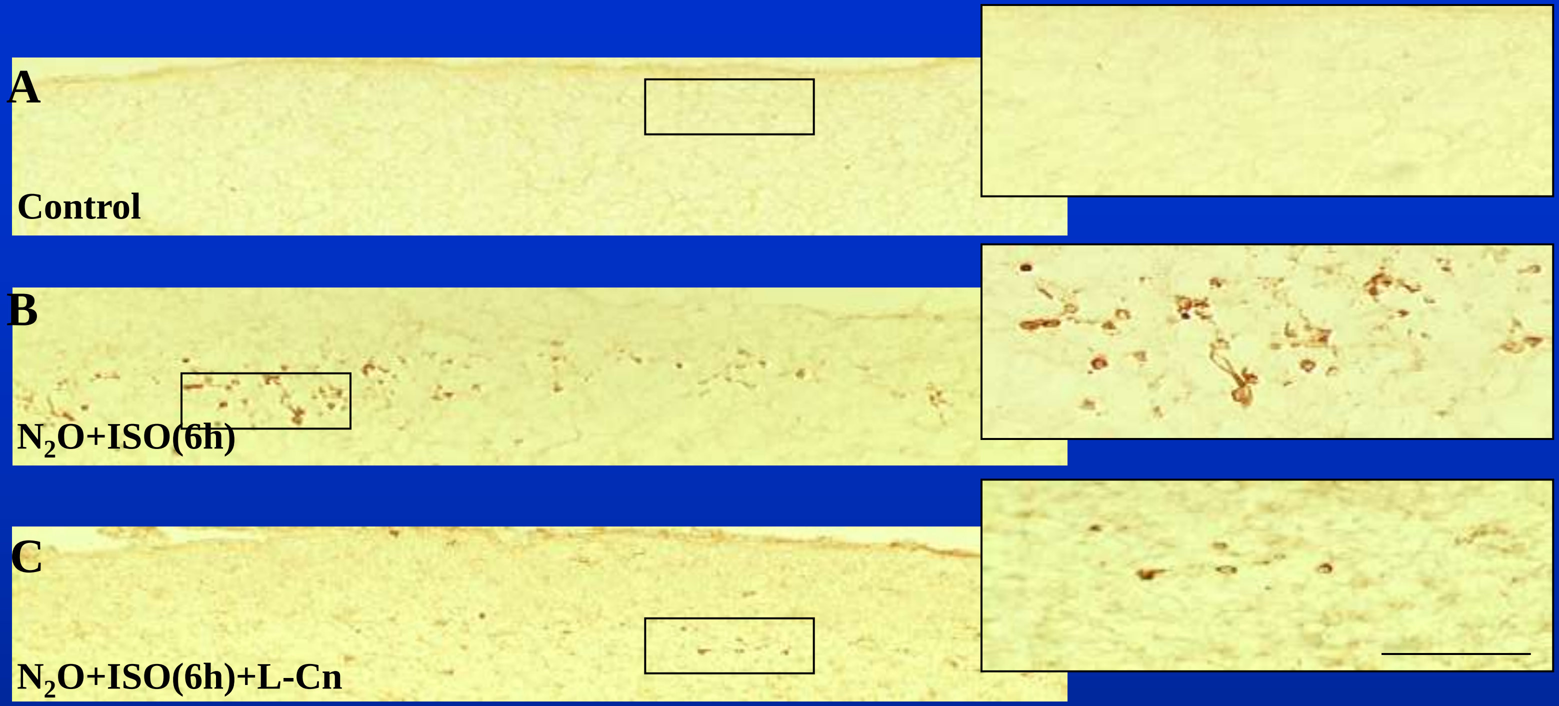


Zou et al. Neuroscience, 2008

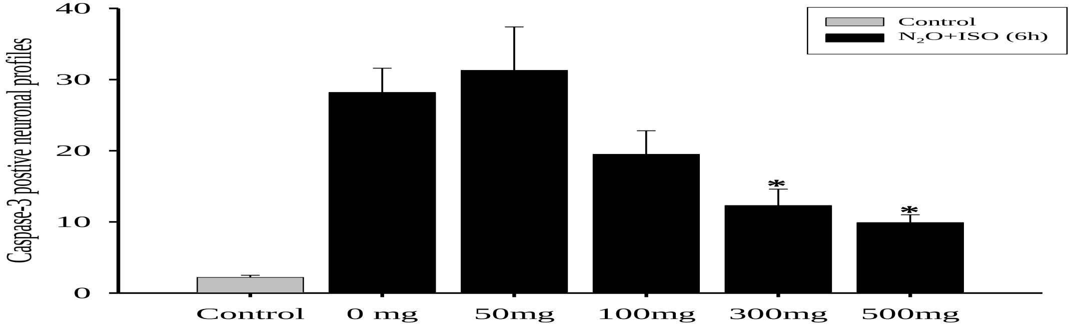




Zou et al. Neuroscience, 2008



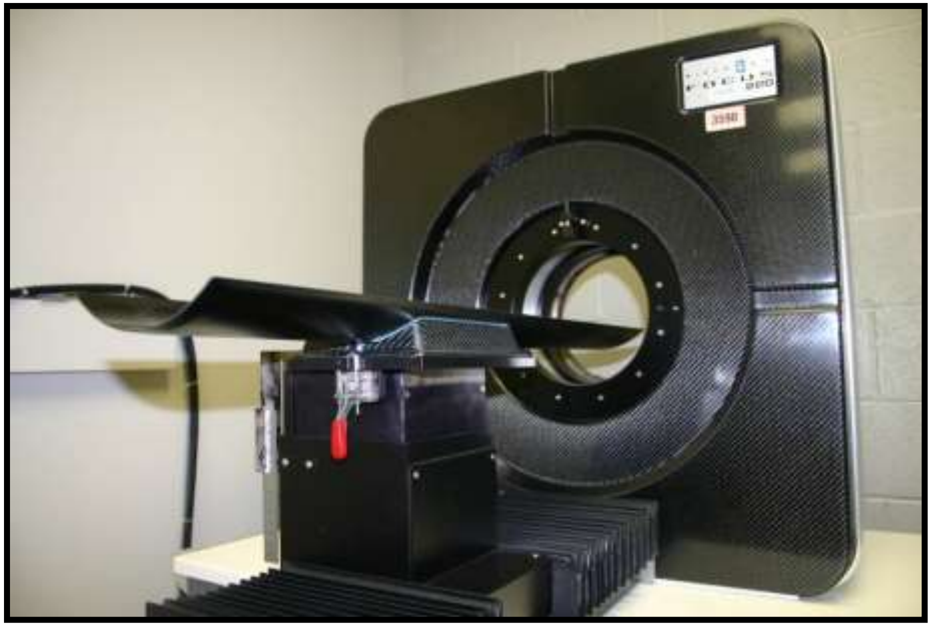
Caspase-3 Positive Neuronal Profiles



L-Carnitine 0 to 500 mg/kg, sc

Zou et al. Neuroscience, 2008

Bio-Imaging at NCTR/FDA



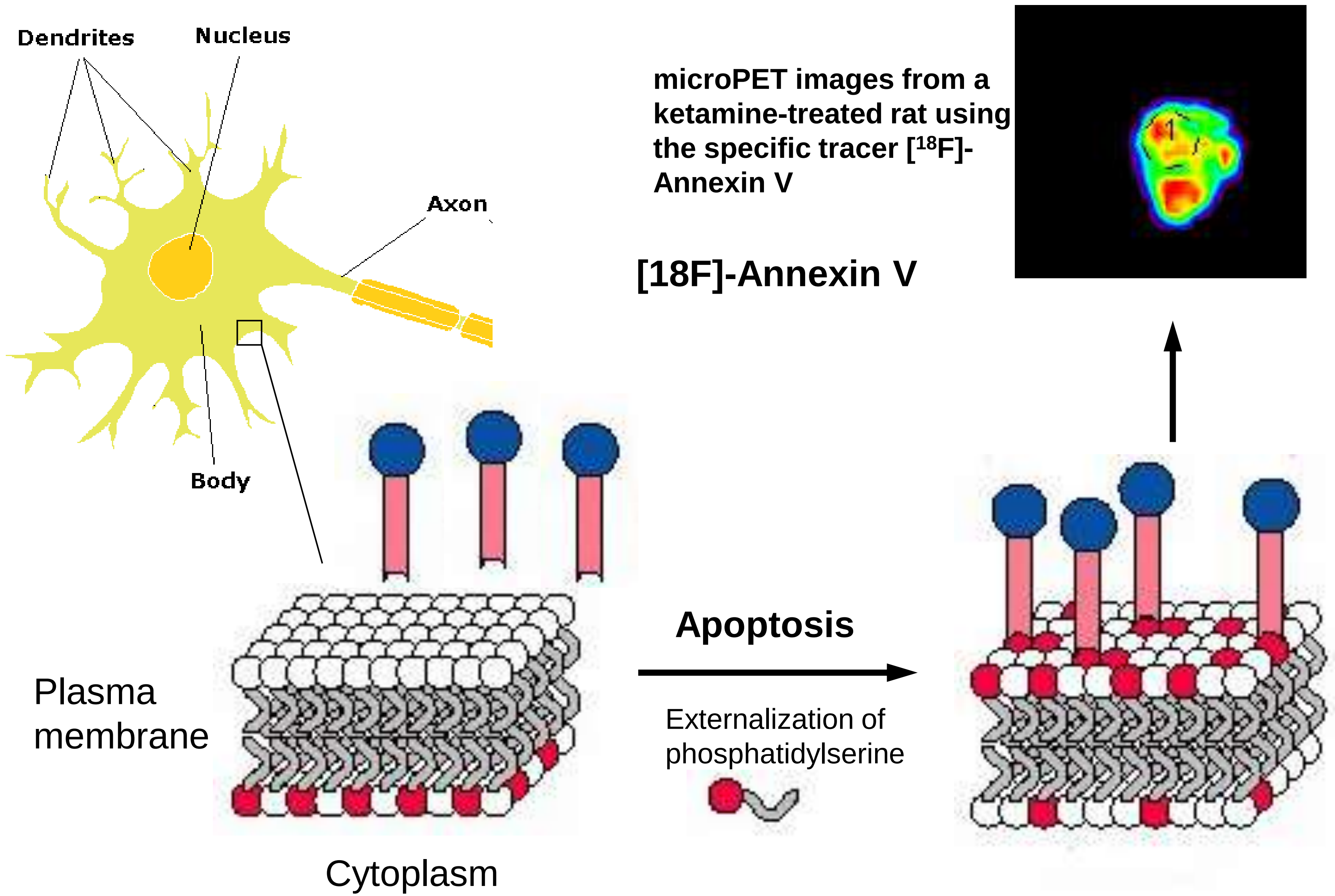
MicroPET
23 cm bore

Biospec MRI
7 Tesla, 30 cm bore

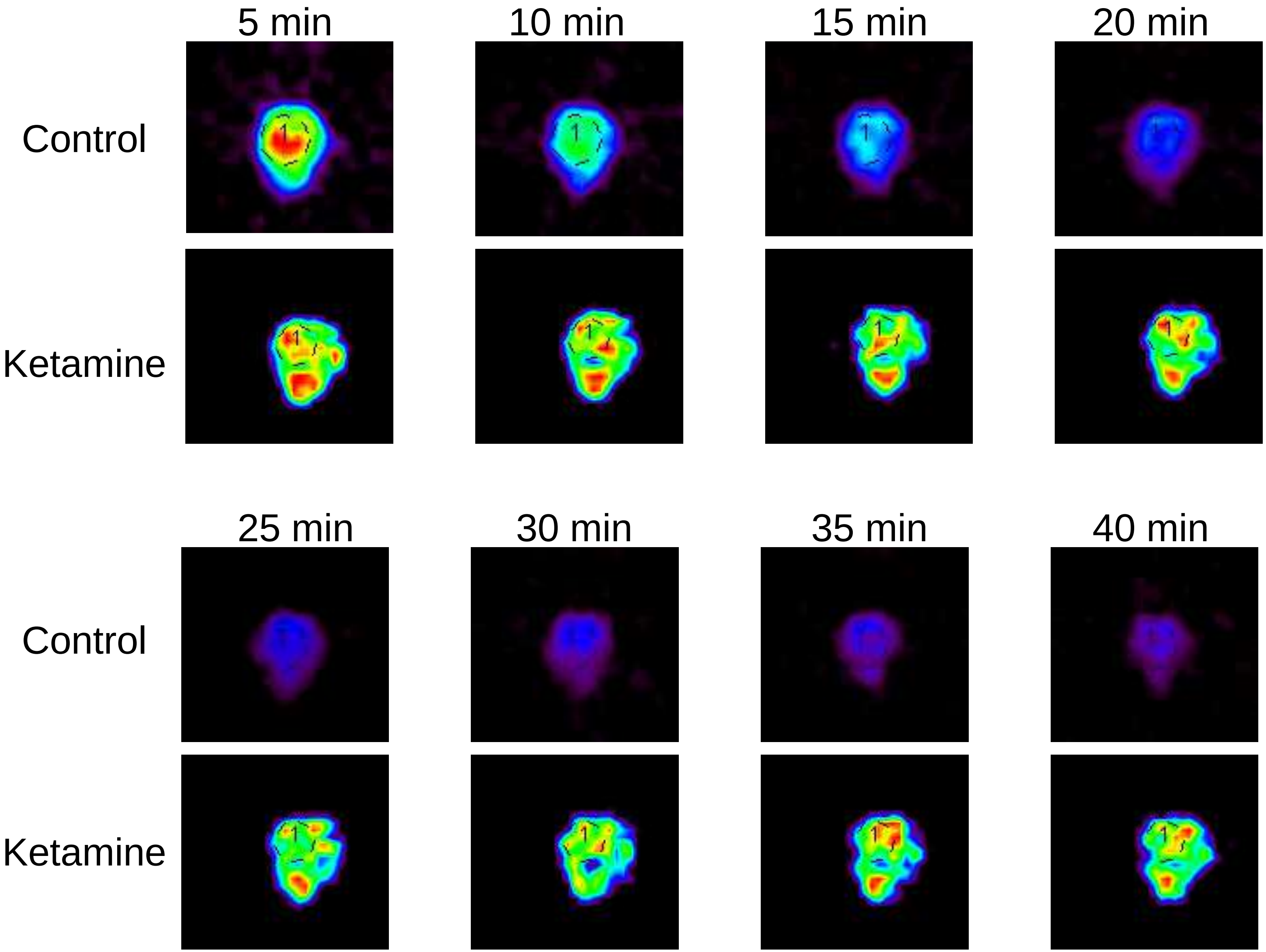
Developmental exposure to ketamine in the rat

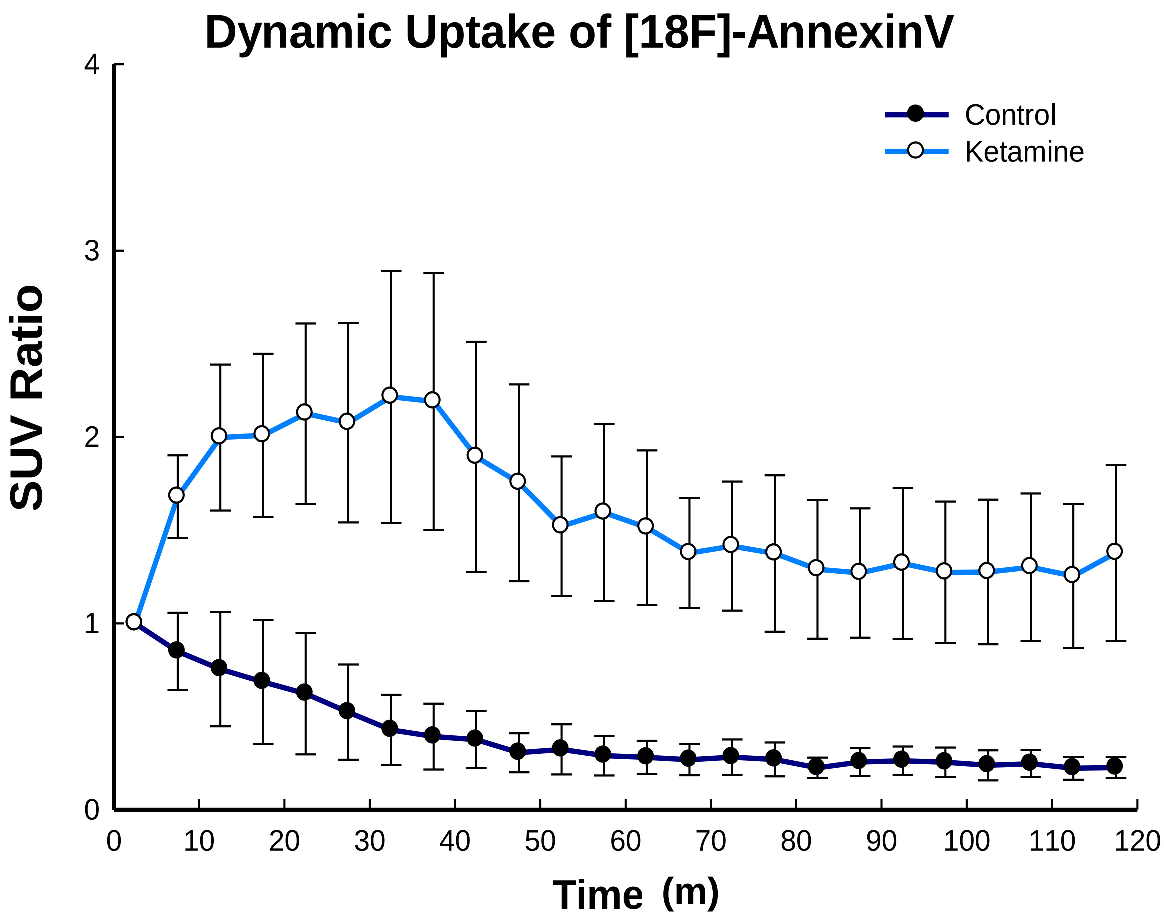
- PND 7: Single episode of anesthesia with ketamine, rat pups in the experimental group were exposed to 6 subcutaneous injections of ketamine (20 mg/kg) and control rat pups received 6 injections of saline.

- MicroPET scan with
  - [^{18}F]-Annexin V: (Zhang et al., Tox Sci, 2009)


Dendrites
Nucleus
Axon
Body
microPET images from a ketamine-treated rat using the specific tracer [^{18}F]-Annexin V
[18F]-Annexin V
Apoptosis
Externalization of phosphatidylserine
Plasma membrane
Cytoplasm

MicroPET Images of Rat Brain after [^{18}F] Annexin V administration



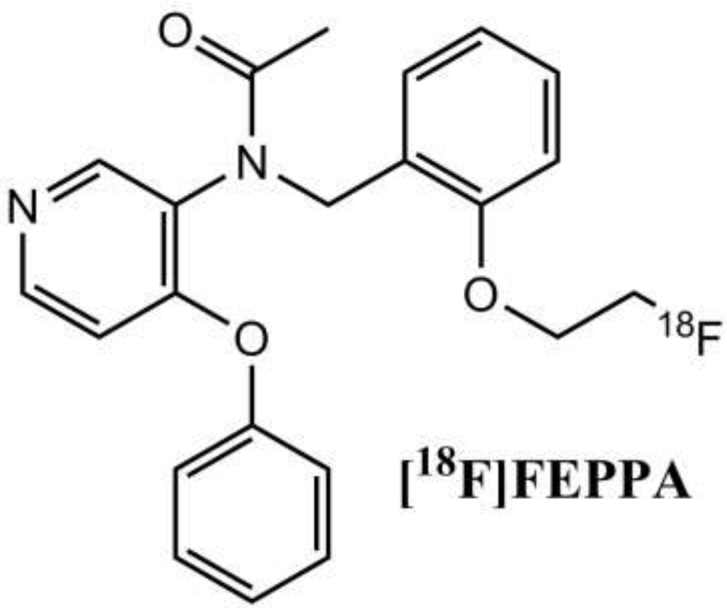


Evidence of neuroapoptosis in vivo without neurohistology

Developmental exposure to ketamine in the rat

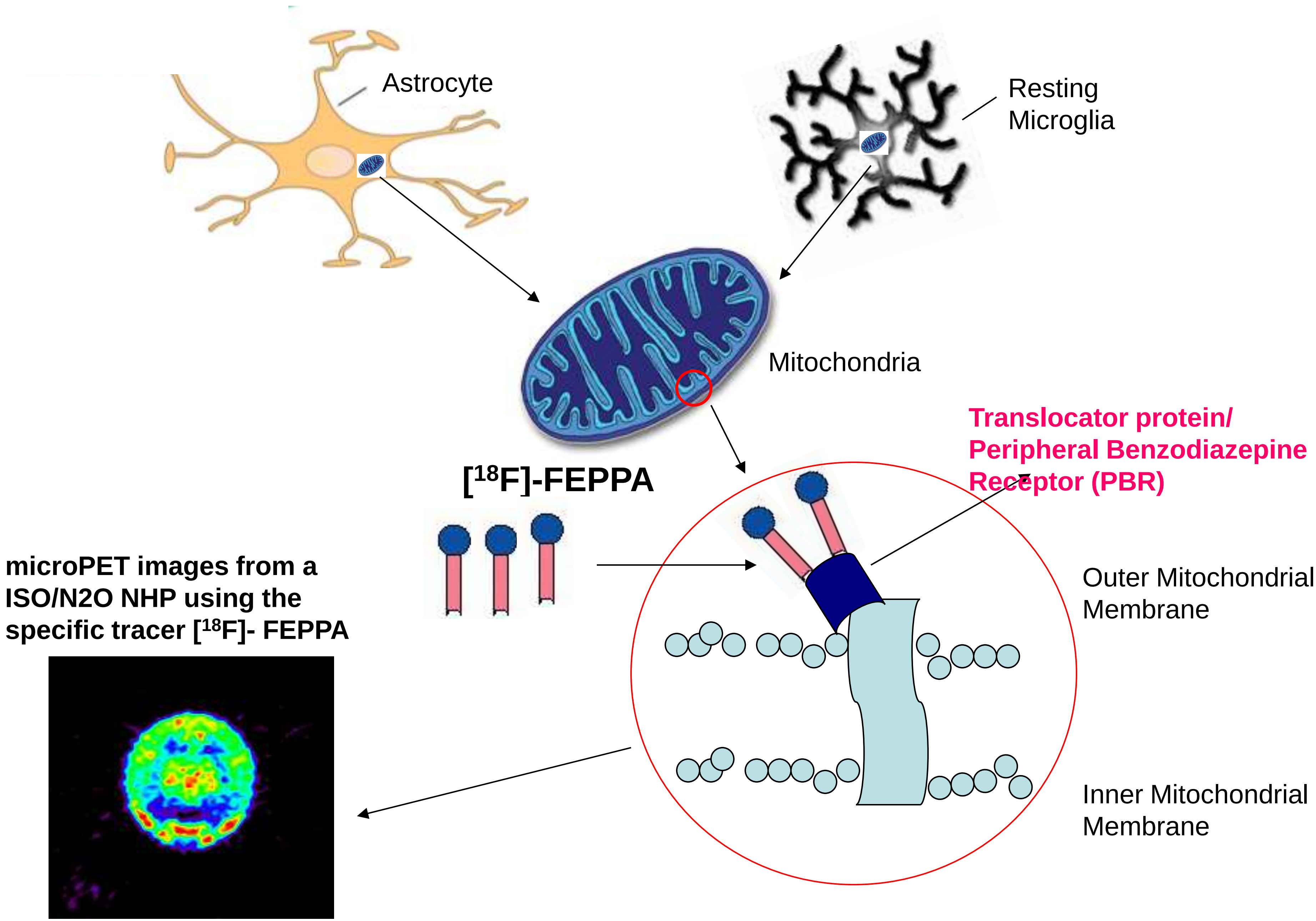
- PND 7: Single episode of anesthesia with ketamine, rat pups in the experimental group were exposed to 6 subcutaneous injections of ketamine (20 mg/kg) and control rat pups received 6 injections of saline.
- microPET scan with
  - [^{18}F]-Annexin V on PND 35: Zhang et al., Tox Sci, 2009
  - [^{18}F]-FEPPA: time course study (ongoing experiment).
    - PND 14: n=4
    - PND 21: n=4
    - PND 28: n=4
    - PND 35: n=7

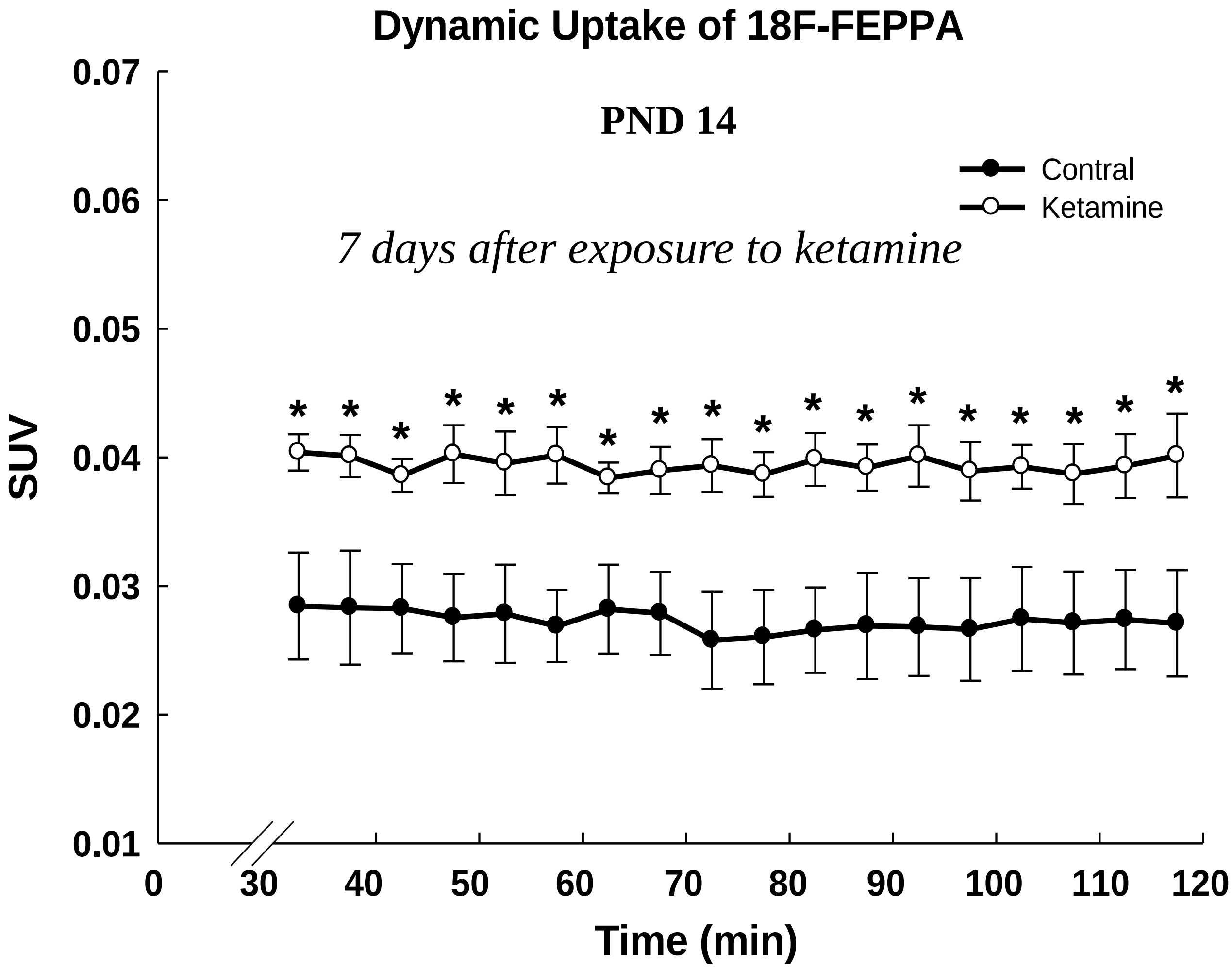
PET ^{18}F labeled ligand

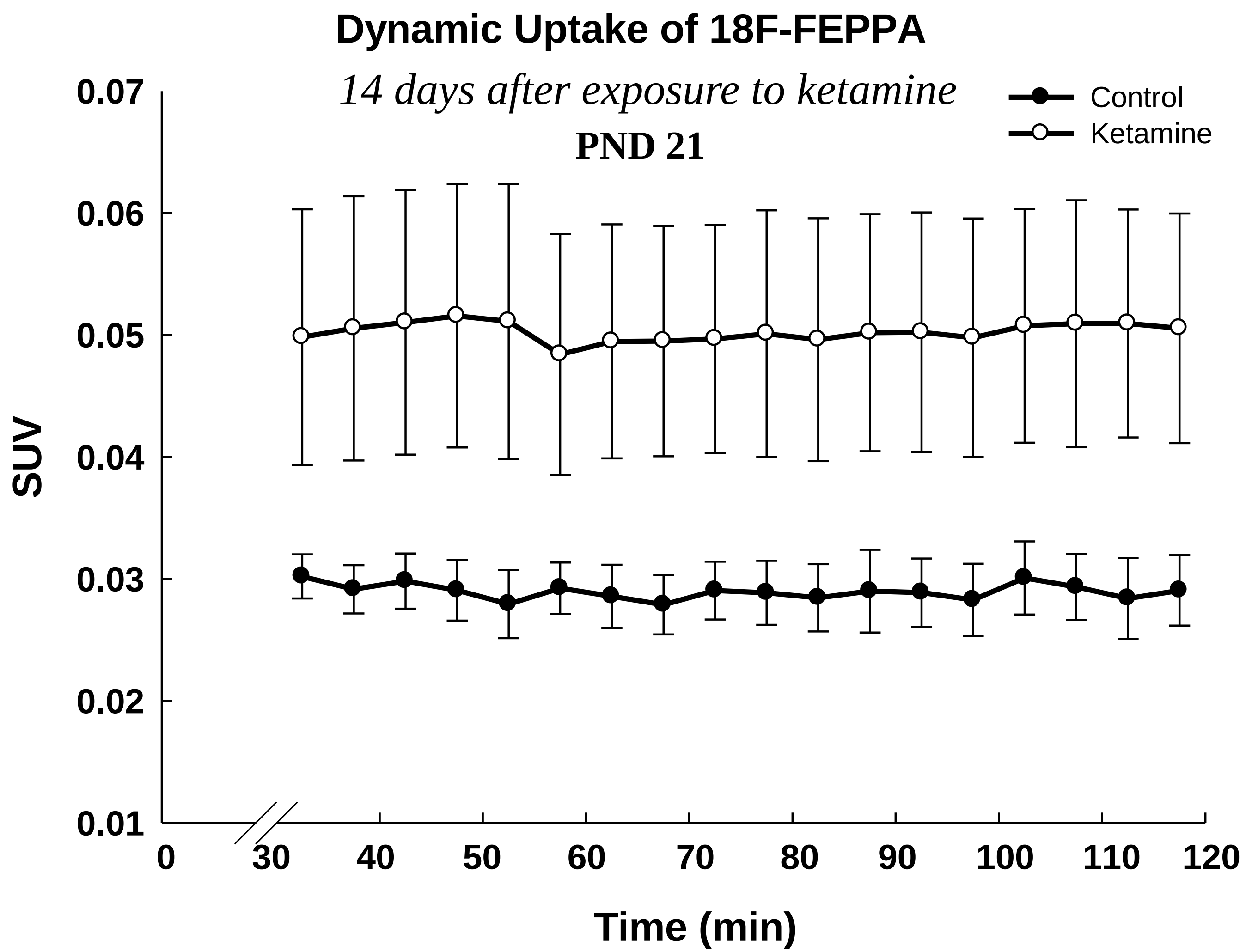


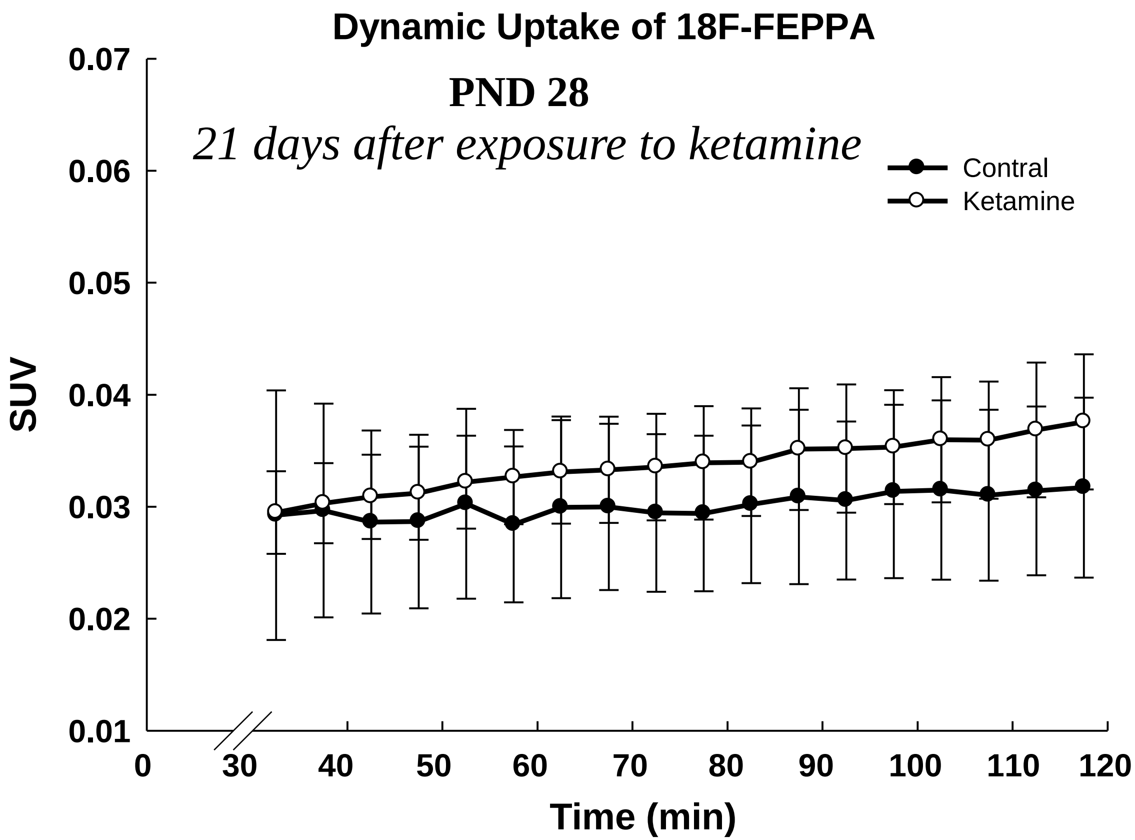
[^{18}F]FEPPA

FEPPA interacts with peripheral benzodiazepine receptors and used as a marker of glial activation in response to neuronal damage and inflammation


Astrocyte
Resting
Microglia
Mitochondria
Translocator protein/
Peripheral Benzodiazepine
Receptor (PBR)
[18F]-FEPPA
Outer Mitochondrial
Membrane
Inner Mitochondrial
Membrane
microPET images from a
ISO/N2O NHP using the
specific tracer [18F]- FEPPA

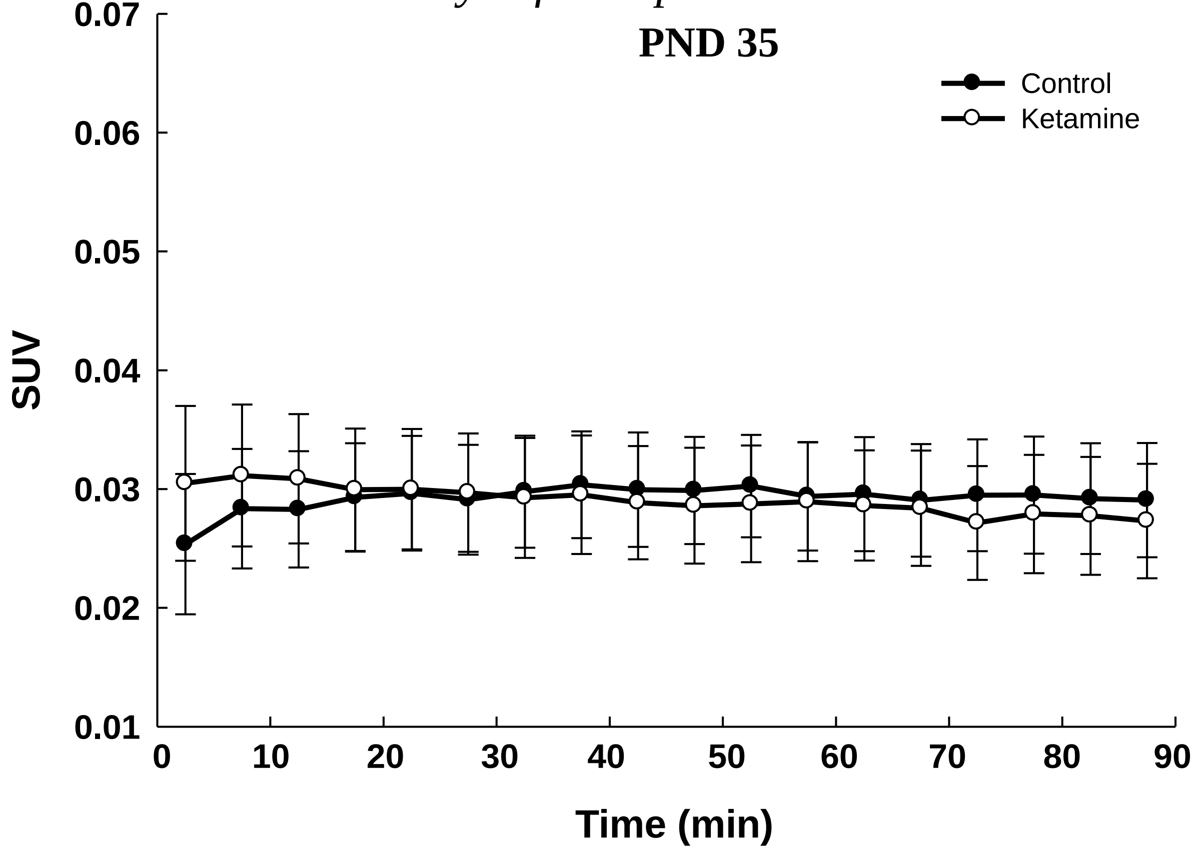

Dynamic Uptake of 18F-FEPPA
PND 14
7 days after exposure to ketamine
Contral
Ketamine
SUV
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
30
40
50
60
70
80
90
100
110
120
Time (min)


Dynamic Uptake of 18F-FEPPA
14 days after exposure to ketamine
PND 21
Control
Ketamine
0.07
0.06
0.05
0.04
0.03
0.02
0.01
SUV
0
30
40
50
60
70
80
90
100
110
120
Time (min)


Dynamic Uptake of 18F-FEPPA
PND 28
21 days after exposure to ketamine
Contral
Ketamine
SUV
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
Time (min)

Dynamic Uptake of 18F-FEPPA

28 days after exposure to ketamine



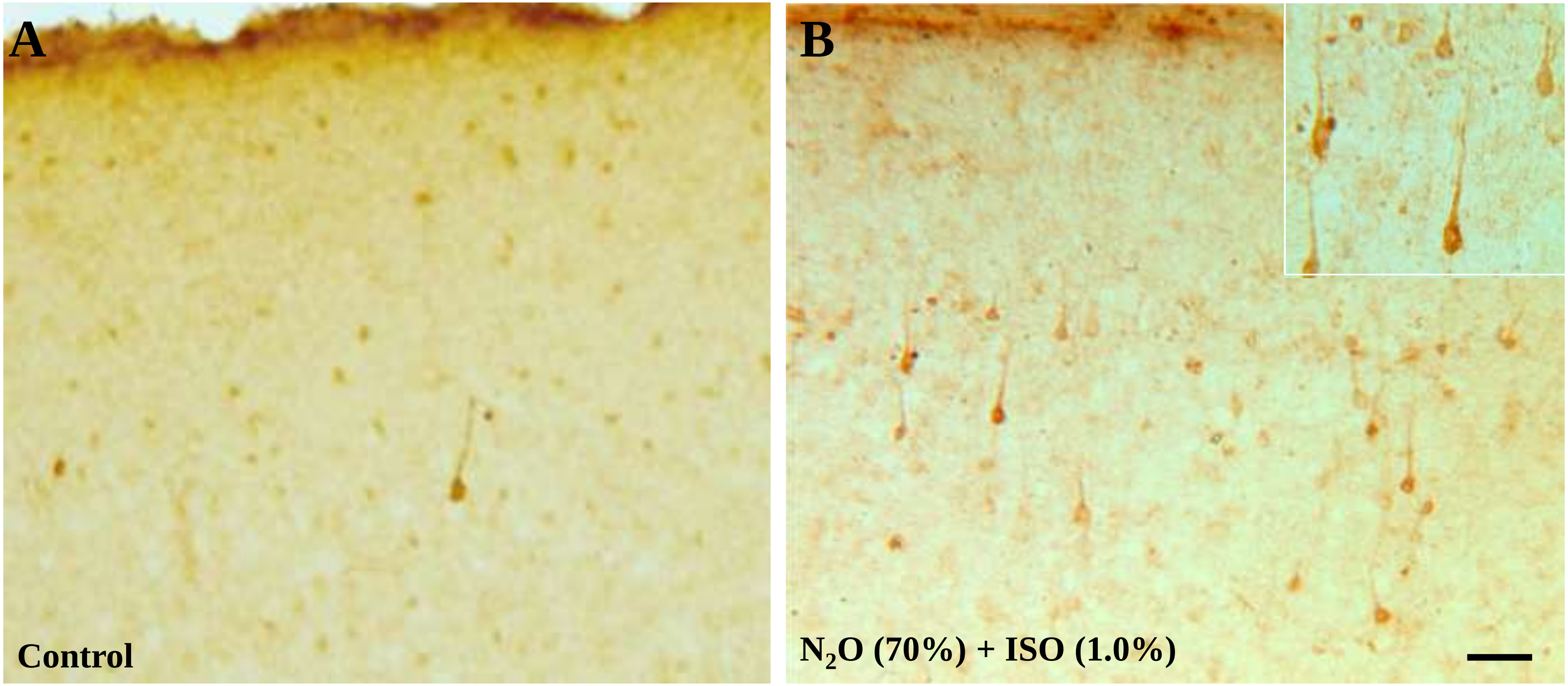
Time course data from the same animals

Physiological Parameters for Infant Monkeys Exposed to Inhaled Gaseous Anesthetics: 1% Isoflurane (ISO) and/or 70% Nitrous Oxide (N_2O) for 8 hrs.

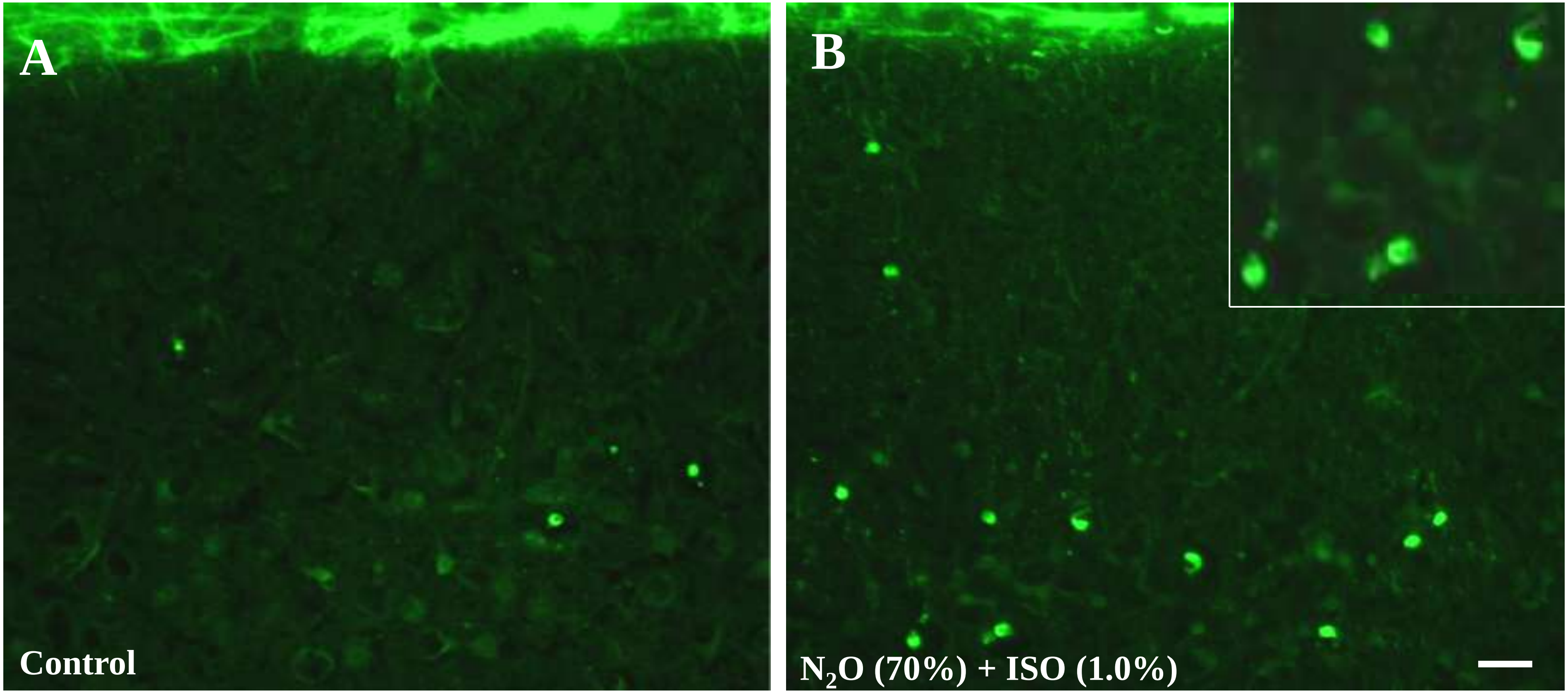
	PND 5/6 Monkeys			
	Control	**ISO**	**N_2O**	**ISO+N_2O**
Respiratory rate (breaths/min)	63±4.8	66±3.1	73±5.5	60±12.5
Heart rate (beats per min)	219±24.1	186±44.6	213±24.7	188±28.5
O_2 saturation (%)	95±2.5	91±1.8	95±3.7	94±0.9
Temperature (ºC)	36.6±0.5	34.8±1.4	36.3±0.6	34.5±1.8
Systolic blood pressure	77±9.5	75±4.5	79±11.7	86±12.1
Diastolic blood pressure	49±4.4	43±3.6	58±10.5	59±13.9
Glucose (mg/dl)	68±13.5	72±13.7	85±17.4	80±10.1
Venous pCO_2	45±9.2	60±1.0	49±6.9	54±10.6
Venous pO_2	26±15.0	28±4.2	27±5.5	28±4.7
Venous pH	7.3±0.1	7.3±0.02	7.2±0.04	7.3±0.08

Zou and Liu et al, Neurotox Teratol, 2011

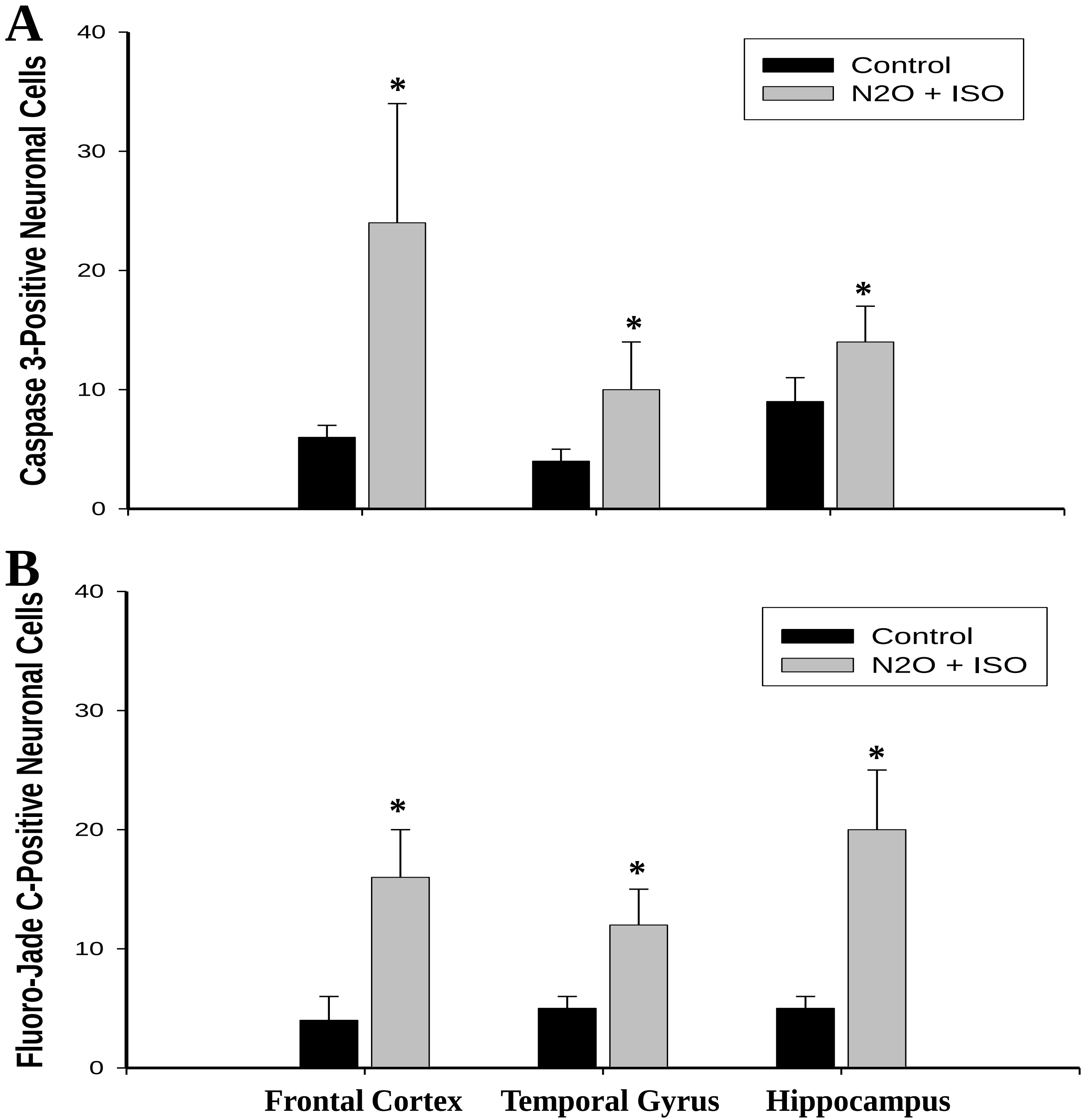
Caspase 3 Immuno-staining (Frontal Cortex, Monkey)



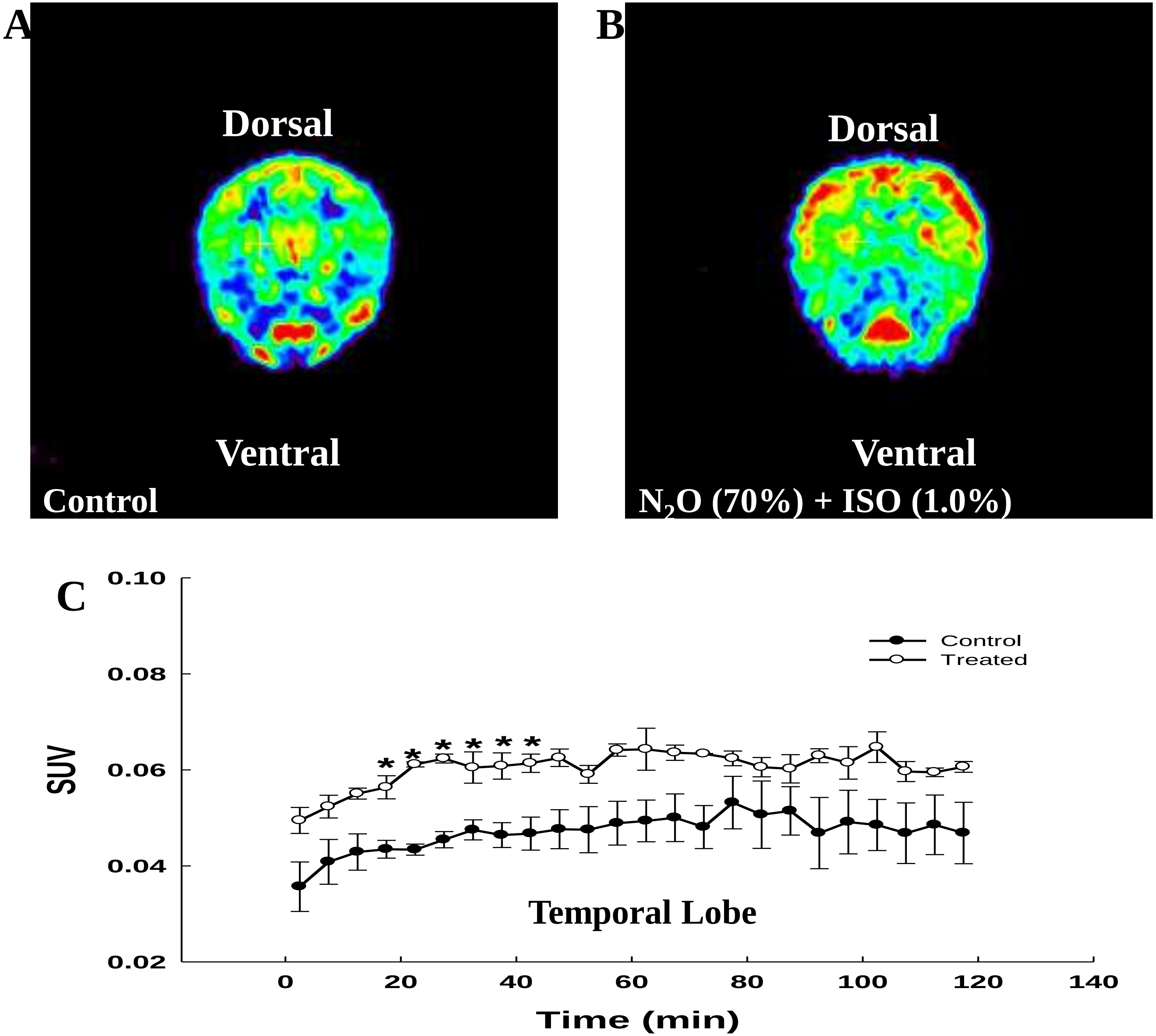
Fluoro-Jade C Staining (Frontal Cortex)



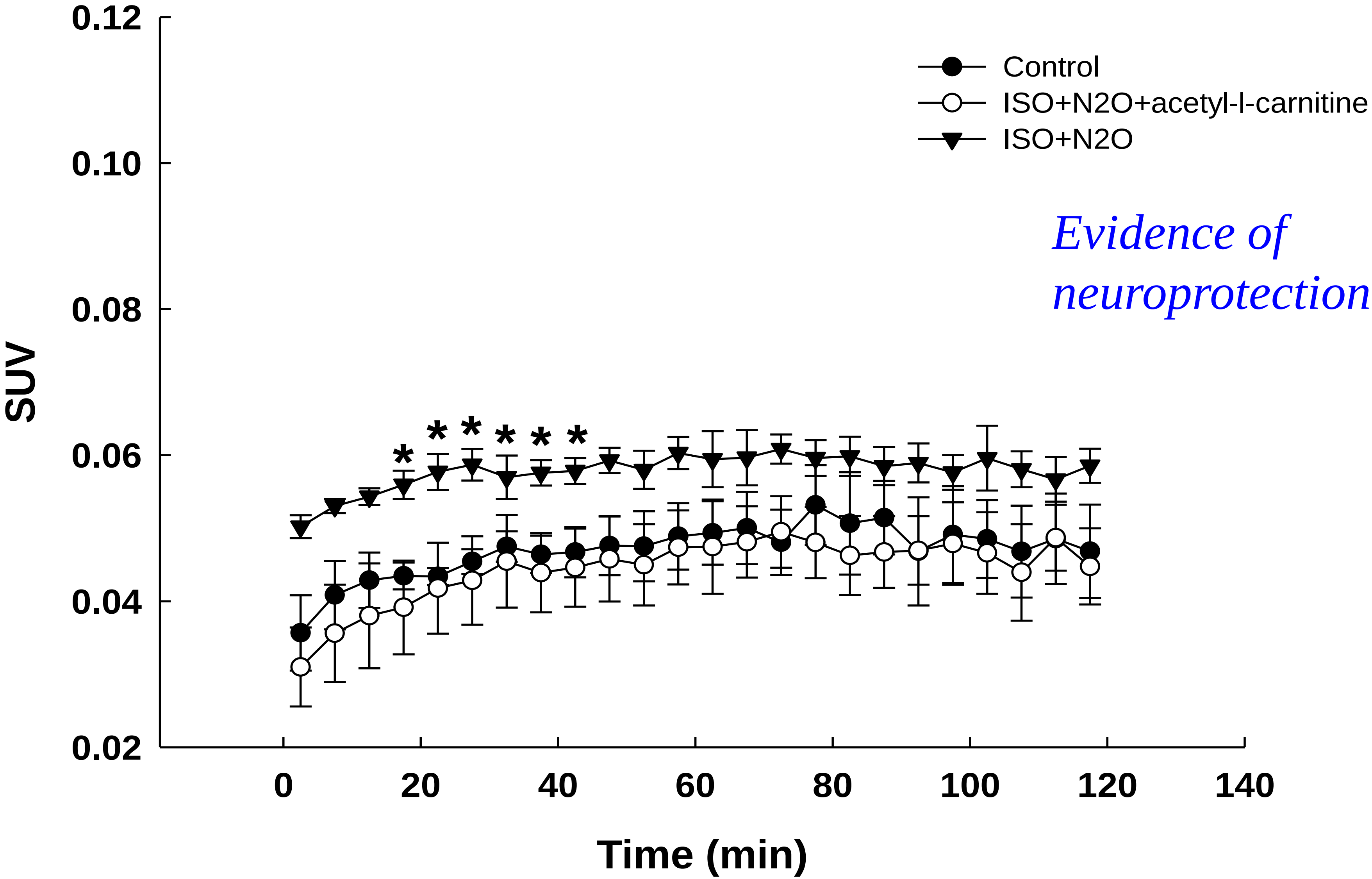
Effects of ISO + N_2O - induced anesthesia (1% Isoflurane (ISO) and 70% Nitrous Oxide (N_2O) for 8 hrs.) in the 5/6 day old monkey



Dynamic Uptake of [^{18}F]-FEPPA (PND 6 Monkeys)



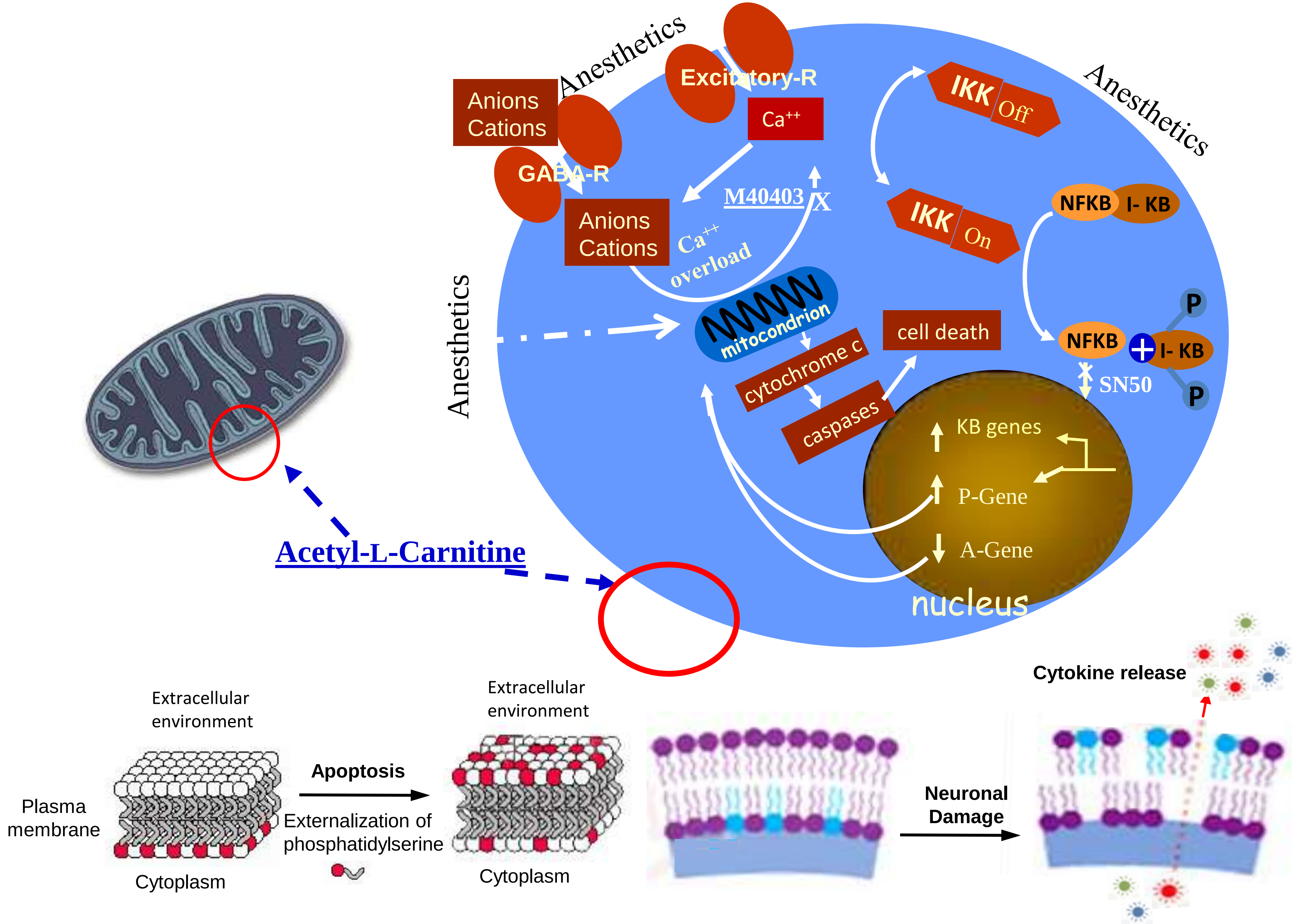
Dynamic Uptake of [^{18}F]-FEPPA on PND 6 (Temporal Lobe)



Evidence of neuroprotection

Advantages of *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

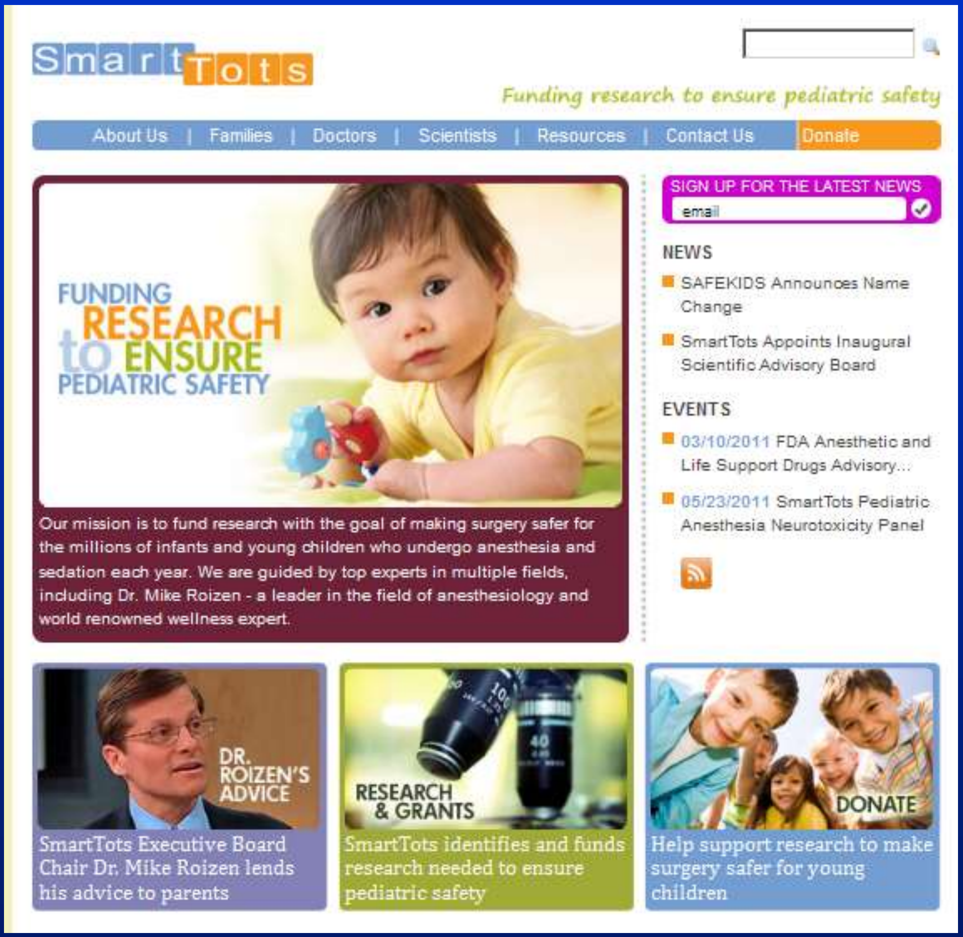
Conclusions

- 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.

SmartTots



Help ensure the safety of anesthetics used in infants and young children, donate today

www.SmartTots.org

Acknowledgements

NCTR/FDA

- Cheng Wang
- Xiaoju Zou
- Andy Scallet
- Becky Divine
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- William Rodriguez

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The Annual Teratology Society
Volleyball Games, 1982-2015
33 Years of NETworking!


Thank you for this honor and your attention.