ANESTHESIA-INDUCED NEUROAPOPTOSIS and OLIGOAPOPTOSIS in the DEVELOPING PRIMATE BRAIN

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All experimental procedures performed at
Oregon National Primate Research Center (ONPRC)

Supported by HD 37100, HD 052664, DA 05072, P30 HD 062171 (Intellectual & Developmental Disabilities Research Center)

PROTOCOL FOR PRIMATE RESEARCH

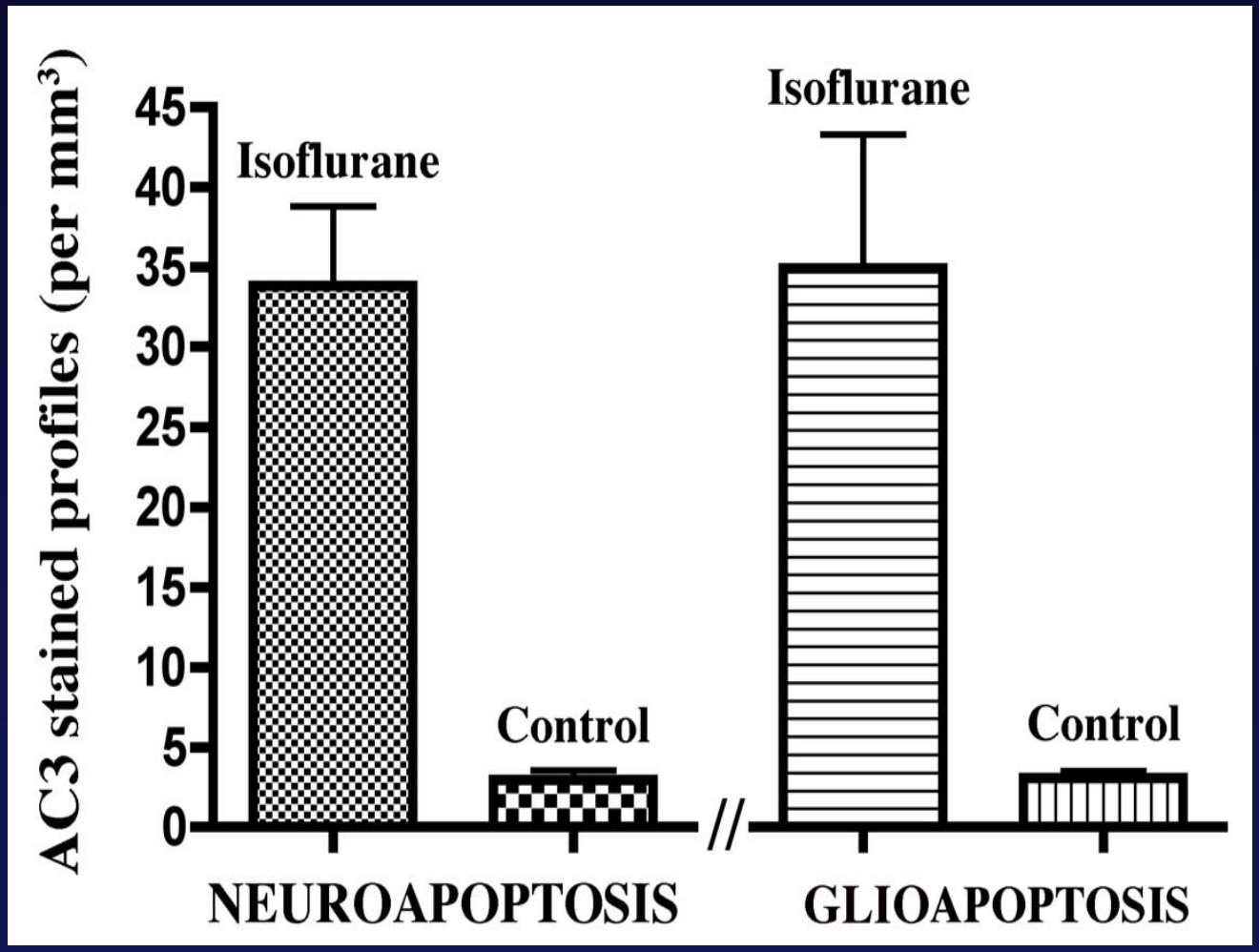
- **Expose fetal (G120) or neonatal (P6) rhesus monkeys to Isoflurane, ketamine or propofol for 5 hours, and examine the brains 3 hrs later for histological documentation of apoptotic cell death.**

- **Fetuses are exposed *in utero* by administering the drug to the pregnant dam. The fetus is delivered by C-Section, then immediately euthanized.**

- **The anesthetic drug is administered to the dam or neonate in an amount sufficient to maintain an intermediate surgical plane of anesthesia.**

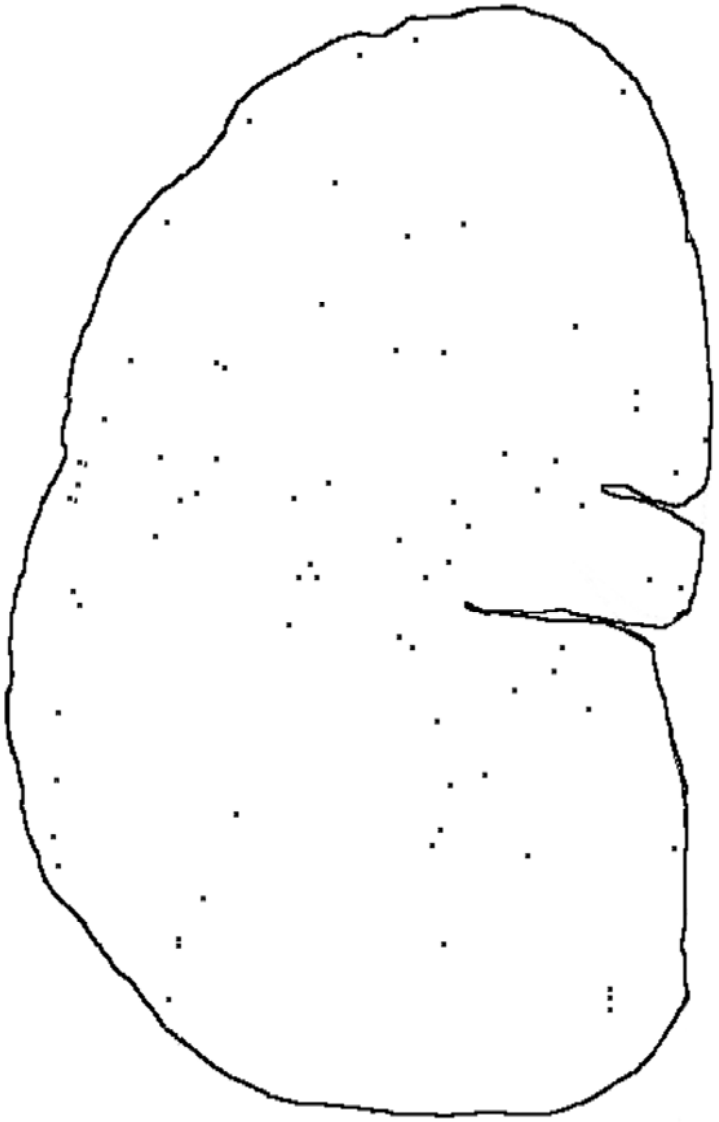
- **Ansgar Brambrink, MD, PhD, Prof of Anesthesiology, OHSU personally supervises the administration of anesthetic drugs. All procedures are IACUC approved and are in full accordance with the PHS Policy on Humane Care and Use of Laboratory Animals.**

NEURO/GLIOAPOPTOSIS RESPONSE TO ISOFLURANE P6 RHESUS NEONATE

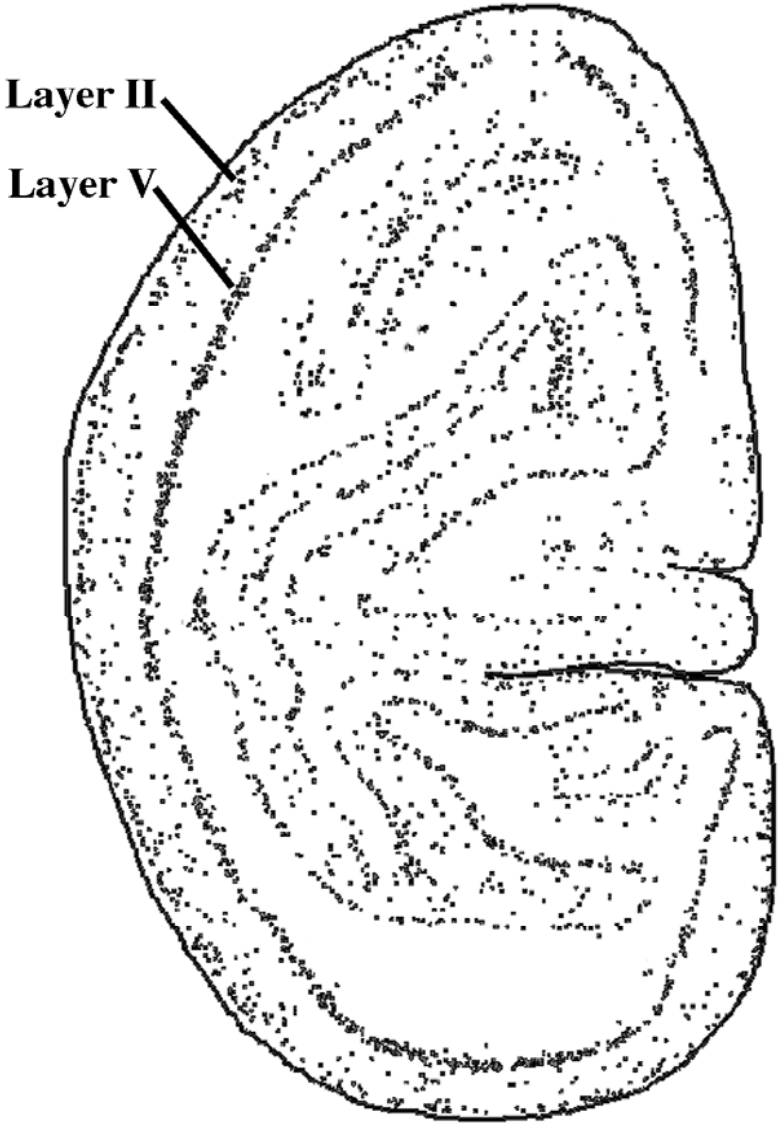


Brambrink et al., Anesth., 2010 Brambrink et al., ASA, 2010

NEUROAPOPTOSIS RESPONSE TO ISOFLURANE
VISUAL CORTEX - P6 RHESUS NEONATE

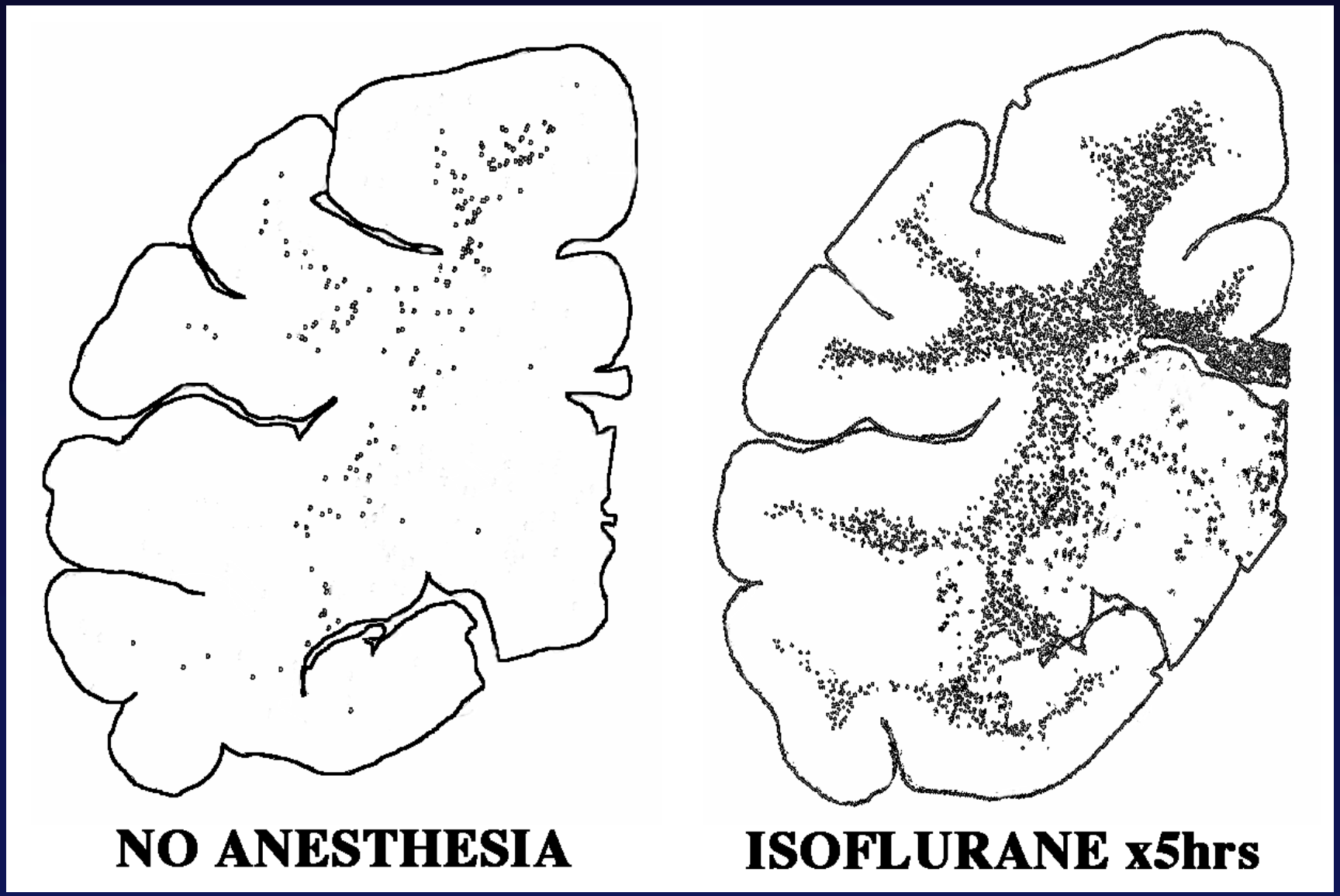


NO ANESTHESIA

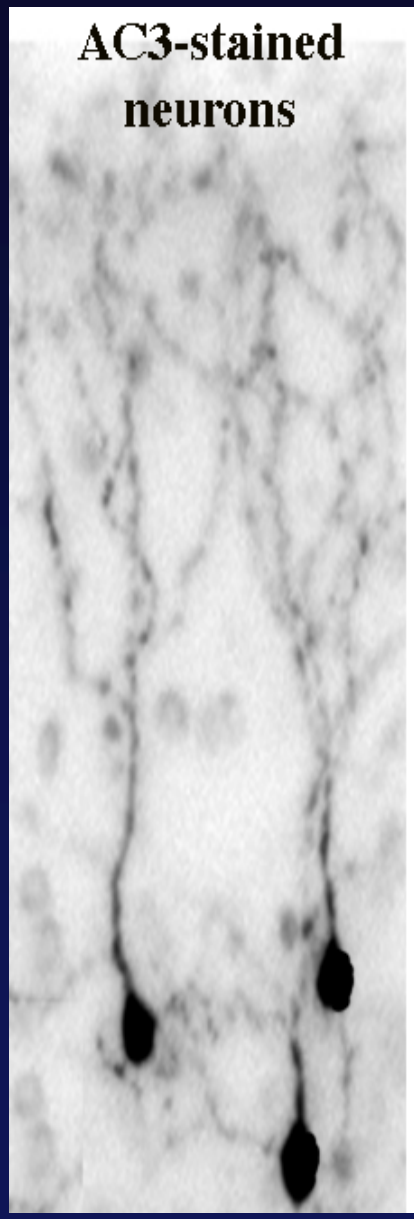


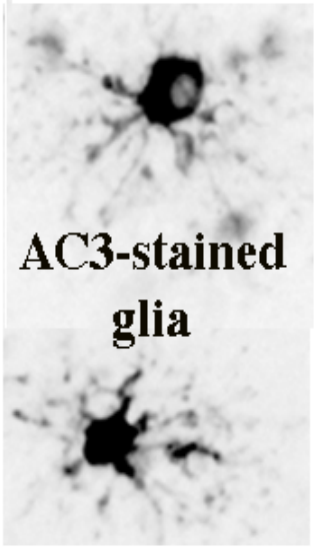
ISOFLURANE x5hrs

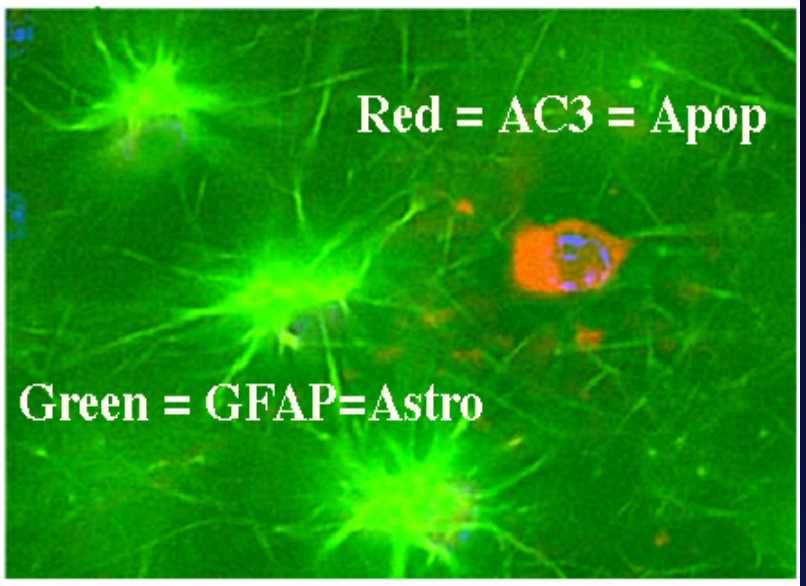
GLIOAPOPTOSIS RESPONSE TO ISOFLURANE
P6 RHESUS NEONATE

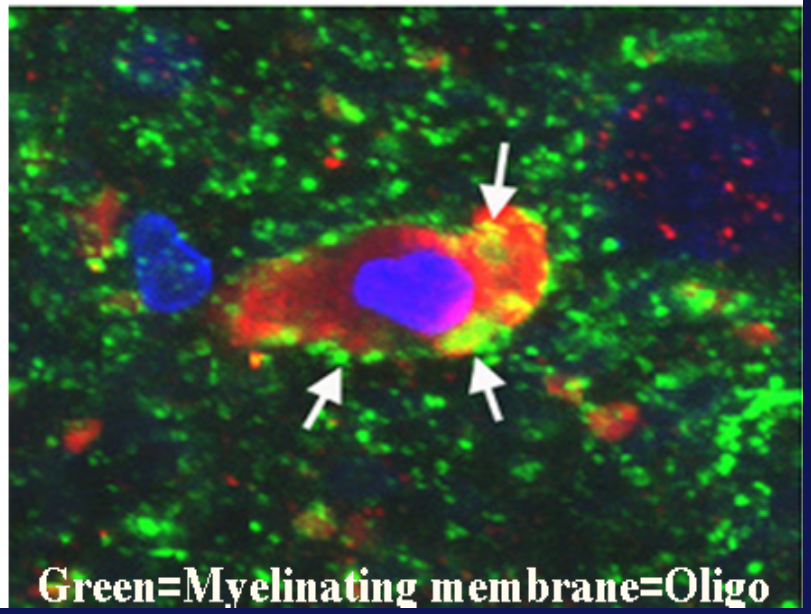


Apoptotic glia are immature Oligos engaged in myelination









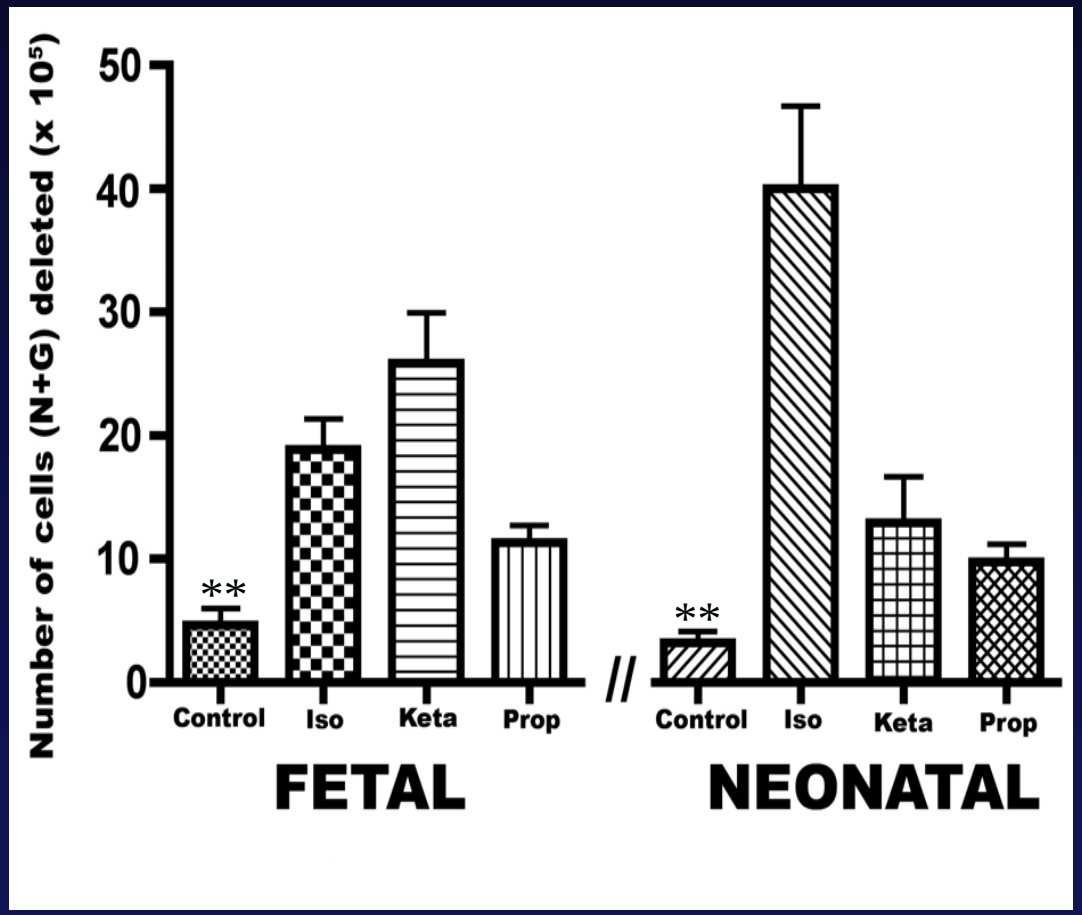
POTENTIAL SIGNIFICANCE OF THE OLIGOAPOPTOSIS PHENOMENON

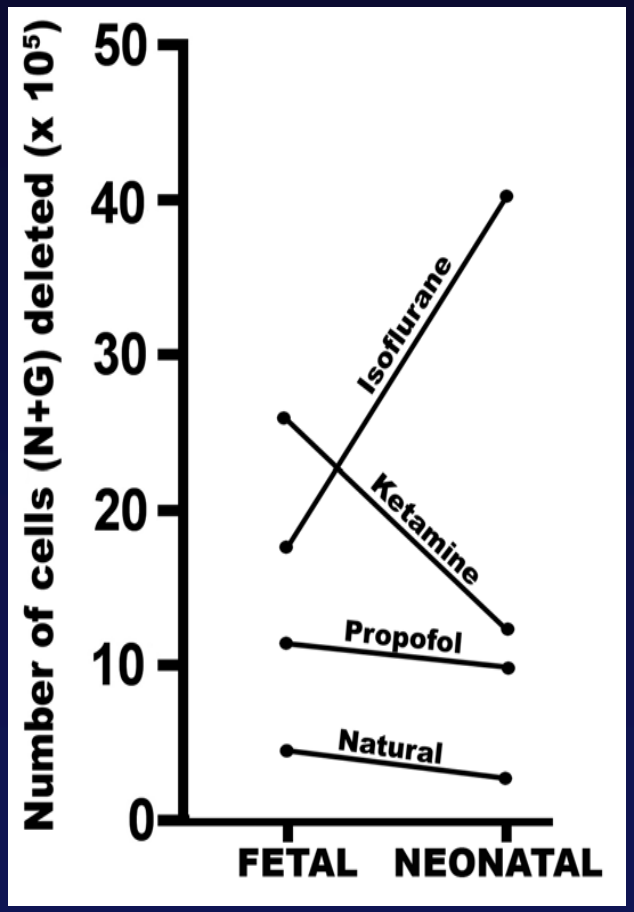
Oligos are vulnerable during a stage when they are just beginning to myelinate axons that interconnect neurons throughout the CNS.

Deletion of oligos at this critical stage may disrupt myelin formation, which could potentially have adverse long term neurobehavioral consequences, that might be additive to the potential consequences of isoflurane-induced neuroapoptosis.

If the window of vulnerability for oligoapoptosis stays open longer than for neuroapoptosis, this may extend the period of risk for brain changes that could have adverse neurobehavioral consequences.

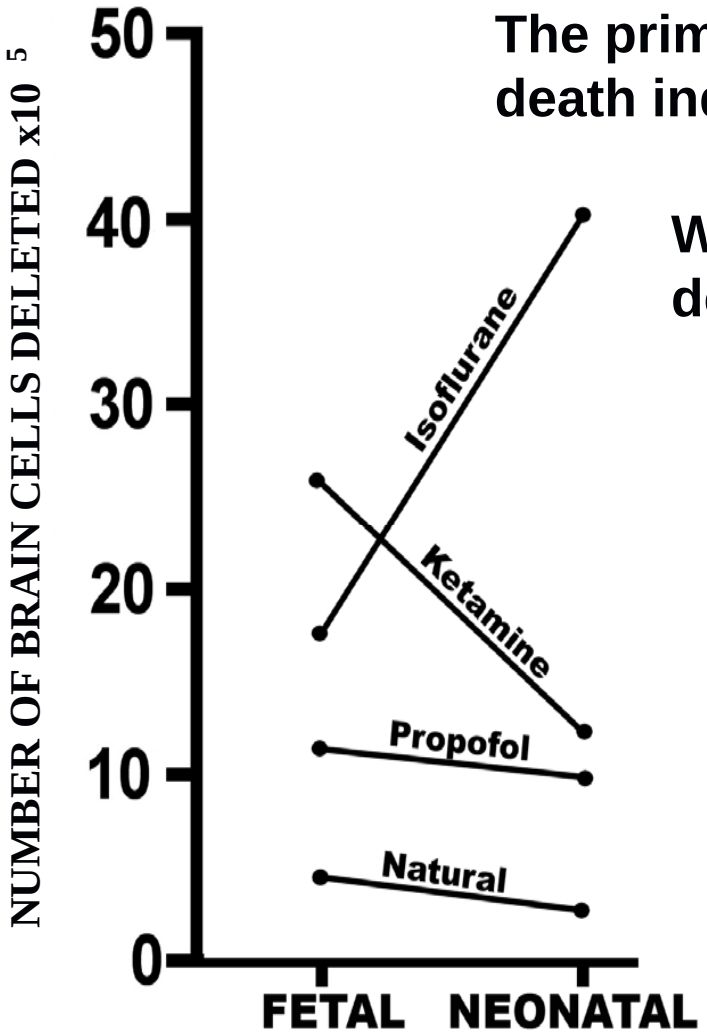
Comparative Toxic impact of three anesthetic drugs in the fetal vs neonatal monkey brain





** n = 4 - 5 per group; $p < 0.02$ for all control vs experimental comparisons

SUMMARY
NON-HUMAN PRIMATE FINDINGS



The primate brain is quite sensitive to brain cell death induced by alcohol* or anesthetic drugs

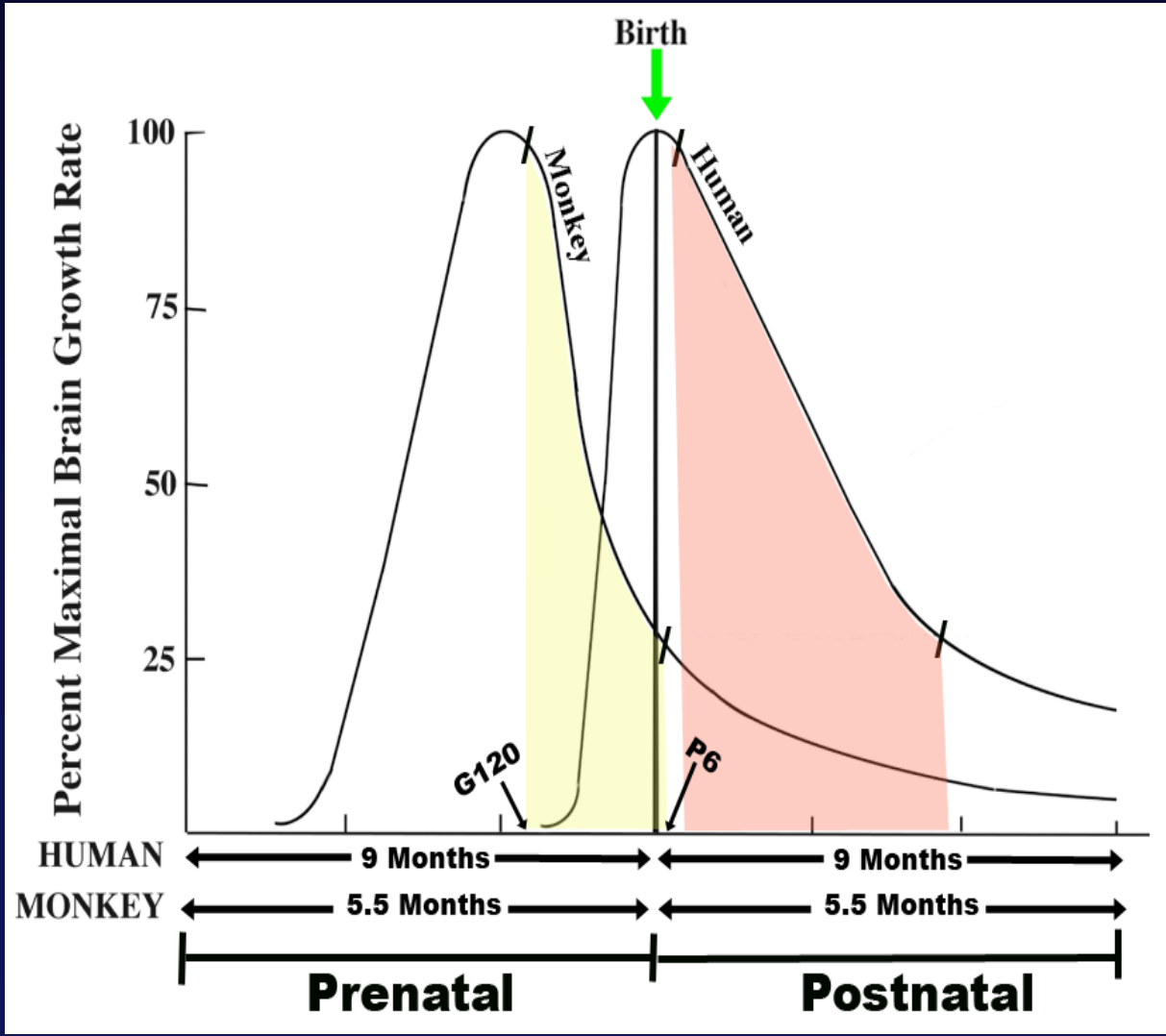
Which anesthetic drug is most toxic depends on the age at time of exposure.

- **Ketamine is most toxic for the fetus**
- **Isoflurane is most toxic for the neonate.**
- **Propofol is least toxic at either age.**

*Farber et al., 2010

Window of Vulnerability

Brain Growth Spurt (BGS)



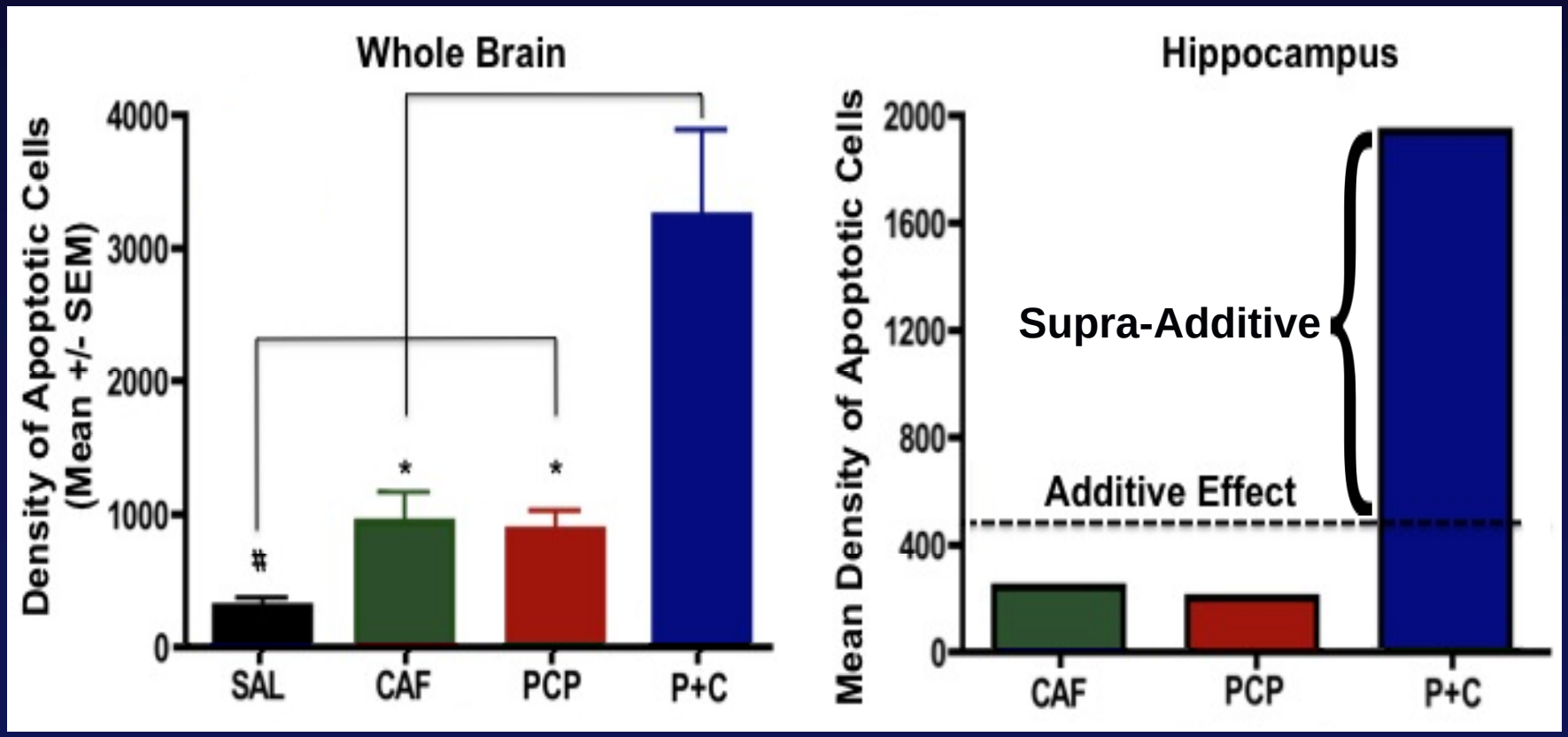
Dobbing & Sands, Early Human Dev., 3, 79, 1979

ARE SINGLE AGENTS MORE (OR LESS) TOXIC THAN MULTI-AGENT COCKTAILS?

There is evidence suggesting that drug combinations containing both an NMDA antagonist and a $GABA_A$ agonist may be more toxic than either agent alone. Alcohol, which acts through both NMDA and GABA receptors, is quite toxic in mice, monkeys and humans.

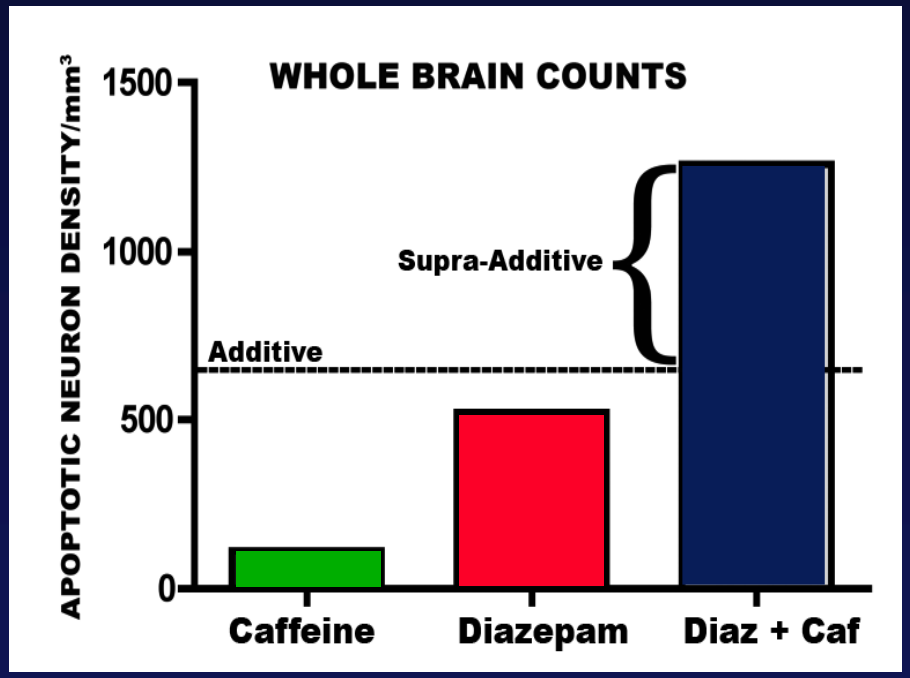
There is also evidence that other types of drug combinations may increase toxicity of anesthetic agents. For example, we have found that caffeine potentiates the toxicity of both NMDA antagonists and $GABA_A$ agonists, and caffeine is used in very high doses to counteract apnea in premature infants who are simultaneously being exposed to anesthetic drugs.

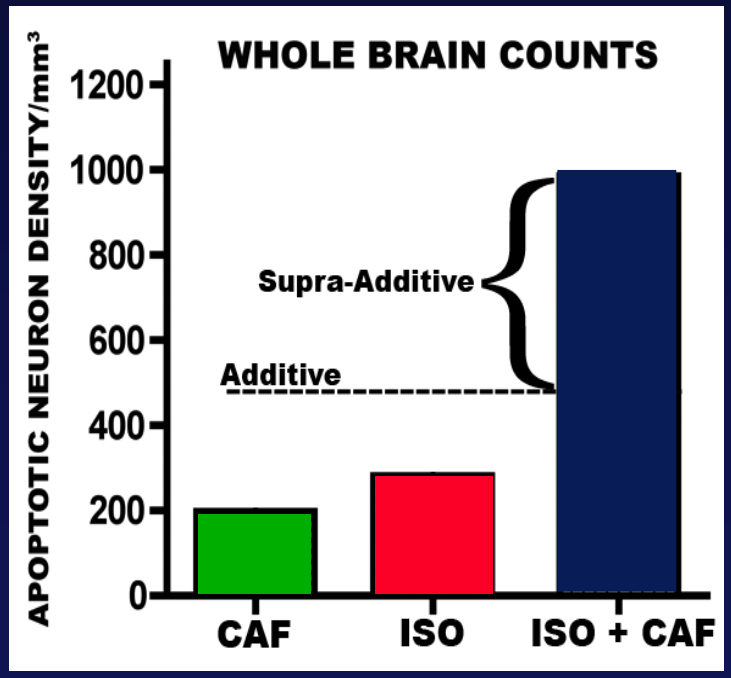
CAFFEINE POTENTIATION of NEUROAPOPTOSIS INDUCED BY PCP (NMDA ANTAGONIST)



Yuede et al., 2010

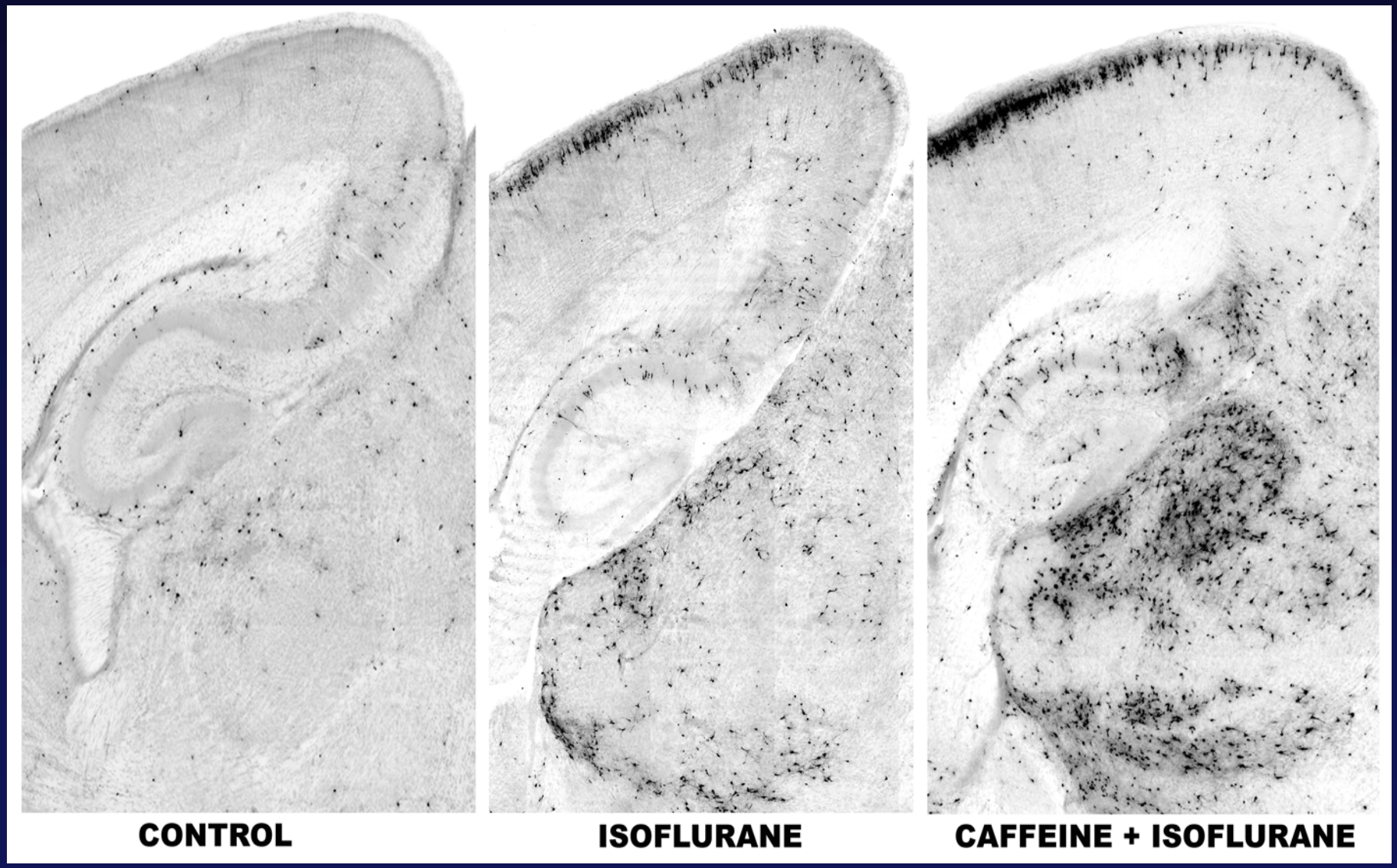
CAFFEINE POTENTIATION of NEUROAPOPTOSIS INDUCED by DIAZEPAM OR ISOFLURANE





Olney et al., 2010

CAFFEINE POTENTIATION OF ISOFLURANE'S APOPTOGENICITY



CAFFEINE

Caffeine is administered prophylactically in the NICU to apnea-prone premature infants to stimulate respiration, which reportedly reduces risk for cerebral palsy.

Premature infants treated with caffeine in the NICU are also exposed semi-chronically to anesthetic drugs. Our preclinical findings* suggest that while caffeine is protecting against hypoxic (excitotoxic) cell death, it may combine with anesthetic drugs to potentiate apoptotic cell death.

Thus, under some circumstances (minimal anesthesia) the effect of caffeine may be beneficial, and under other circumstances (maximal anesthesia) it may be detrimental. Unfortunately, the detrimental effect can happen very quietly without waving red flags.

*** Caffeine blood levels in our infant mice are in the range considered safe and therapeutic for premature human infants.**

INTERESTING PARADOX

Our infant mouse studies support the following conclusions:

Caffeine stimulates respiration, enhances brain oxygenation, counteracts hypoxia and prevents excitotoxic neurodegeneration.

Isoflurane, at 75% MAC, suppresses respiration and renders mouse pups apneic, hypoxic and cyanotic unto death within about 2 hrs.

If we co-administer caffeine with isoflurane (75% MAC), the mouse pups are pink, healthy and happy at 2 hrs.

When we examine the brains, the pale, cyanotic pups exposed to isoflurane alone have a modest amount of neuroapoptosis, whereas the pink and happy caffeine + isoflurane pups have a massive display of neuroapoptosis.

What is caffeine trying to tell us?

Translating Developmental Time From Experimental Species To Humans: An Interdisciplinary Approach

Barbara Clancy PhD
University of Central Arkansas
University of Arkansas for Medical Sciences
University of Arkansas at Little Rock

Barbara Finlay PhD
Richard Darlington PhD
Cornell University

Model systems are required
DIFFERENT MODELS, GOOD REASONS

	gestation (d)	% of studies
Precocial		
Macaques	165	1%
Cats	65	<1%
Guinea pigs	69	<1 %
Spiny mice	39	<1%
Altricial		
Rabbits	31	<1%
Hamsters	16	1%
Ferrets	41	1%
Rats	22	50%
Mice	19	45%

Birth/gestation % may not be ideal milestones for comparisons

Addressing the Challenge

- Proportions development — Robinson & Dreher 1990
- Anatomical/stages comparisons — Carnegie stages, Bayer et al. 1993
- Vulnerability patterns — Dobbing & colleagues 1970s

•RULES OF THUMB

Rat brain PN 5-7 = Human newborn?

•Vague

•Restricted rat/primate/human

•All neural regions grouped together

But different vulnerability patterns
for different brain regions?

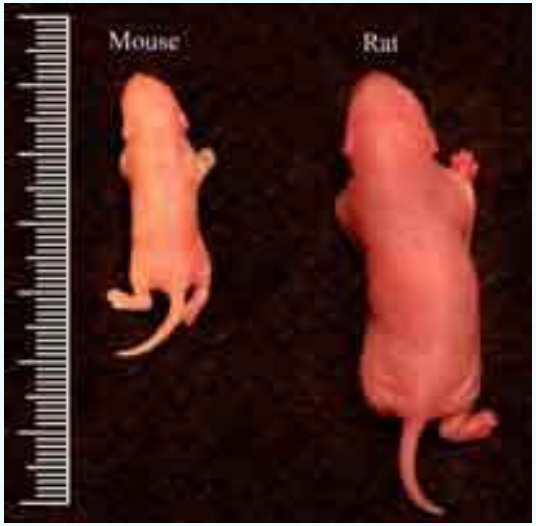
Comparisons using “anchor” events

	RAT	MONKEY	HUMAN
conception	**1**	**1**	**1**
Event 1 (example: corpus callosum appears)	**18**		
Event 2 (example: rod neurogenesis peaks)		**85**	
eye opening	**36**	**126**	**160**

A novel approach for this critical challenge

EVOLUTIONARY PRINCIPLES

Development



* constrained

* conserved

HOW DO BRAINS GET BIGGER?

Striking conservation in the order of neural development

$$\ln (PC\ day - k) = SP + ST$$

derived to understand brain evolution

Finlay and Darlington, 1995, Science 268:1537-1668

Clancy, Darlington and Finlay 2000, Neuroscience, 105:7-17
Clancy, Darlington and Finlay, 2001, Developmental Science, 3:57-66
Clancy and Finlay, 2001, Readings in Language Development, Blackwell Publishers
Finlay, Clancy and Kingsbury, 2003 Advances in Infancy Research, Praeger Press

Many mathematical methods tested

General Linear Model

Scores derived from regression coefficients

quantifies “goodness” of fit

- Events – late score high

- Species – slow developing score high

Darlington 1995
Darlington et al. 1999

- Express PC days on log scale (Y)
  - “dog years” do not work
- Adjust using constant (4.34)
- Predict Y using “dummy” variables
  - 1 for event or species, 0 for all others

$$\ln(\text{PC day} - k) = SP + ST$$

2000 - 9 mammalian species

90 different developmental events

Gleaned from empirical literature

Currently

11 mammalian species

>225 different developmental events

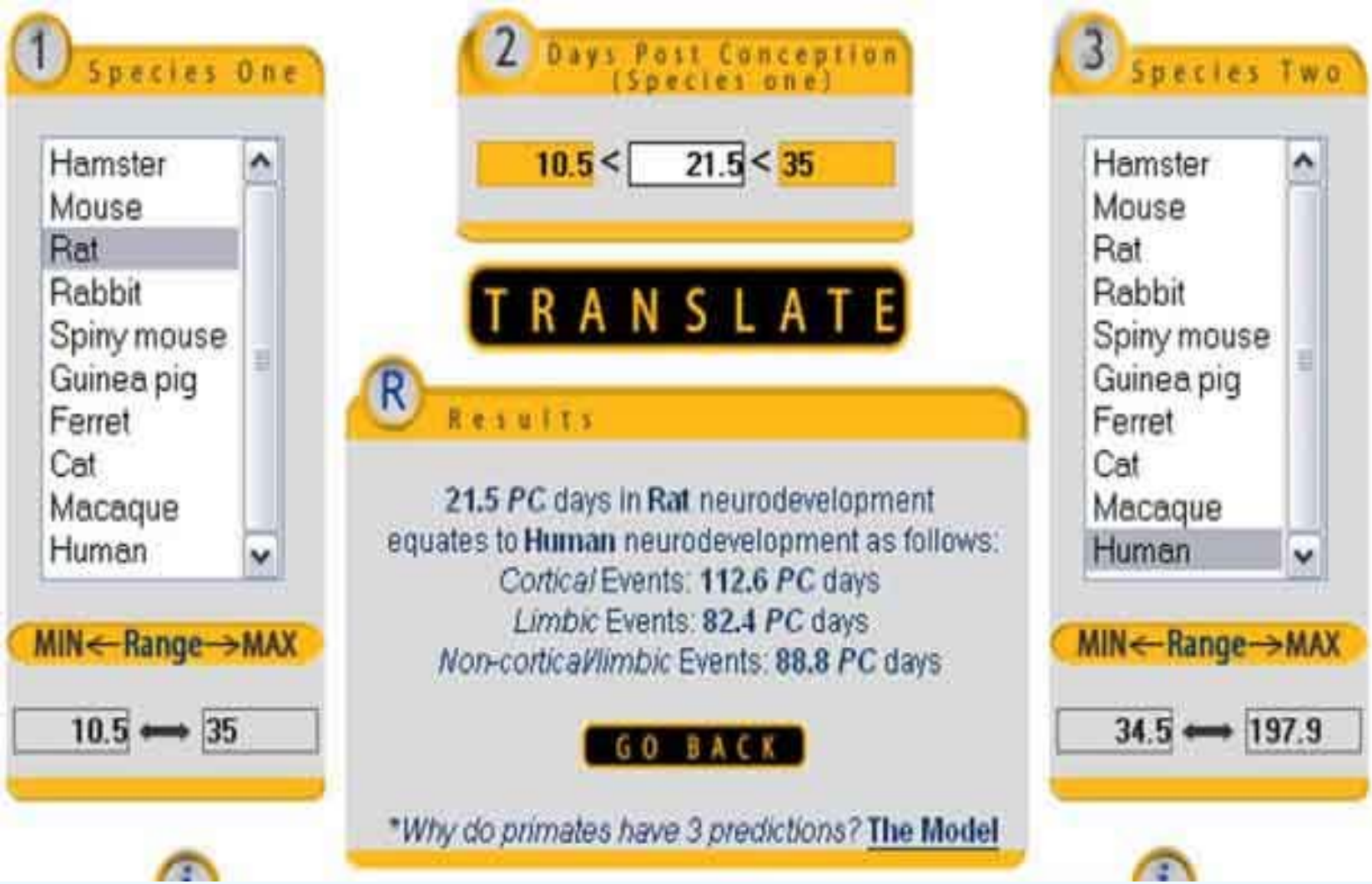
model takes <u>all</u> relationships into account

	post conception day		
	RAT	MONKEY	HUMAN
conception	**1**	**1**	**1**
Event 1 (example: corpus callosum appears)	**18**		
Event 2 (example: rod neurogenesis peaks)		**85**	
eye opening	**36**	**126**	**160**

	RAT	MONKEY	HUMAN
conception	**1**	**1**	**1**
Event 1 (example: corpus callosum appears)	**18**	?	?
Event 2 (example: rod neurogenesis peaks)	?	**85**	?
eye opening	**36**	**126**	**160**

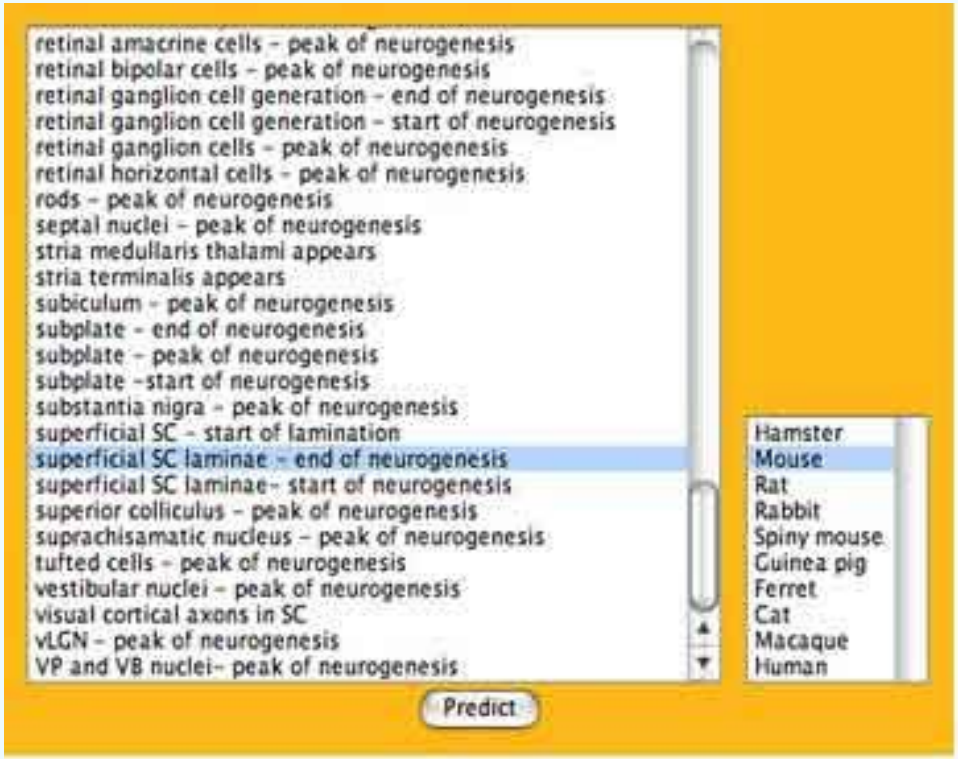
	RAT	MONKEY	HUMAN
conception	**1**	**1**	**1**
Event 1 (example: corpus callosum appears)	**18**	**73**	**91***
Event 2 (example: rod neurogenesis peaks)	**25**	**85**	**107**
eye opening	**36**	**126**	**160**

* PC88 Ashwell et al 1996 – date of conception?



Clancy, et al NeuroToxicology, 2007; Clancy, et al Neuroinformatics, 2007
Site accessed > 50,000 by >600 universities, medical centers, research centers ; Citations>125

predict specific events



What can “translations” from experimental species tell us about anesthesia, and human brain development?

1) Is modeling useful/preferable for humans?

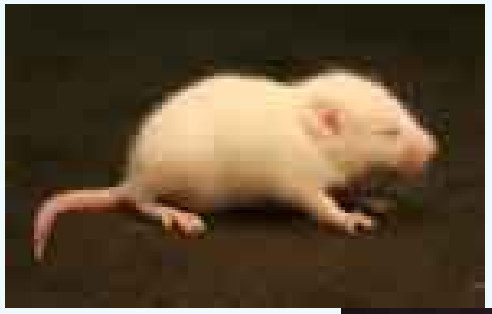
2) Are primates different?

3) Does somatic development detach from neural?

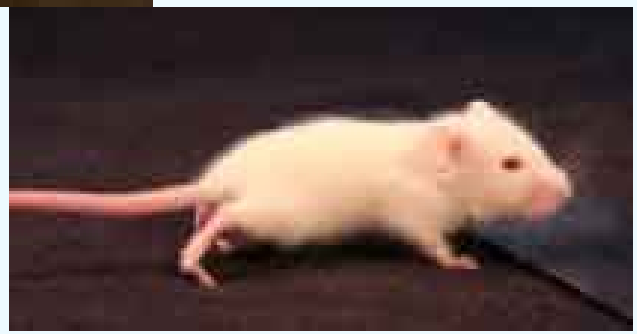
4) How to relate anesthesia ages and timing? Are there different times for different region?

Is modeling useful for humans?
(Preferable? controls, conception dates, sampling intervals)

Example EYE OPENING



Mouse PN 12.5



Rat PN 14.5

Humans 28 weeks?
Model 22 weeks?
4D sonogram imaging – model was right

bootstrap effect

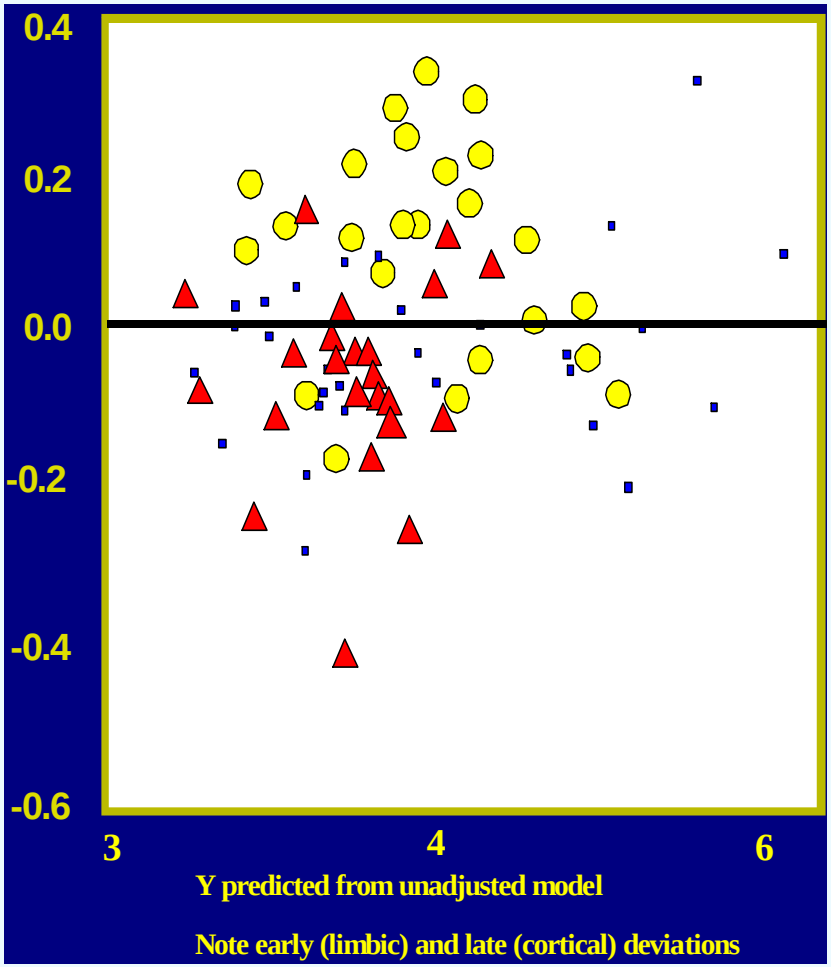
PRIMATES
ARE
DIFFERENT

cortical events

limbic events

Modeling allows specific adjustments

Cortical events – add
Limbic events – subtract



PRIMATES ARE DIFFERENT

GABA?

Modeling will allow translations

Humans	Empirical	Unadjusted Model
GABAergic Events	**PC day**	**PC day**
MGE produces Lhx6 in GABAergic cells - start		61
GABAir cells lower intermediate zone/subventricular border	77 [1]	71
Lhx6 first in cortex in GABAergic cells		75
GABA cells in subplate/ cells from MGE reach cortex	140 [2]	82
MGE produces Lhx6 in GABAergic cells - end		92
GAD activity adult-like	280 [3]	197

1 Zecevic and Milosevic, 1997. 2 Letinic et al., 2002. 3 Romijn et al., 1991

Clancy, Teague-Ross & Nagarajan (2009) Frontiers in Neuroanatomy
Clancy, DeFelipe, Espinosa, Fairen, Jinno, Rockland, Tamamaki, & Yan (2010) Frontiers in Neuroanatomy.

NOT EVERYTHING FITS

Some events need adjustments Limbic/Cortical/ GABA

Some events cannot be included (in THIS model)

- BIRTH
- SYNAPTOGENESIS SURGE
- WEANING

Differences in maternal/ fetal interactions

POSTNATAL EVENTS

Reflexes, behavior

Does somatic development detach from neural?

Currently testing somatic/motor

Onset:

- Rooting
- Crawling
- Social play
- Walking *(Garwicz et al. 2009)*

- *Sexual maturation may NOT fit*
- *Onset of walking may fit*

HOW TO RELATE ANESTHESIA INTERVALS

WHEN DATA IS BASED ON EXPERIMENTAL SPECIES

SIMILAR NEURAL EVENTS

Mouse PN 11 = Rat PN13 = Human

Limbic PC 142
Cortical PC196
Other PC154

Post conception	**Rat**	**Macaque**			**Human**		
		Limbic	Other	Cortex	Limbic	Other	Cortex
Ikonomidou et al. 1999 **Jevtovic-Todorovic et al. 2003**	27	85	92	116	107	116	147
Fredriksson, et al. 2004	30	95	103	131	121	131	166

Timing of rodent brain events may not be not comparable to primate dates

rat	human		
20	77	83	100
birth	84	91	110

timing??

2-4 hours in rats = 1-2 DAYS in humans?

LENGTH OF EXPOSURE TO STRESS, DRUGS, TOXINS, ETC.

METABOLIC DEVELOPMENT?

Modeling useful similarities (and difference) across species in any process constrained to occur in sequence

- METABOLIC RATES
- MALE/FEMALE DIFFERENCES
- CRITICAL WINDOWS?
- GENE EXPRESSION
- AGES (birth – puberty)
- ORGANS (heart/lung)
- NEW STRAINS, NEW SPECIES (cows? need somatic data)
- TEST NEW SYSTEMS (GABA, NMDA receptors)

VALUE OF AN INTERDISCIPLINARY APPROACH

- Individual Event - does it fit?

- Is any system different?
  - Systems - visual, limbic, cortical)
  - Systems - GABAergic, dopamine, cholinergic

(Zhou et al. 2011)

•PRIMATE/RODENT SIMILARITIES AND DIFFERENCES

•DISPARITIES IN DEVELOPMENT OF BRAIN REGIONS

•COMPARISIONS NEURAL /MOTOR/SOMATIC DEVELOPMENT

•SAVINGS IN RESOURCES (TIME, FUNDING, ETC.)

•APPROXIMATIONS, BUT CURRENTLY FEW OTHER OPTIONS

NEEDED:
•DATABASE FROM EMPIRICAL LITERATURE
•MATHEMATICAL ANALYSES

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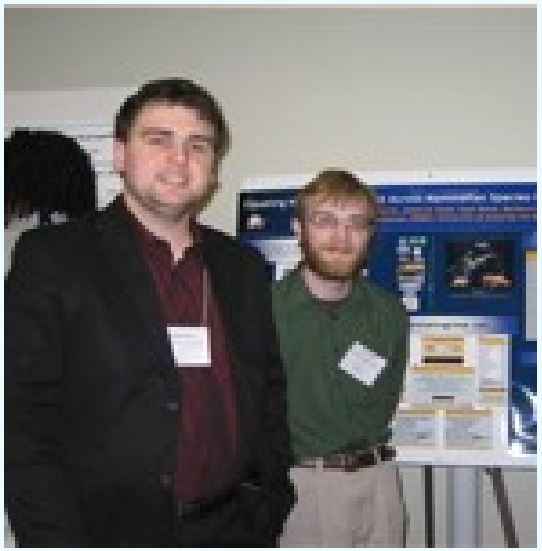

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Ketamine and the neonatal brain: rat pups vs. human babies

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Methodist Healthcare Family

THE UNIVERSITY of TENNESSEE HEALTH SCIENCE CENTER

The New England
Journal of Medicine

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Volume 326 JANUARY 2, 1992 Number 1

HALOTHANE–MORPHINE COMPARED WITH HIGH-DOSE SUFENTANIL FOR ANESTHESIA AND POSTOPERATIVE ANALGESIA IN NEONATAL CARDIAC SURGERY

K.J.S. Anand, M.B., B.S., D.Phil., and P.R. Hickey, M.D.

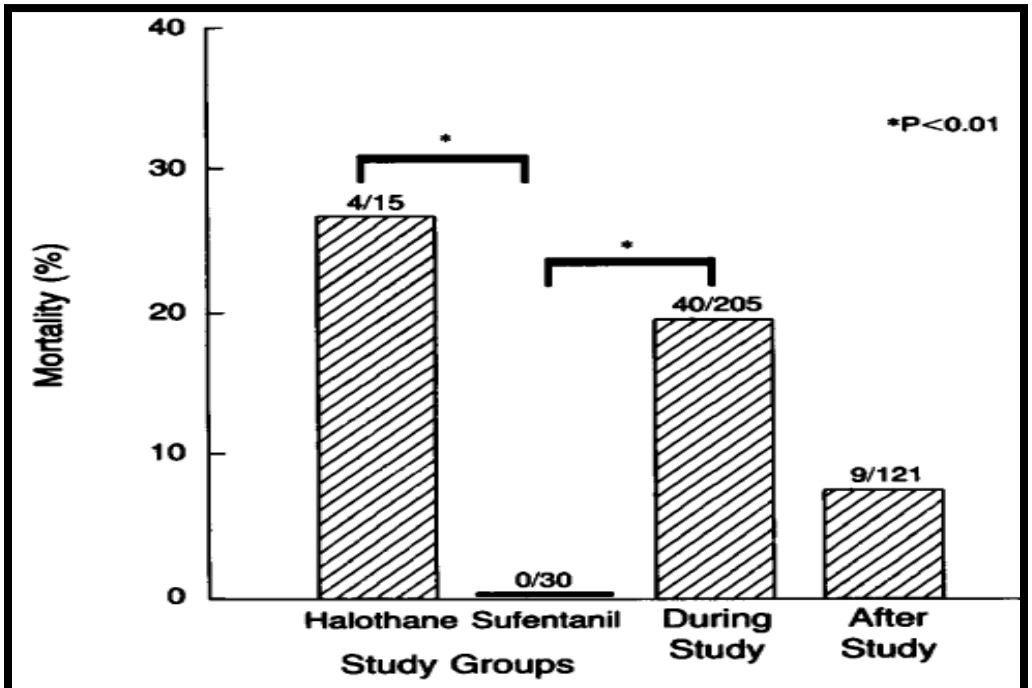

Figure 5. Mortality in the Study Groups and Hospital Mortality

Le Bonheur Children's Hospital
Methodist Healthcare Family
THE UNIVERSITY of TENNESSEE HEALTH SCIENCE CENTER

Best Practice & Research Clinical Anaesthesiology 24 (2010) 475–484

Contents lists available at ScienceDirect

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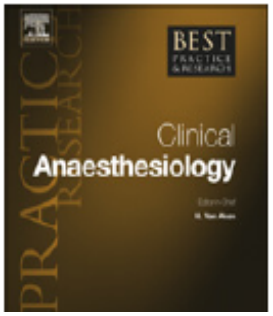

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REVIEW ARTICLE

Use of Analgesic and Sedative Drugs in the NICU: Integrating Clinical Trials and Laboratory Data

XAVIER DURRMEYER, LASZLO VUTSKITS, KANWALJEET J. S. ANAND, AND PETER C. RIMENSBERGER

Neonatal Intensive Care Unit [X.D.], Centre Hospitalier Intercommunal de Créteil, Créteil 94000, France; Departments of Pediatrics [X.D., P.C.R.], Anesthesiology, Pharmacology, and Intensive Care [L.V.], and Neuroscience [L.V.], University Hospital of Geneva, Geneva 1211, Switzerland; Department of Pediatrics [K.J.S.A.], University of Tennessee Health Science Center, Memphis, Tennessee 38103

Can Adverse Neonatal Experiences Alter Brain Development and Subsequent Behavior?

Biology of the Neonate 2000; 77: 69-82

K.J.S. Anand Frank M. Scalzo

Department of Pediatrics, University of Arkansas for Medical Sciences, and Pain Neurobiology Laboratory, Arkansas Children's Hospital Research Institute, Little Rock, Ark., USA

Pain, plasticity, and premature birth: a prescription for permanent suffering?

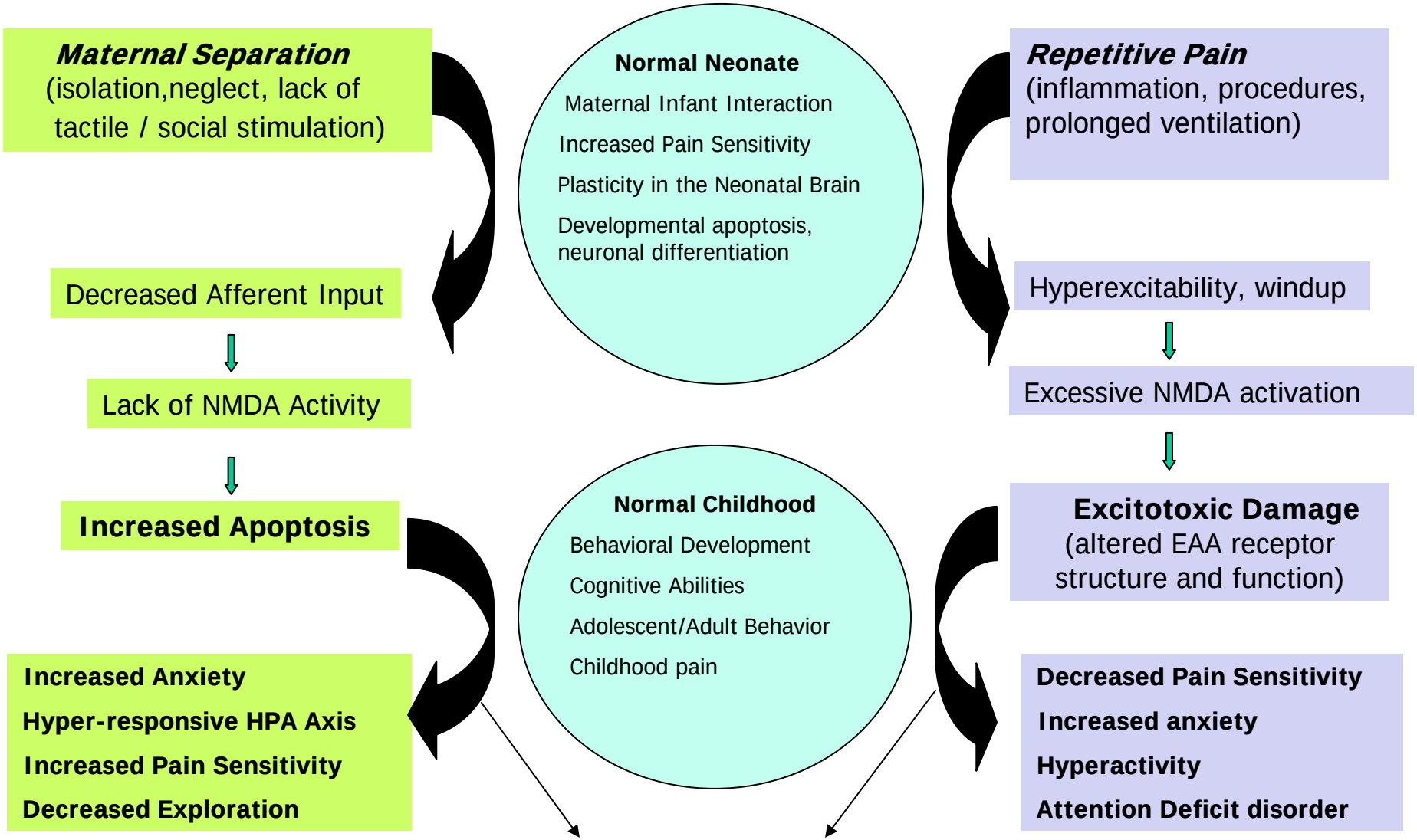
A collection of clinical and animal studies suggest that exposure to pain during the neonatal period leads to long-term changes in neural circuitry and behavior, contradicting the theory that infants don't 'remember' painful experiences.

ABOUT 11,000 NEWBORN infants are receiving intensive care in the U.S. today.

K.J.S. ANAND

sponse to tactile or noxious stimuli. This led to an increase in pain response behav-

Anand & Scalzo. ***Biology of the Neonate*** 77:69-82, 2000



Cognitive Impairment

Behavioral problems

Poor Socialization Skills

NMDA receptor antagonists induce widespread neurodegeneration in developing rat brains

Blockade of NMDA Receptors and Apoptotic Neurodegeneration in the Developing Brain

Chrysanthy Ikonomidou,* Friederike Bosch, Michael Miksa, Petra Bittigau, Jessica Vöckler, Krikor Dikranian, Tanya I. Tenkova, Vanya Stefovska, Lechoslaw Turski, John W. Olney

1999 VOL 283 SCIENCE www.sciencemag.org

Ketamine and neurodegenerative mechanisms

(Anand et al., *Anesthesiology*, 2004, 2005, 2007

Bhutta, … Anand et al. *Acta Pædiatrica*, 2007)

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A DIFFERENT VIEW

Anaesthetic neurotoxicity in rodents: is the ketamine controversy real?

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Ontogeny of NMDA receptor subunits in the Rat Brain

Subunit	Fetal	PND 6	PND 19	Adult	
NR1		+	++	+	Cortex
NR2B		+++	+		Cortex
NR1	+	+	+	+	Cere-bellum
NR2A		+	++	+++	Cere-bellum
NR2B		+++	++	+	Cere-bellum
NR2C		+	++	+++	Cere-bellum
NR2D		++			Cere-bellum

Haberny et al., *ToxSci*, 68:9-17, 2002

Ontogeny of NMDA receptor subunits in the Human Cortex

Subunit	Fetal	Neonatal	Adult
NR1	++	+	+
NR2A	+	++	+++
NR2B	++	+	
NR2C	+	++	+++
NR2D	+++	++	

Haberny et al., *ToxSci*, 68:9-17, 2002

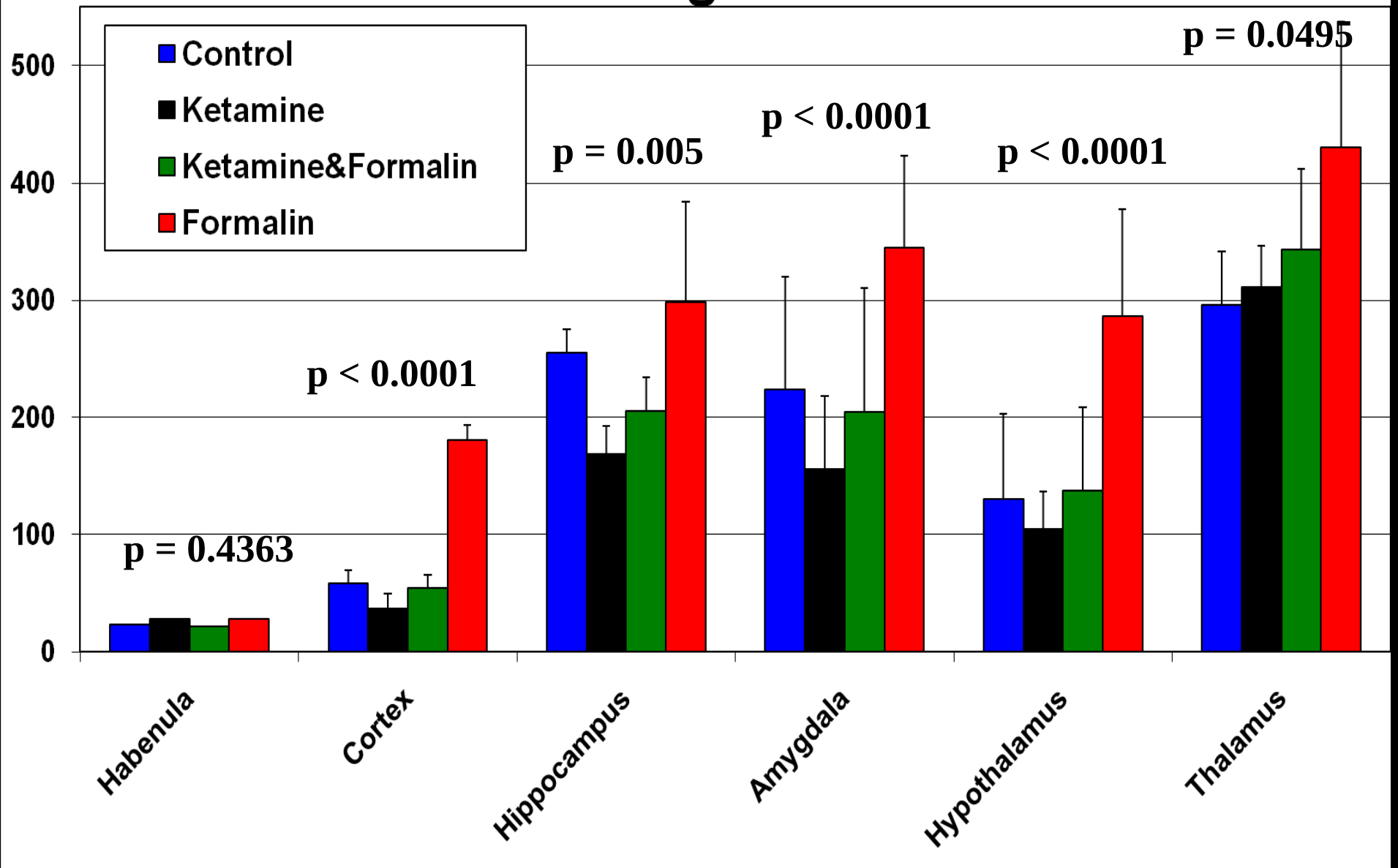
"All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy"

Philipus Aureolus Theophrastus
Bombastus von Hohenheim-Paracelsus

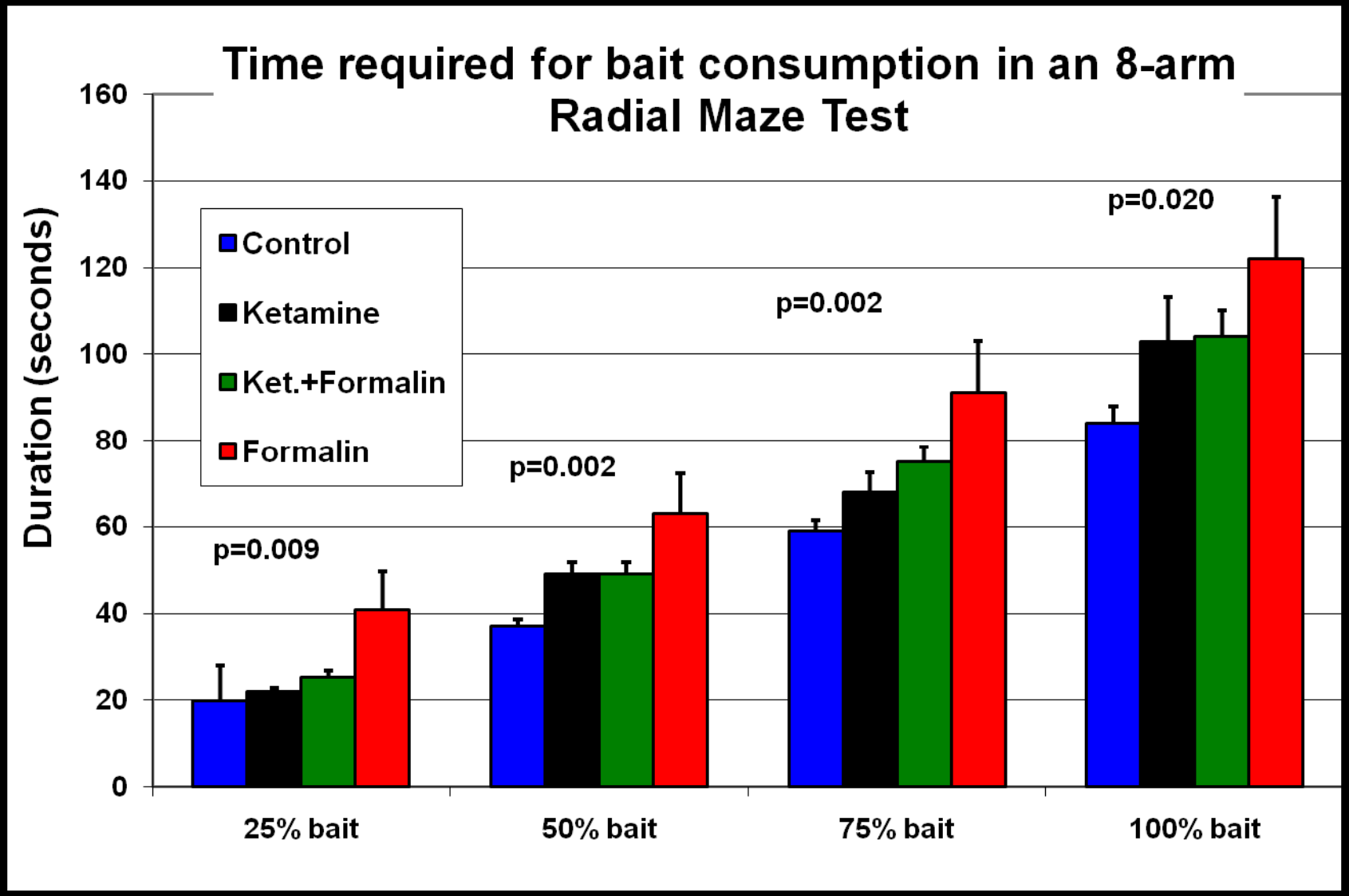
Supraspinal responses to repetitive inflammatory pain

- Long-Evans rat pups (n=62), cross-fostered at P0, stimulated daily from P1 to P4
- Randomly assigned to Control gr. *vs.* 4%formalin *vs.* Ketamine (5mg/kg) *vs.* Ketamine + formalin
- Sacrificed on P5, perfused with 4% PFA (0-4°C)
- Cryostat sections (20µm) immunostained with FluoroJade-B, a specific marker for cell death
- Cell counts were made using MCID software (Imaging Research Inc., St. Catharines, Ont., Canada)
- Counts verified by two blinded observers

Neuronal Cell Death in selected brain regions



Impaired long-term visual-spatial memory following Repetitive Neonatal Pain



Conclusions

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Ketamine Reduces the Cell Death Following Inflammatory Pain in Newborn Rat Brain

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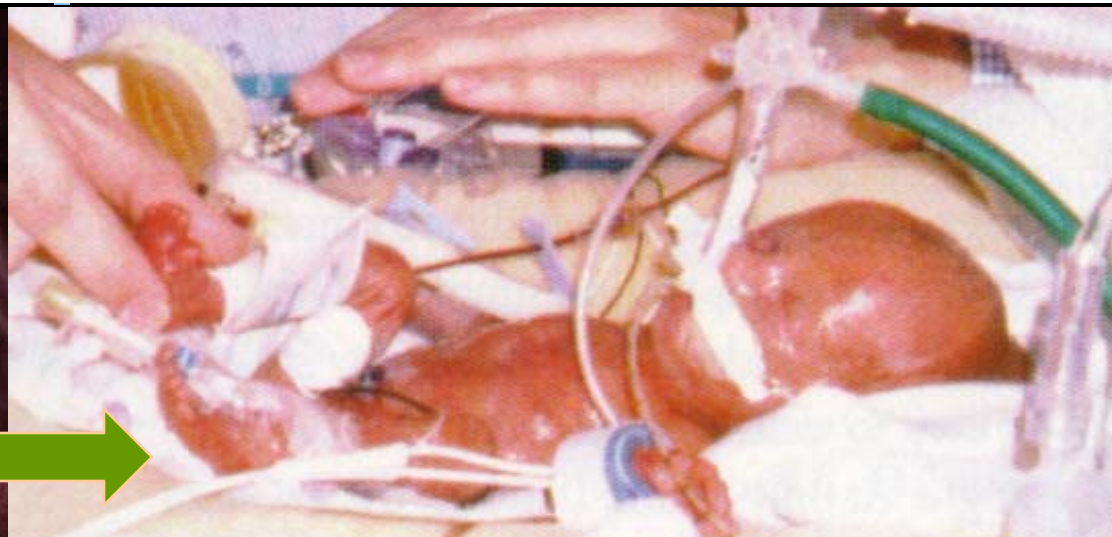
Pain Neurobiology Lab, AR Children's Hospital Research Institute, Little Rock, Arkansas 72202; Department of Pediatrics, University of Arkansas for Medical Sciences, College of Medicine, Little Rock, Arkansas 72205



Pilot RCT: Background

- Significant organ dysfunction follows cardiopulmonary bypass (CPB) →Affects all major organs including brain
- CPB-induced cellular injury is mediated via Glutamate excitotoxicity *(Redmond et al, 1994, 1995)*
- Immature neurons are potentially more susceptible to excitotoxic cell death

Pilot RCT: Hypothesis

- Ketamine (2 mg/kg), via NMDA receptor blockade and anti-inflammatory effects, will block excitotoxic neuronal cell death and inflammation, thereby offering neuroprotective effects during CPB

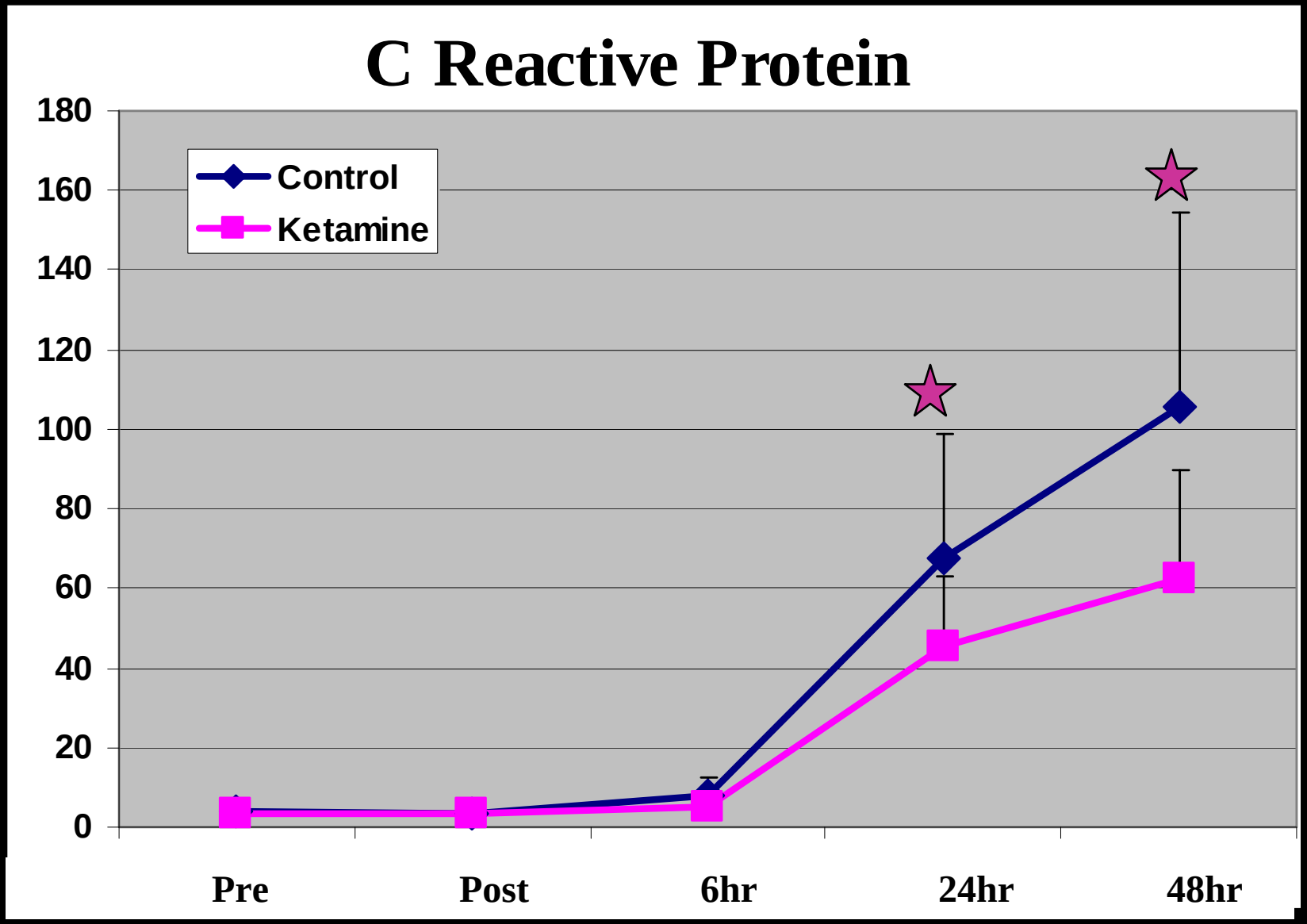
Pilot RCT: Methods

(Bhutta, Anand, et al, PCCM, 2011)

- Blinded, placebo-controlled RCT in children age < 1 year, with no chromosomal abnormalities, undergoing surgical VSD repair
- Cases received Ketamine 2 mg/kg pre-CPB (n=13), Controls received Saline (n=11); same anesthetic & surgical protocols
- Plasma markers of inflammation and CNS injury measured at end of surgery, 6 hrs, 24 hrs, and 48 hrs after surgery
- MRI/MR-Spectroscopy before CPB and at hospital discharge in cases and controls (n=5 in each group)
- Plasma ketamine enantiomers measured during/after surgery
- Bayley Scales for Infant Development (BSID-II) assessed before and 2-3 weeks after surgery

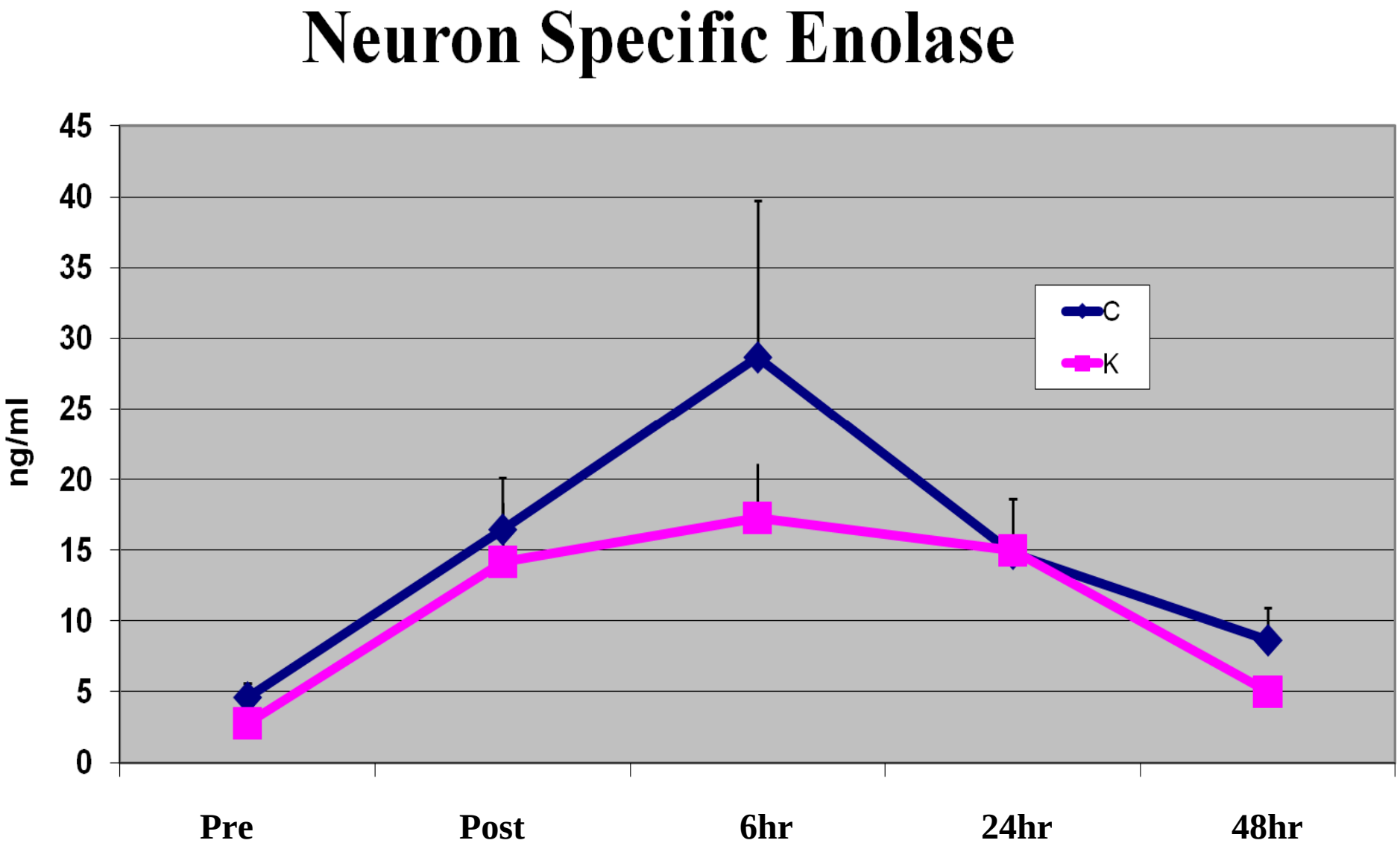
Pilot RCT: Effects on inflammation

(Bhutta, Anand, et al, PCCM, 2011)



Pilot RCT: Effects on Neuronal injury

(Bhutta, Anand, et al, PCCM, 2011)



Pilot RCT: MR Spectroscopy

(Bhutta, Anand, et al, PCCM, 2011)

- ***TE 35 data:*** Glx levels lower in the Ketamine group vs. Control group (p=0.02) in the Frontal White Matter (FWM) postop. Glx decreased in Ketamine group pre- vs. post-surgery (p=0.02)

- ***TE 144 data:*** Creatine (Cr) levels increased in the Control group pre- vs. post-surgery in the Basal Ganglia (BG) (p=0.02)

- In the BG and FWM, Choline (Cho) levels decreased pre- vs. post-surgery ($p<0.03$) in the Ketamine group, but no changes occurred in the control group

Pilot RCT: Conclusions

- Ketamine (2 mg/kg) prior to CPB may reduce CNS injury and inflammation following CPB in infants
- Infants receiving Ketamine show less glutamate release, lower choline (decreased neuronal metabolism) and creatine levels (less energy requirements) in postoperative MR Spectroscopy scans
- NIRS signal postop shows greater chaos/complexity in the Control group
- We propose a large, multicenter RCT to confirm these results and examine the safety of Ketamine use

TOXICOLOGICAL SCIENCES **108(1)**, 149–158 (2009)
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Potential Neurotoxicity of Ketamine in the Developing Rat Brain

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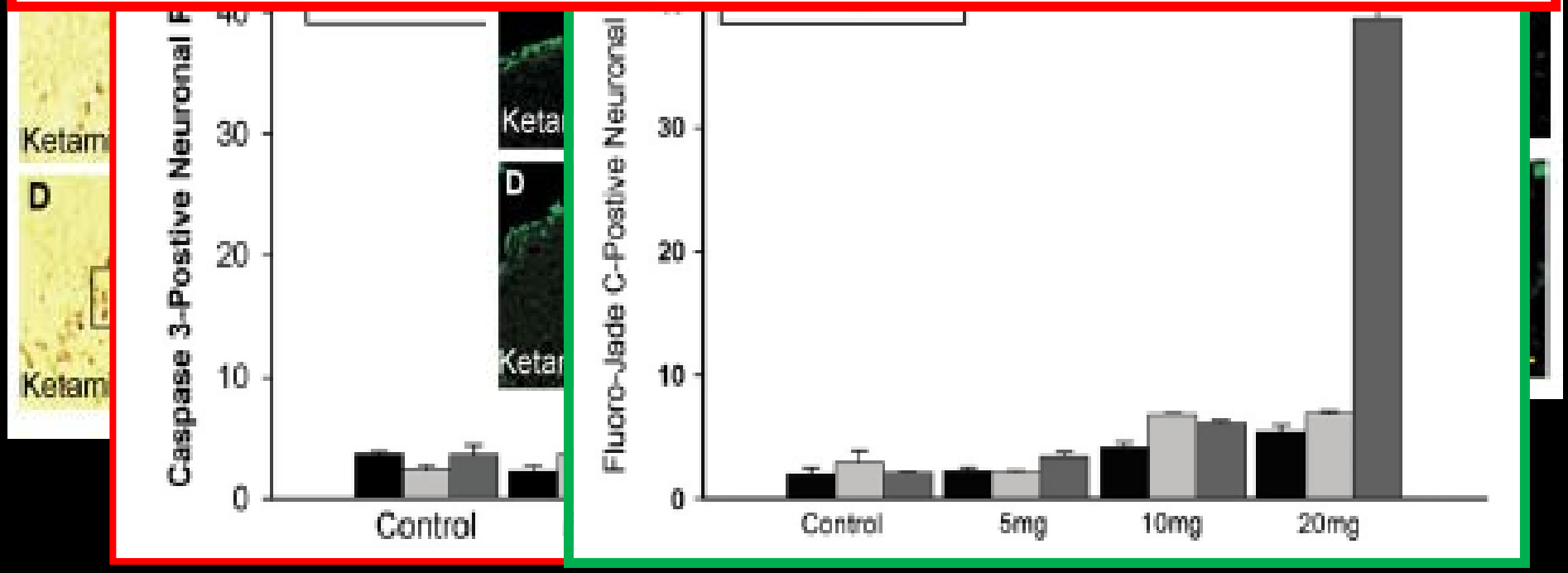


Effects of high-dose Ketamine

- **Effects on non-NMDA receptor populations**
- **Roles of synaptic vs. extra-synaptic NMDA receptor activity**
- **Effects on Neural-Stem Progenitor Cells (NSPCs)**

<u>Non-NMDA receptor effects</u>:

- Agonist activity on dopamine D_2, serotonin 5-HT_2 receptors, μ- and κ-opioid receptors (Kapur & Seeman, 2002)
- Non-competitive antagonism of nicotinic Ach receptors

Effects of high-dose Ketamine

- Extra-synapt[illegible] NMDARs on

REVIEWS

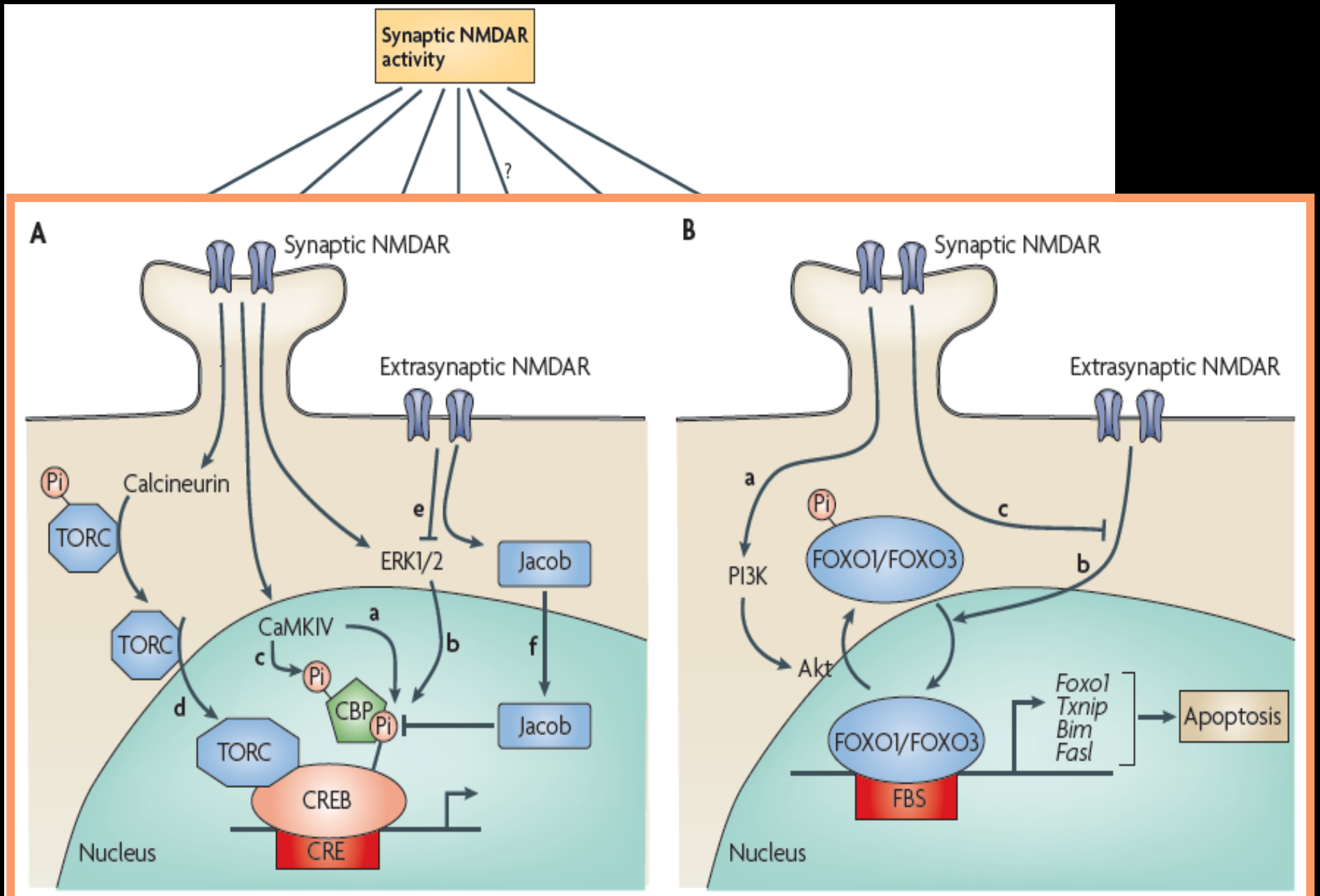
NATURE REVIEWS | NEUROSCIENCE

682 | OCTOBER 2010 | VOLUME 11

Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders

Giles E. Hardingham and Hilmar Bading‡*

Abstract | There is a long-standing paradox that NMDA (*N*-methyl-D-aspartate) receptors (NMDARs) can both promote neuronal health and kill neurons. Recent studies show that NMDAR-induced responses depend on the receptor location: stimulation of synaptic NMDARs, acting primarily through nuclear Ca^{2+} signalling, leads to the build-up of a neuroprotective 'shield', whereas stimulation of extrasynaptic NMDARs promotes cell death.

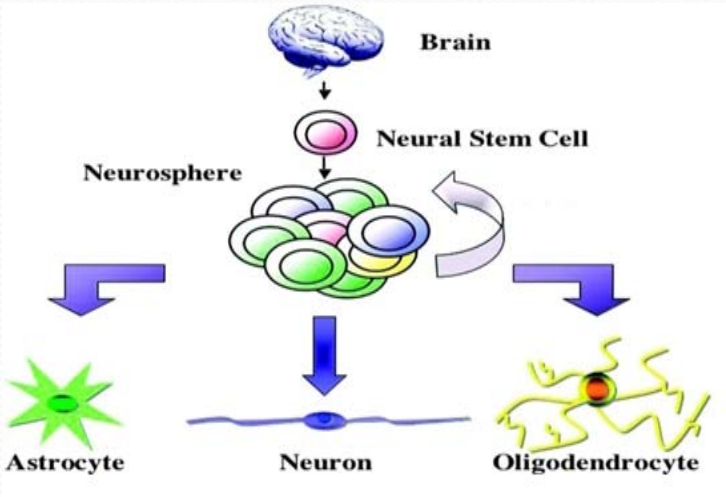


Opposing effects of synaptic and extrasynaptic NMDAR signalling on gene expression.

Hardingham GE, Bading H. Nature Reviews Neuroscience 11: 682-696, 2010.

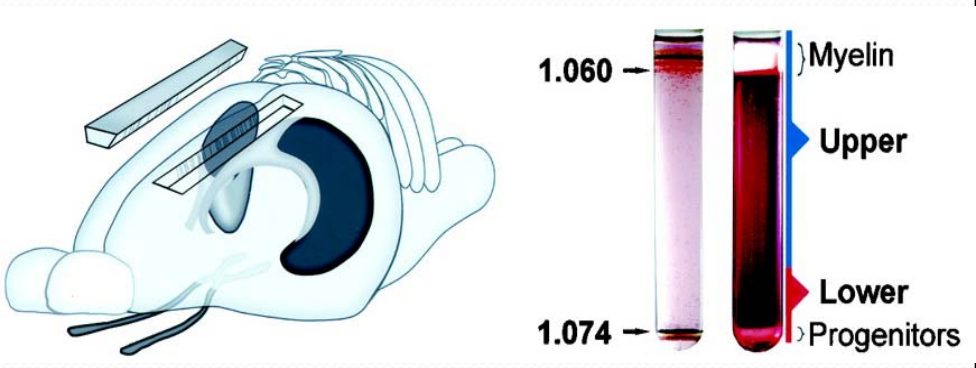
Isolation of NSPCs from the cortex of the embryonic brain at E17

A



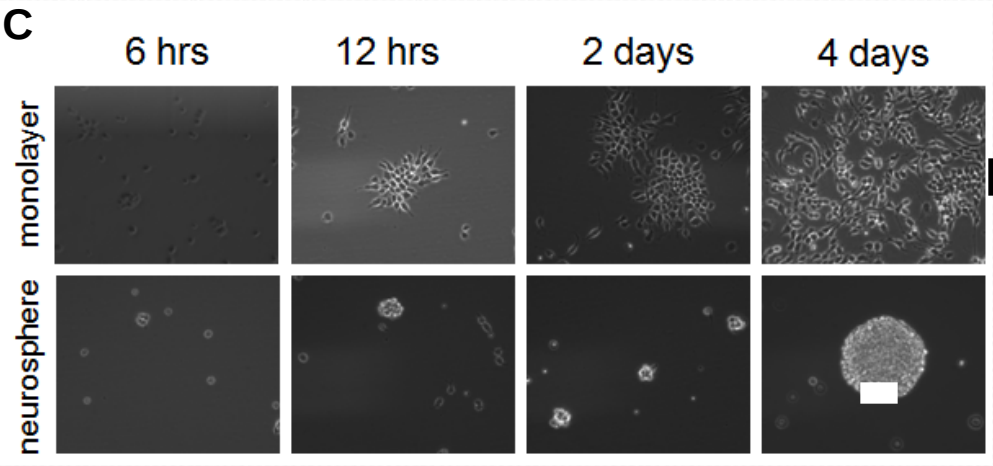
Kosaka N, 2006

B



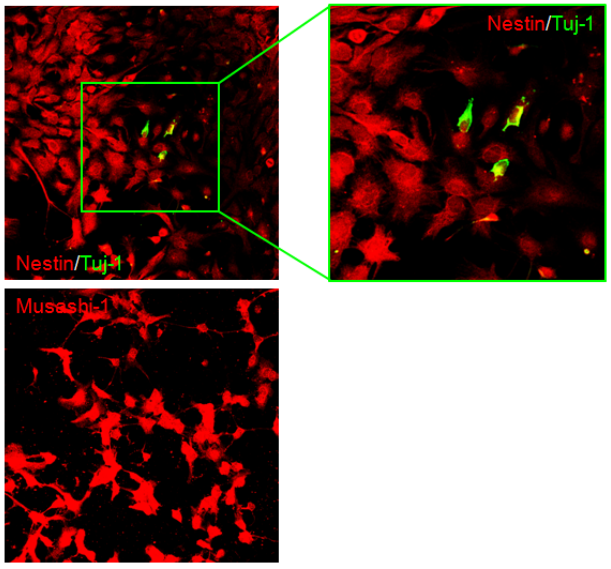
Palmer TD, 1999

C

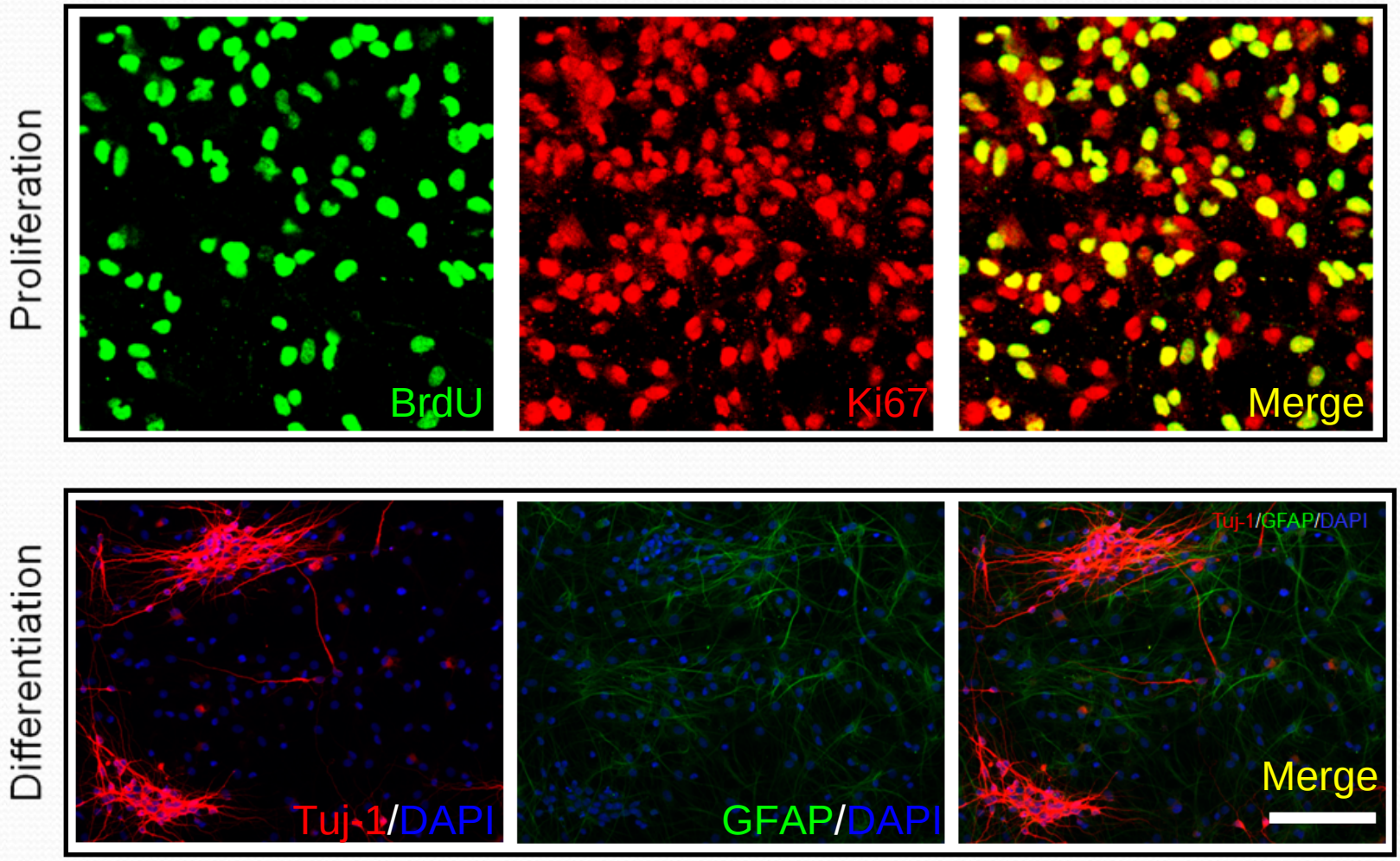


Scale bar: 100 µm

D



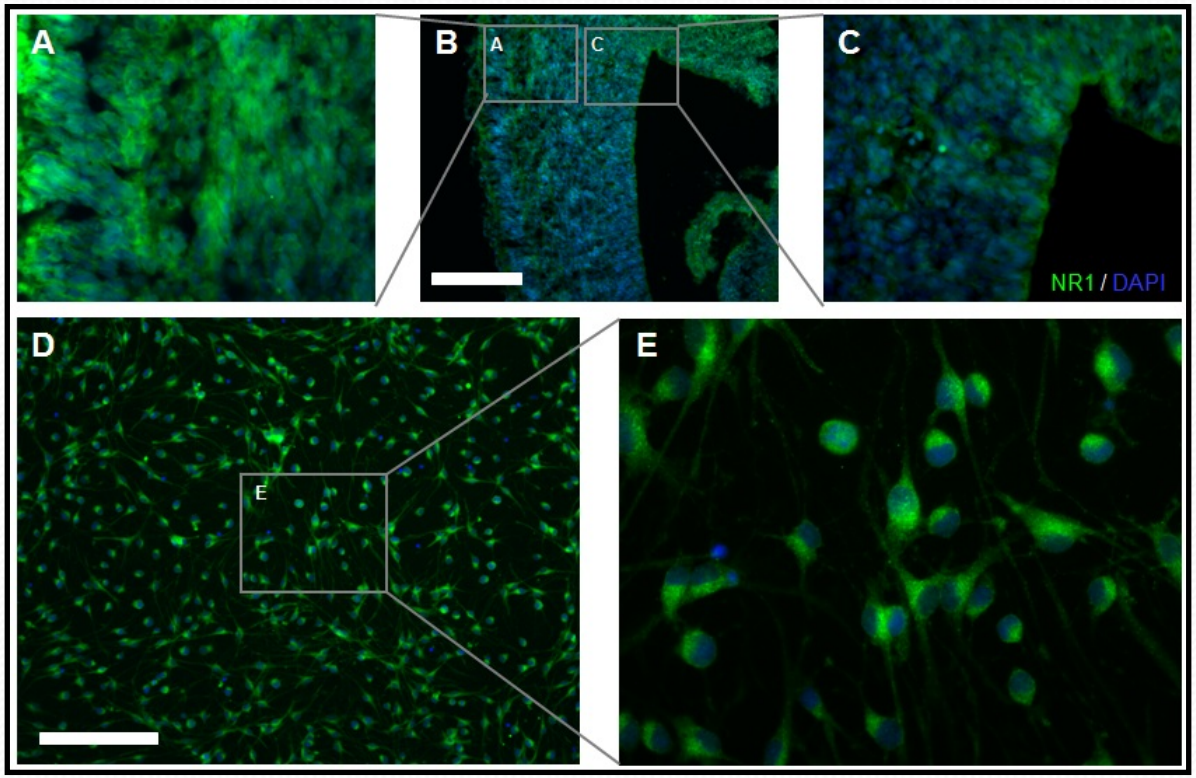
Proliferation and differentiation of NSPCs



Scale bar: 50 μm

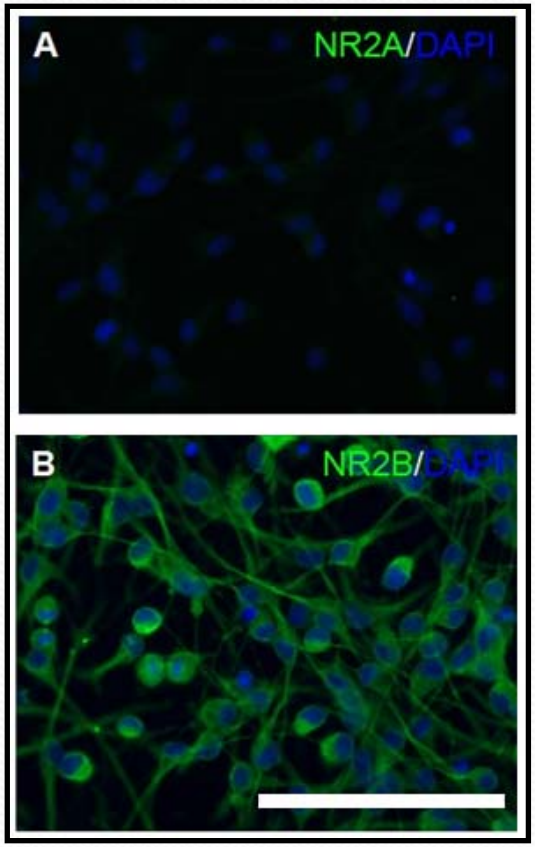
NMDA receptor subunits expressed in NSPCs

NR1



Scale bar: 100 µm

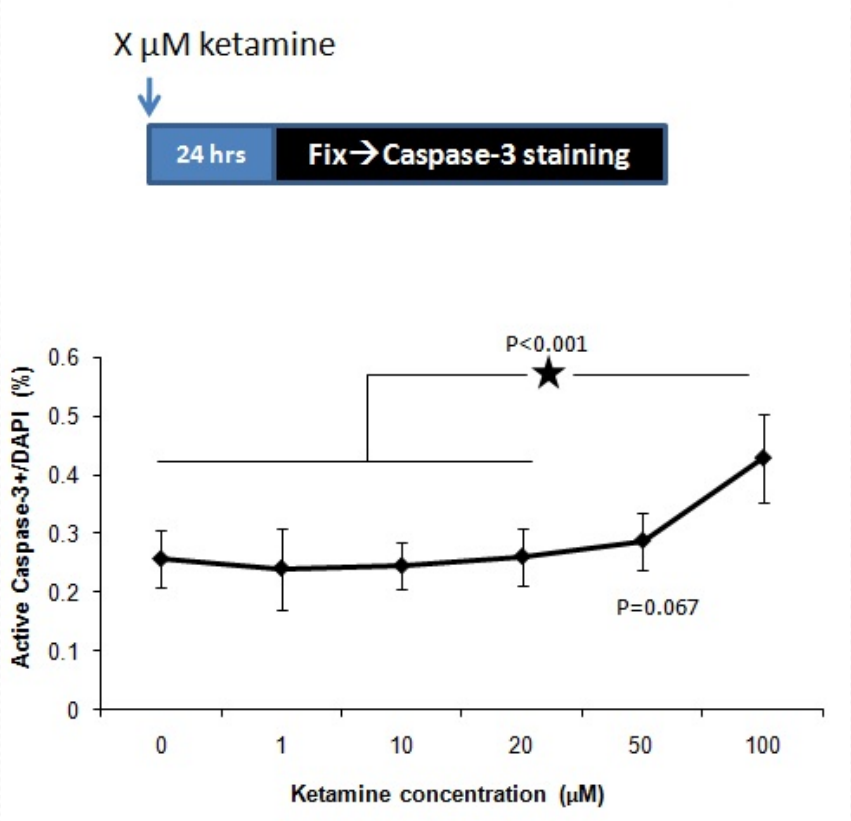
NR2A/B



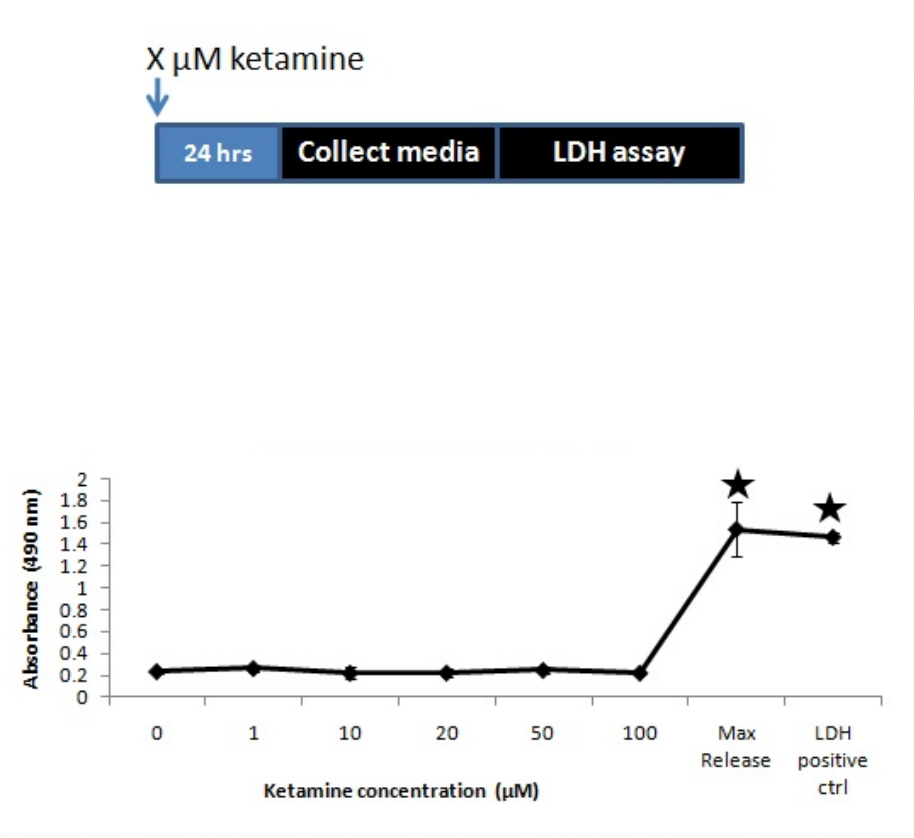
Scale bar: 50 µm

NSPCs are resistant to the neurotoxic effects of Ketamine

Dose Response - Apoptosis

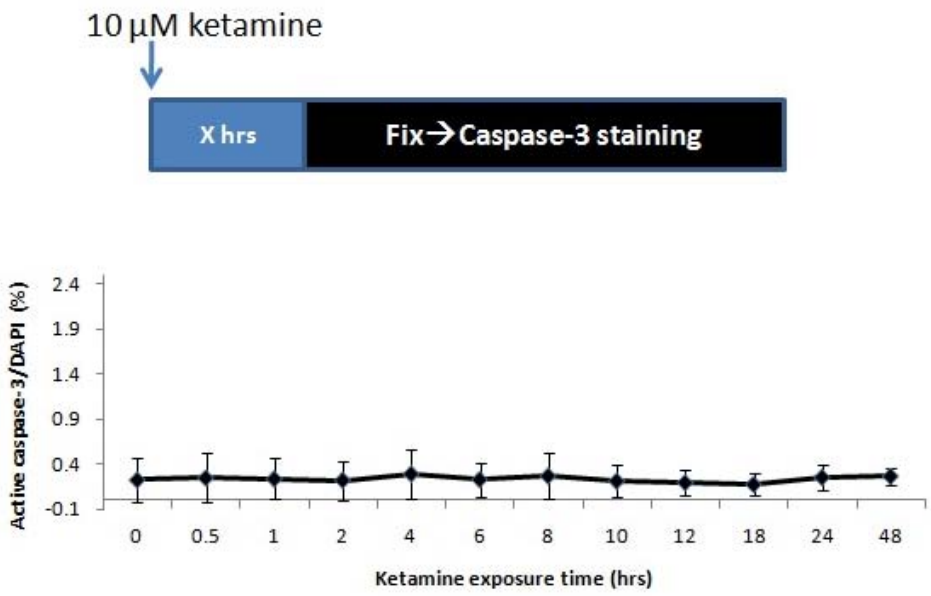


Dose Response - Necrosis

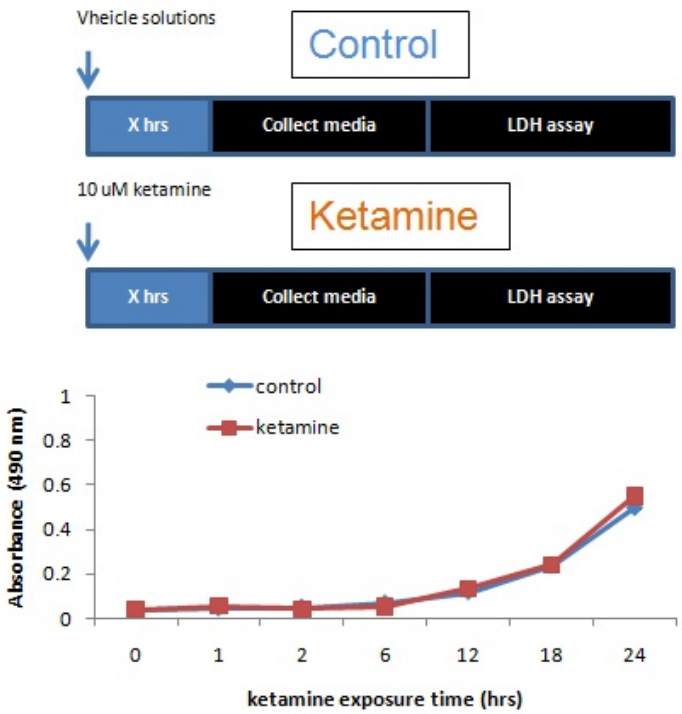


NSPCs are resistant to the neurotoxic effects of prolonged Ketamine exposure

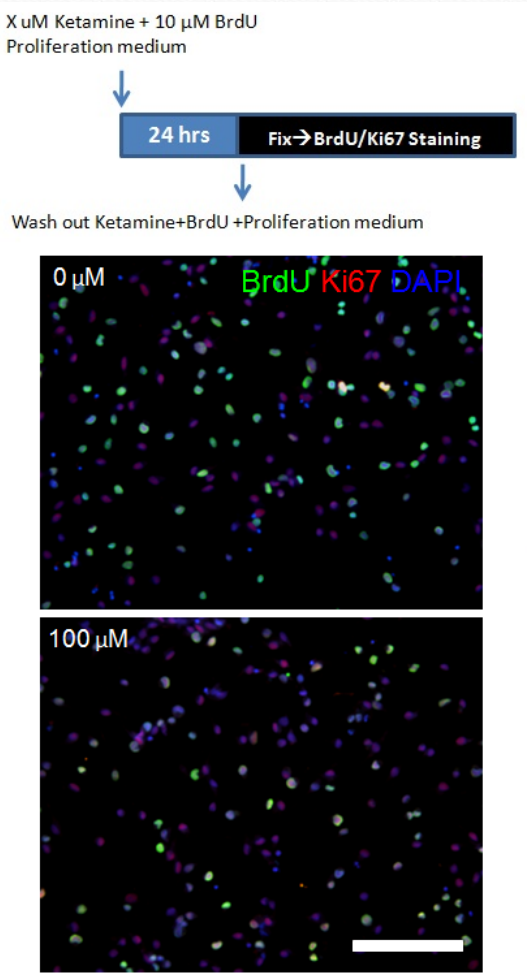
Time Response - Apoptosis



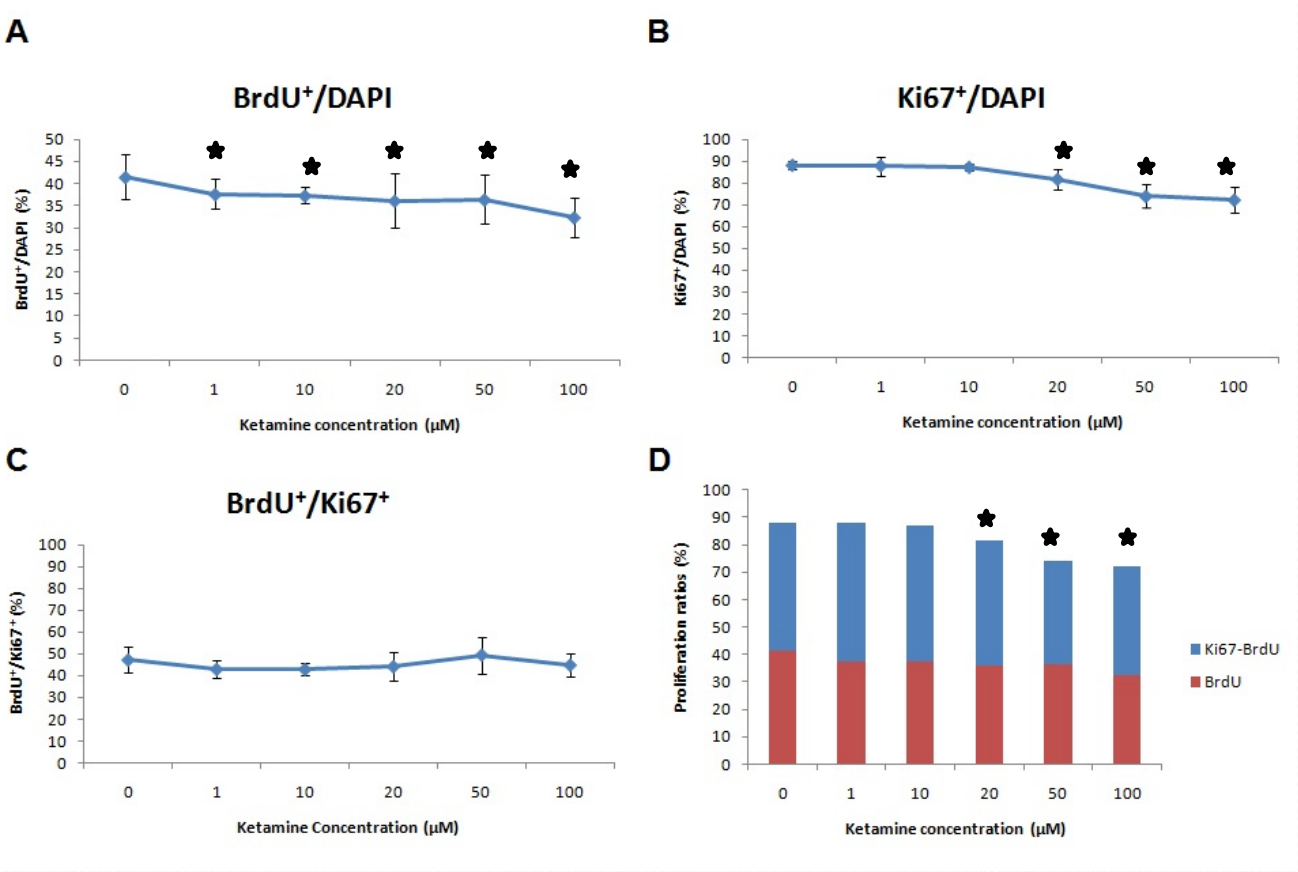
Time Response - Necrosis



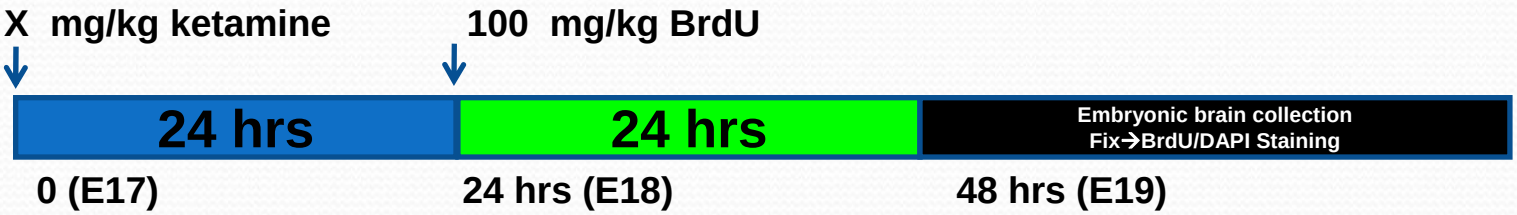
Ketamine inhibits the proliferation of NSPCs

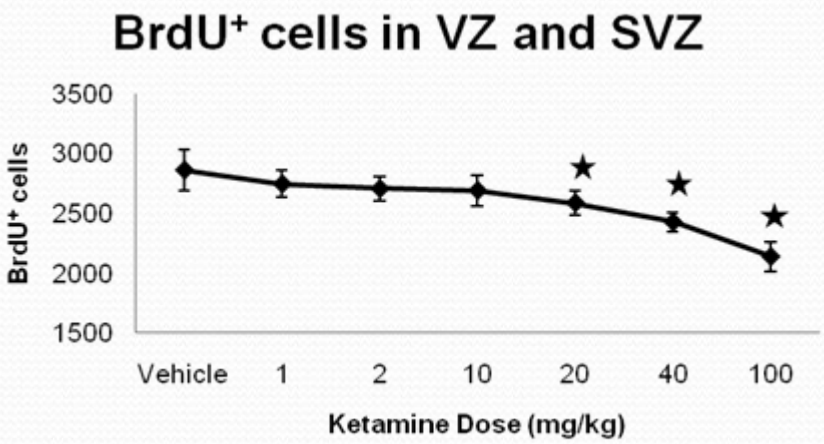


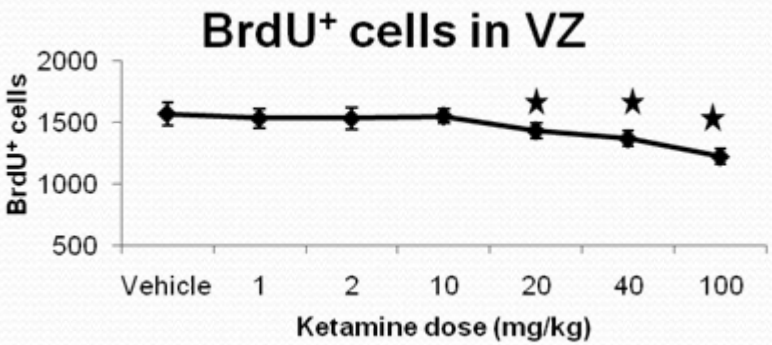
Scale bar: 50 μm

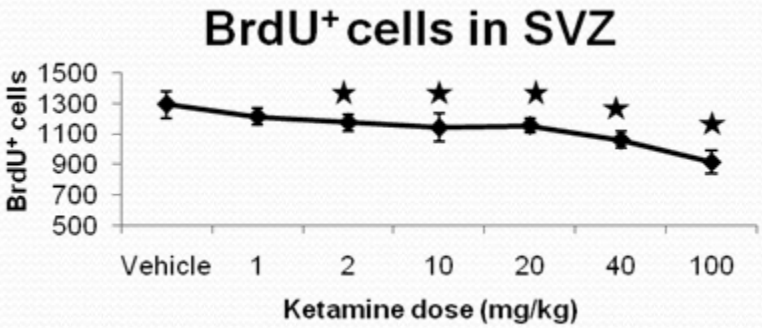


Ketamine reduces proliferative cells in the VZ/SVZ regions of rat fetal brain









Prolonged Ketamine exposure inhibits proliferation of NSPCs

Control:

10 uM BrdU + Proliferation medium

↓

X hrs	Fix→BrdU/Ki67 Staining

↓

Wash out BrdU + proliferation medium

Ketamine:

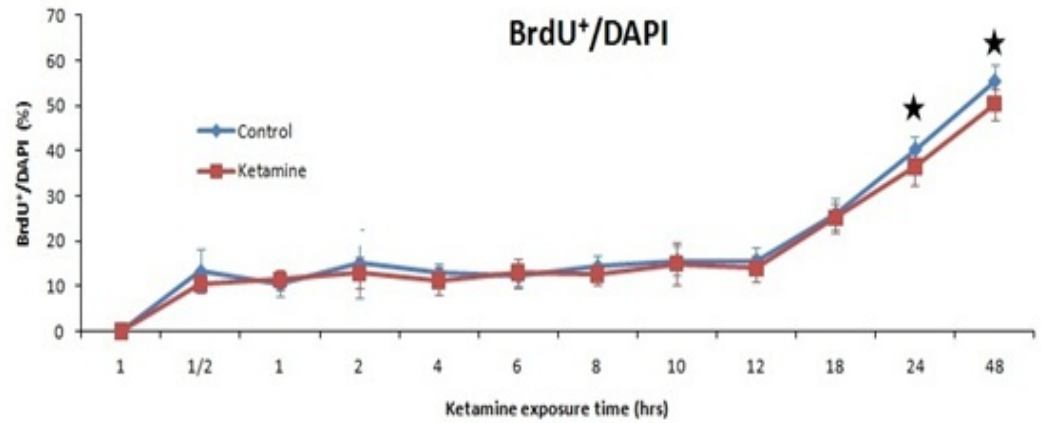
10 uM BrdU + 10 uM ketamine + Proliferation medium

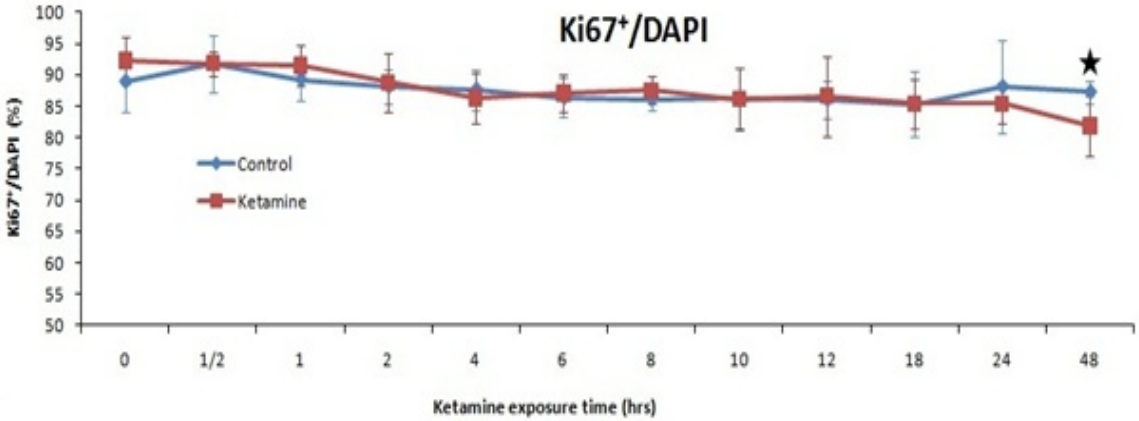
↓

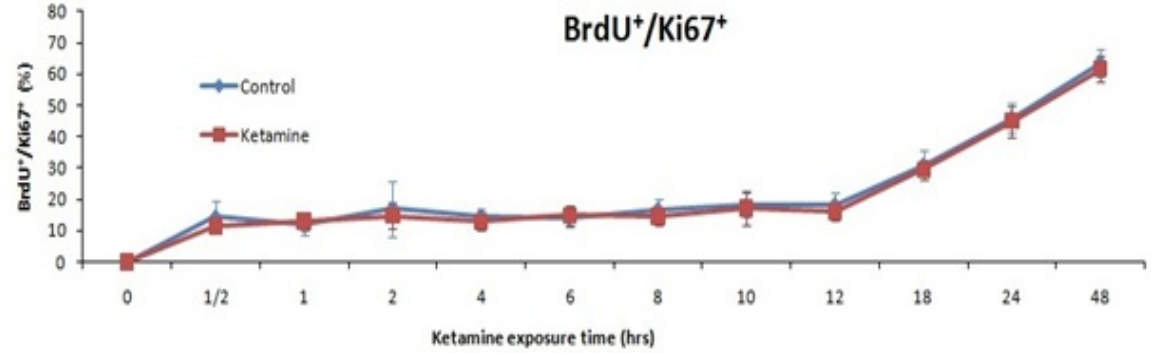
X hrs	Fix→BrdU/Ki67 Staining

↓

Wash out BrdU+ ketamine + proliferation medium

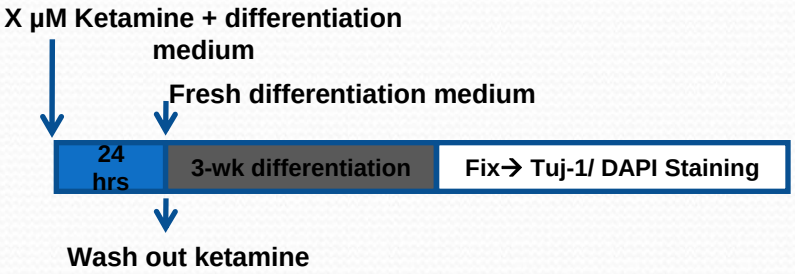


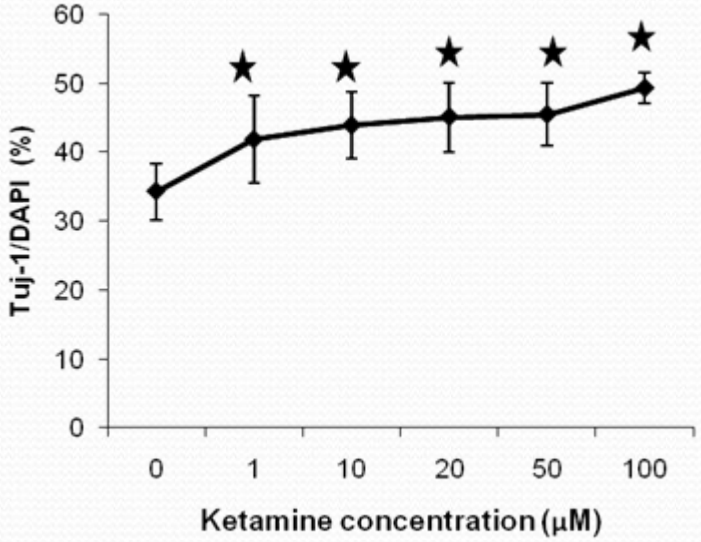




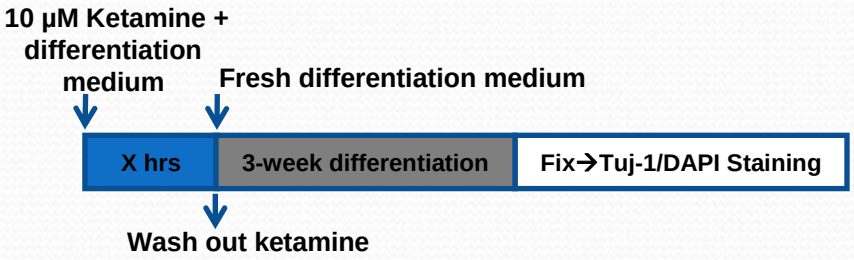
Ketamine promotes neuronal differentiation of NSPCs

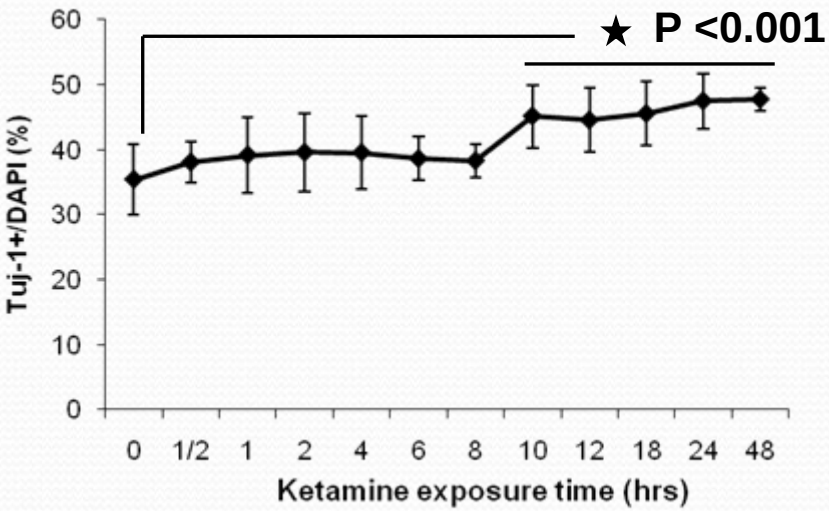
Dose response





Time response





Conclusions: In vitro studies

- NSPCs are resistant to the neurotoxic effects of ketamine at a low or clinically relevant high doses
- Proliferation of NSPCs is inhibited by ketamine in a concentration- and time-dependent manner (from *in vivo* and *in vitro* experiments)
- Ketamine enhances the neuronal differentiation of NSPCs in a concentration- and time-dependent manner

Thank you for your attention