Phenotype-Based Mechanistic Studies for the Assessment of Drug Safety and Drug-Drug Interactions in Zebrafish: Efficacy of Dietary Supplements

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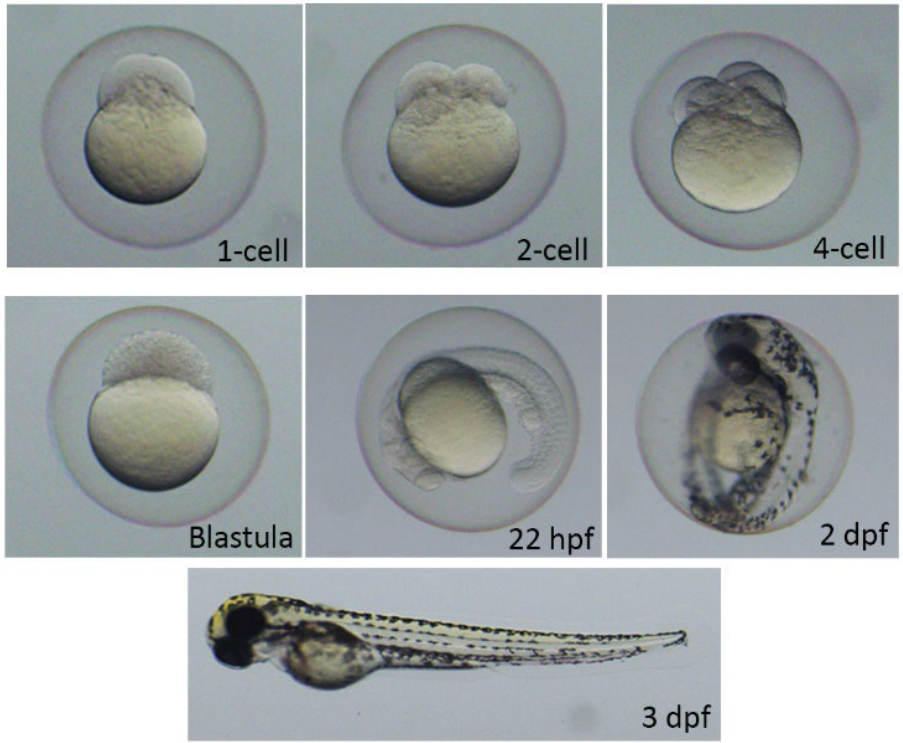
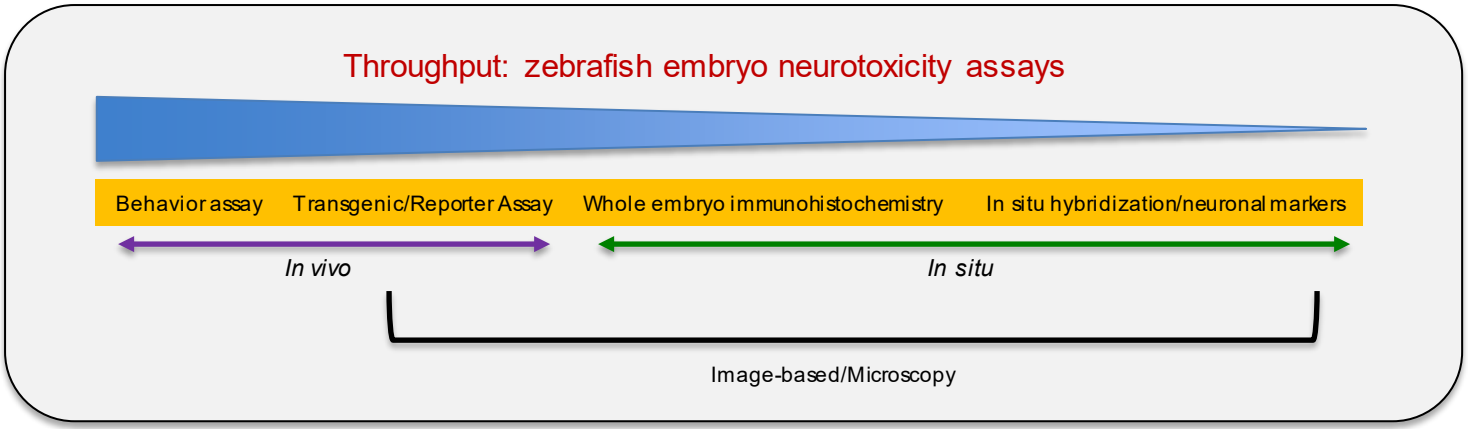
Zebrafish Embryos



- A vertebrate with rapid development
- Transparent body
- Anatomic/physiologic and genomic similarity with humans
- Expression of CYP metabolic enzymes
- Aligns well in the context of the 3Rs (Reduce, Refine and Replace)


Throughput: zebrafish embryo neurotoxicity assays
Behavior assay
Transgenic/Reporter Assay
Whole embryo immunohistochemistry
In situ hybridization/neuronal markers
In vivo
In situ
Image-based/Microscopy

Why study ketamine,
a pediatric anesthetic and
an NMDA receptor antagonist?

Food and Drug Administration (FDA) supports the Pediatric Anesthesia Safety Initiative (PASI). The goal of PASI is to bridge the scientific and clinical gaps in the field of pediatrics to ensure the safe use of anesthetic and sedative agents in children.

https://www.fda.gov/drugs/information-drug-class/pediatric-anesthesia

FDA Drug Safety Communication: FDA approves label changes for use of general anesthetic and sedation drugs in young children

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This is an update to the FDA Drug Safety Communication: FDA review results in new warnings about using general anesthetics and sedation drugs in young children and pregnant women issued on December 14, 2016.

Safety Announcement

[4-27-2017] The U.S. Food and Drug Administration (FDA) is notifying the public that we have approved previously announced label changes regarding the use of general anesthetic and sedation medicines in children younger than 3 years. These changes include:

- A new Warning stating that exposure to these medicines for lengthy periods of time or over multiple surgeries or procedures may negatively affect brain development in children younger than 3 years.
- Addition of information to the sections of the labels about pregnancy and pediatric use to describe studies in young animals and pregnant animals that showed exposure to general anesthetic and sedation drugs for more than 3 hours can cause widespread loss of nerve cells in the developing brain; and studies in young animals suggested these changes resulted in long-term negative effects on the animals' behavior or learning.

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A DIFFERENT VIEW

Anaesthetic neurotoxicity in rodents: is the ketamine controversy real?

Adnan T Bhutta[1], Ajay K Venkatesan[2], Cynthia R Rovnaghi[2], K J S Anand[3]
1.UAMS College of Medicine – Department of Pediatrics, Little Rock, Arkansas, USA

2007

INTRODUCTION

Different animal models have confirmed the neurotoxic effects of anaesthetics, such as ketamine, but these only occur following large doses or prolonged exposures in very young animals. These findings have questionable clinical relevance and should not preclude the clinician from using

cally. We suggest that animal models in which large, repeated doses are given to neonatal rats have relevance to clinical use *only* as models for toxicity and accidental overdose, but not for the routine clinical use of ketamine anaesthesia or analgesia

Paediatr Drugs. 2012 Feb 1;14(1):13-21. doi: 10.2165/11592840-000000000-00000.

Anesthetic-related neurotoxicity and the developing brain: shall we change practice?

Vutskits L[1].

1 Pediatric Anesthesia Unit, Department of Anesthesiology, Pharmacology and Intensive Care, University Hospital of Geneva, Geneva, Switzerland. laszlo.vutskits@unige.ch

2012

potentially confounding factors. Thus, despite significant advances in the field, there is still insufficient evidence to determine whether anesthetics are harmful to the developing human brain. Consequently, no change in clinical practice can be recommended.

Review > BMJ. 2019 Dec 9;367:l6459. doi: 10.1136/bmj.l6459.

Does general anesthesia affect neurodevelopment in infants and children?

2019

Mary Ellen McCann [1 2], Sulpicio G Soriano [1 2]

Affiliations – collapse

Affiliations

1 Department of Anesthesia, Harvard Medical School, Boston, MA, USA.
2 Department of Anesthesiology, Critical Care and Pain Medicine, Boston Children's Hospital, Boston, MA, USA.

non-human primates. The possibility of anesthetic induced neurotoxicity occurring in children has led to concerns about the safety of pediatric anesthesia. A spectrum of behavioral changes has been documented after general anesthetic exposure in young children, including emergence delirium, which may be evidence of toxicity. Most clinical studies are retrospective; specifics about medications or monitoring are unavailable and many of the outcomes may not be sensitive to detect small neurocognitive deficits. Some of these retrospective studies have shown an association between anesthesia exposure at a young age and neurocognitive deficits, but others have not. Practitioners and families should be reassured that although general anesthetics have the potential to induce neurotoxicity, very little clinical evidence exists to support this.

Science, 2014

BIOMEDICAL RESEARCH

Researchers struggle to gauge risks of childhood anesthesia

Neurological warning signals in animals drive push for new studies and public outreach

By **Kelly Servick**, *in Silver Spring, Maryland*

Anesthesiologists and surgeons who operate on children have been dogged by a growing fear—that being under anesthesia can permanently damage the developing brain. Although the few studies of children knocked out for surgeries have been inconclusive, evidence of impaired development in nematodes, zebrafish, rats, guinea pigs, pigs, and monkeys given common anesthetics has piled up in recent years. Now, the alarm is reaching a tipping point. "Anything that goes from [the roundworm] *C. elegans* to nonhuman primates, I've got to worry about," Maria Freire, co-chair of the U.S. Food and Drug Administration (FDA) science advisory board, told attendees at a meeting the agency convened here last month to discuss the issue.

The gathering came as anesthesia researchers and regulators consider several moves to address the concerns: a clinical trial of anesthetics in children, a consensus statement about their possible risks, and an FDA warning label on certain drugs. But each step stirs debate. Many involved in the issue are reluctant to make recommendations to parents and physicians based on animal data alone. At the same time, more direct studies of anesthesia's risks in children are plagued by confounding factors, lack of funding, and ethical issues.

"We have to generate—very quickly—an action item, because I don't think the status quo is acceptable," Freire said at the 19 November meeting. "Generating an action item without having the data is where things become very, very tricky."

Concerns first arose after a 1999 study, in which drugs that block *N*-methyl-D-aspartate (NMDA) receptors in the brain, including the common anesthetic ketamine, appeared to trigger the death of neurons in newborn rats. Similar results emerged for drugs that act on γ-aminobutyric acid (GABA) receptors, including sevoflurane, one of the most commonly used anesthetics in children. A 2011 FDA-led study also found that rhesus monkeys exposed to certain anesthetics in the first 6 days of life had permanent cognitive deficits, as measured by lab tests of learning and motivation.

Annual anesthesia use in the U.S.

6 million
Children under 15 years old

1.5 million
Infants under 12 months

Commonly used anesthetics in children have been associated with brain damage in animal studies.

Most surgeries performed in children under 3—when animal data suggest the developing brain is most vulnerable—are not optional, however. And there is no tried-and-true alternative to the drugs that have raised safety concerns. "Based on the current knowledge, we're kind of stuck," says David Warner, a pediatric anesthesiologist at the Mayo Clinic in Rochester, Minnesota, whose recent analyses of a database of local children point to increased risk of learning disabilities and lower test scores after multiple anesthesia exposures.

Further work has not been easy to fund. FDA can often compel companies to do safety studies of new drugs because they have massive investments at stake. But most current anesthetics have lost patent protection and are sold at low prices by generics manufacturers, says Russ Altman, a biomedical informatics specialist at Stanford University in Palo Alto, California, and the FDA science board's outgoing chair. Regulatory pressure on these companies "would just scare them away from creating these drugs."

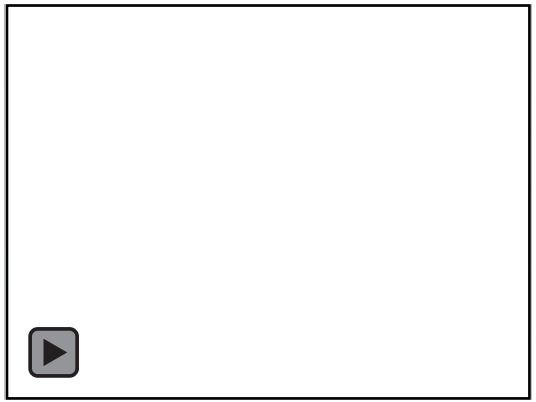
To help fill the information gap, FDA in 2010 formed Strategies for Mitigating Anesthesia-Related neuroToxicity in Tots (SmartTots), a partnership with the International Anesthesia Research Society (IARS). The initiative has struggled to scrape together money from foundations, professional societies, and private donors. It has given out $600,000 to four ongoing projects that evaluate children previously given anesthesia.

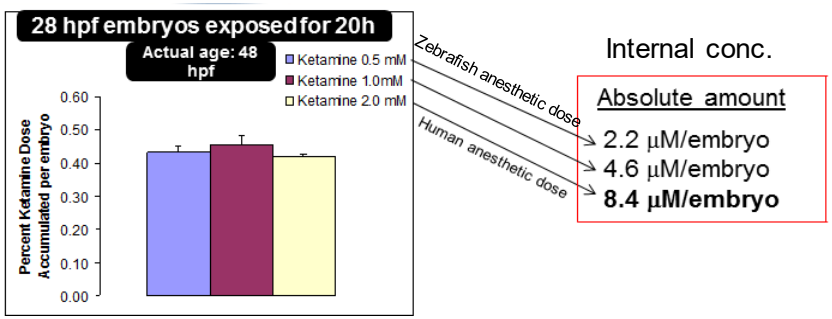
But such studies can't separate the effects of anesthesia from other factors that might cause neurological damage, such as the stress of the surgery itself or the child's underlying medical condition. SmartTots is now shifting its focus to designing a large multicenter trial, which would randomize kids to receive different types of anesthesia, then follow up with cognitive testing over several years.

A task force assembled this spring proposed that the trial compare sevoflurane with a sedative called dexmedetomidine—an agent that in animal studies does not appear to be neurotoxic and that may even mitigate the damaging effects of other drugs. Anesthesiologist and task force member Dean Andropoulos of Baylor College of Medicine in

CREDITS: (IMAGE) JASON EDWARDS/NATIONAL GEOGRAPHIC SOCIETY/ODYSSEY; (DATA) NATIONAL HOSPITAL DISCHARGE SURVEY (2006); AN NATIONAL INPATIENT SAMPLE; U.S. AGENCY FOR HEALTHCARE RESEARCH AND QUALITY; VIA SUN, *BRITISH JOURNAL OF ANAESTHESIA*, 2010

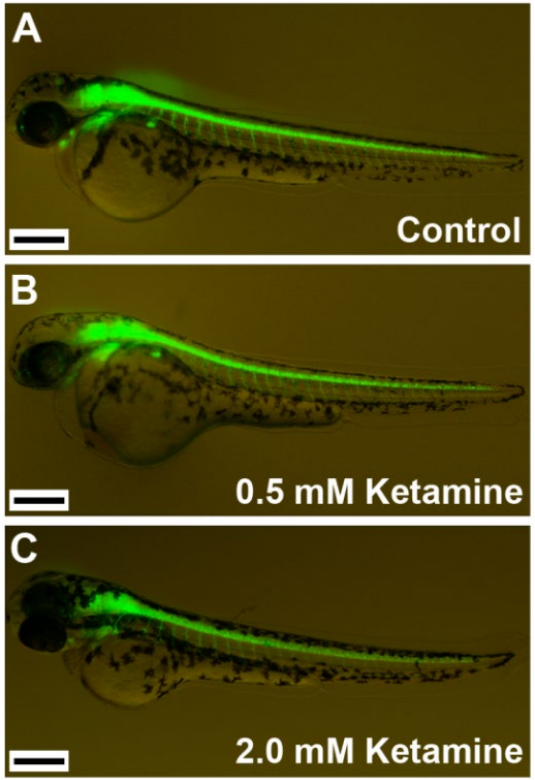
Internal concentration of ketamine is much lower than the original dose added to water



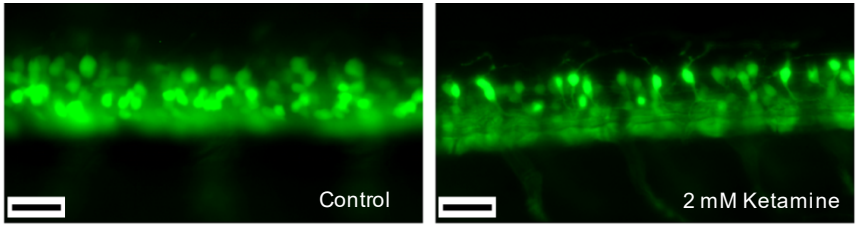


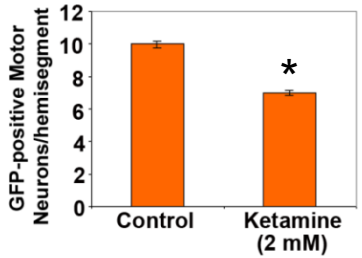
J Appl Toxicol., 2014

Ketamine-induced neurotoxicity *in Vivo* (*hb9:GFP* transgenic zebrafish embryos: Motor neurons and axons)



hb9-GFP Transgenic Live Embryos

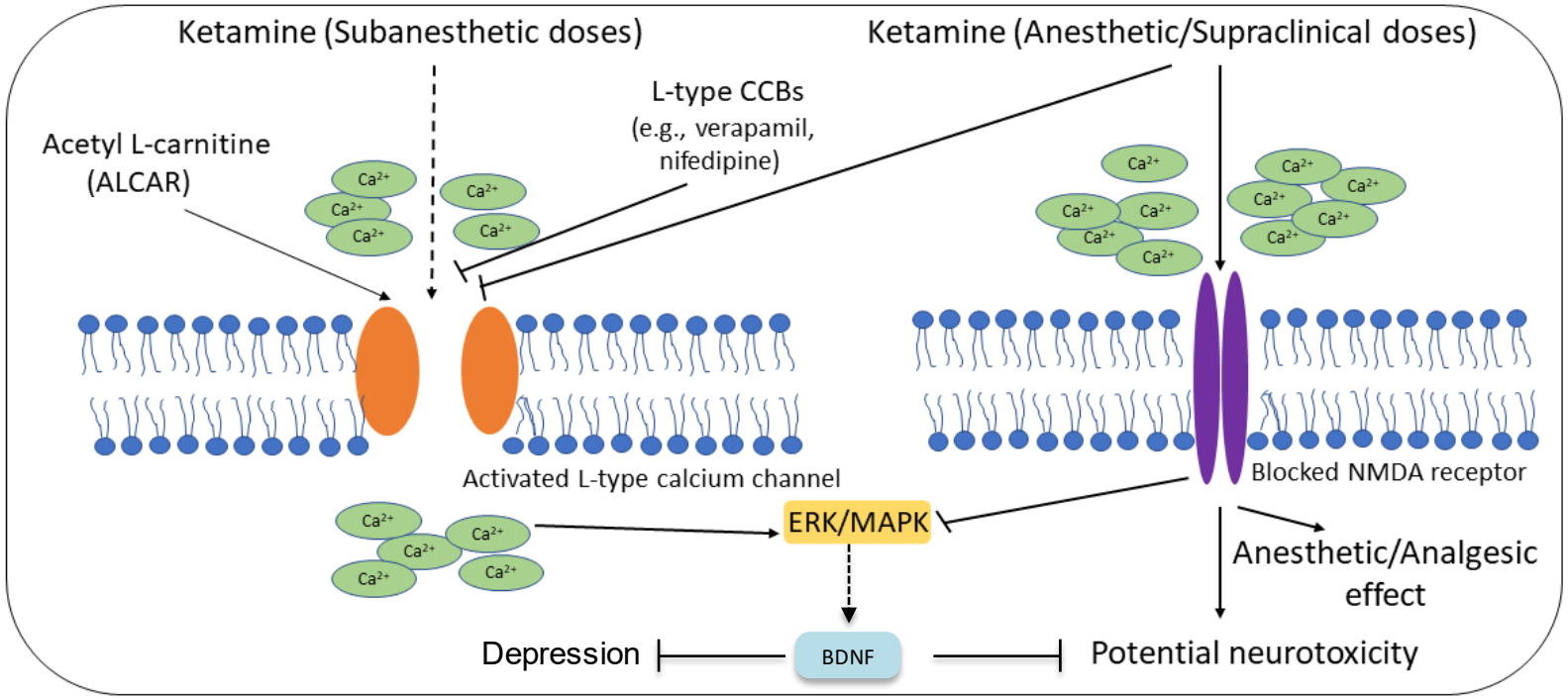




J Appl Toxicol., 2013

Akeju et al. (2014) Neurotoxicol Teratol. Ketamine reduced the number of hb9:GFP mouse motor neurons during differentiation of mESC.

Critical role of calcium in ketamine-induced effects on the nervous system



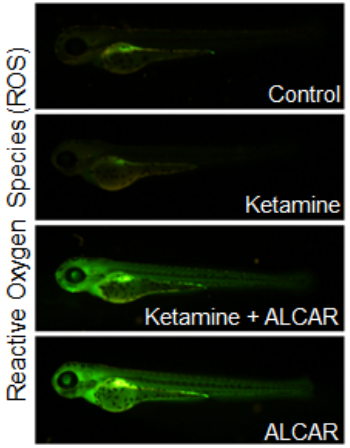
Adpated from:

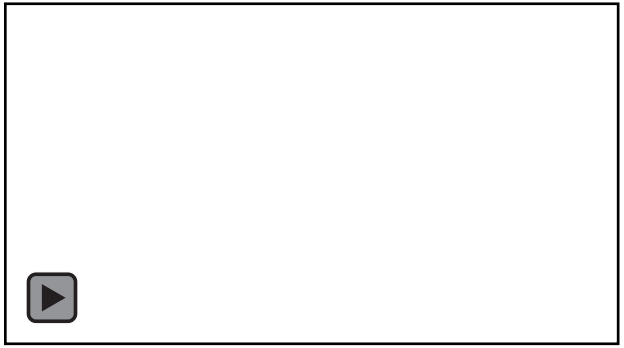
Antidepressant actions of ketamine: Potential role of L-type calcium channels.
B. Robinson, Q. Gu and J. Kanungo. *Chem Res. Toxicol*. 2021

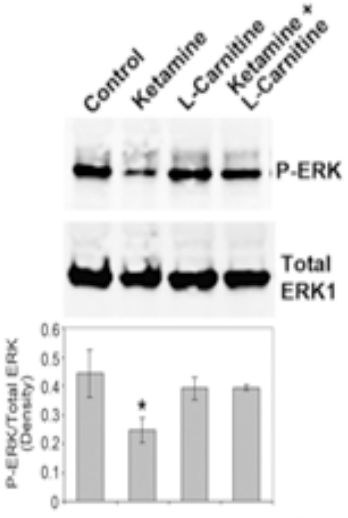
Acetyl l-carnitine (ALCAR) reverses ketamine-induced effects on targets of calcium signaling (heart rate, MAPK and ROS)

- Ketamine reduces heart rates in newborn human infants and in pregnant and infant non-human primates.
  Saarenmaa et al., Arch Dis Child Fetal Neonatal Ed., 85:F53–6, 2001.
  Hotchkiss et al., J Am Assoc Lab Anim Sci. 46:21-28, 2007.

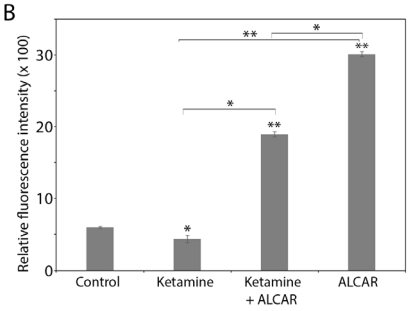
- Ketamine suppresses MAPK/ERK activation in mice brains. Straiko et al., Anesthesiology, 110: 862-868, 2009



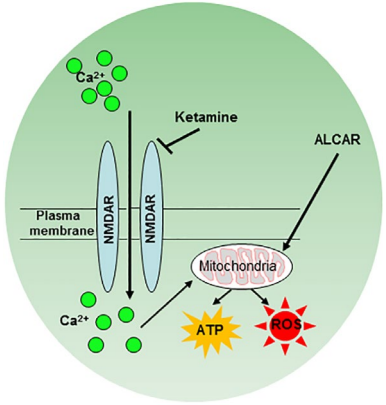


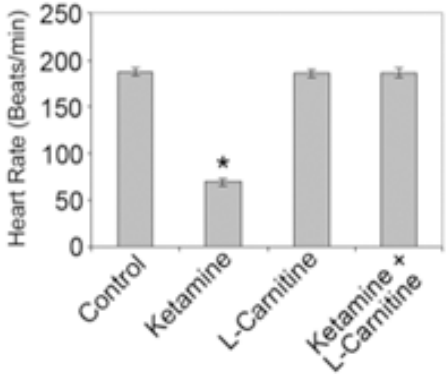


Reprod Toxicol., 2012



Neurosci Lett., 2019

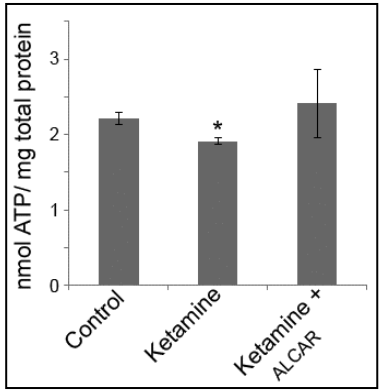




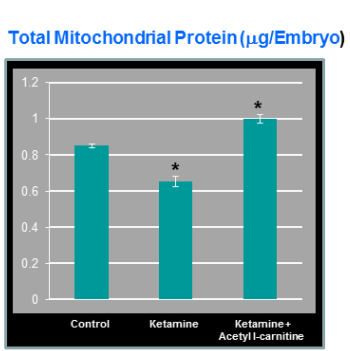
Ketamine's effect on ATP levels & mitochondria in zebrafish embryos

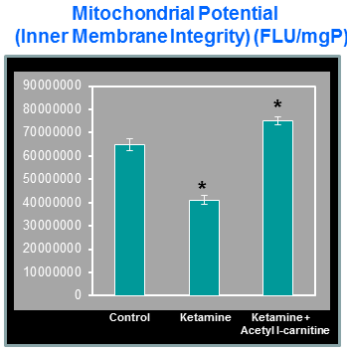
- Ketamine decreases ATP level in human induced pluripotent stem cell-derived neurons but does not induce ROS production. (Ito et al., 2015, PLoS One)

- Ketamine suppresses ATP biosynthesis in HepG2 cells. (Chang et al., 2009, Drug Metabolism & Disposition)



- Ketamine causes reduction in mitochondrial membrane potential in human induced pluripotent stem cell-derived neurons. (Ito et al., 2015; PLoS One)

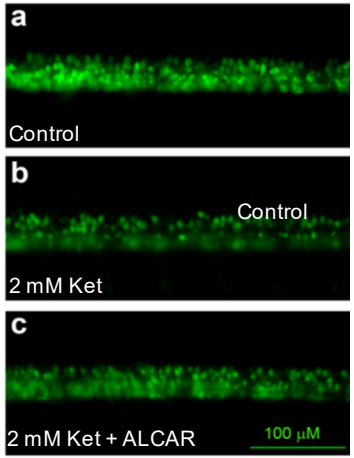


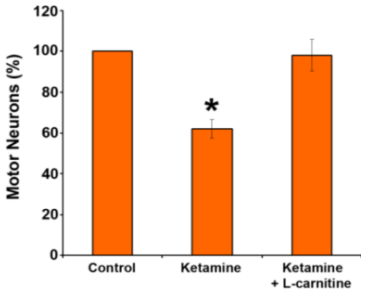


Neurotoxicol Teratol., 2018

Acetyl L-carnitine (ALCAR) protects zebrafish embryos from ketamine-induced neurotoxicity

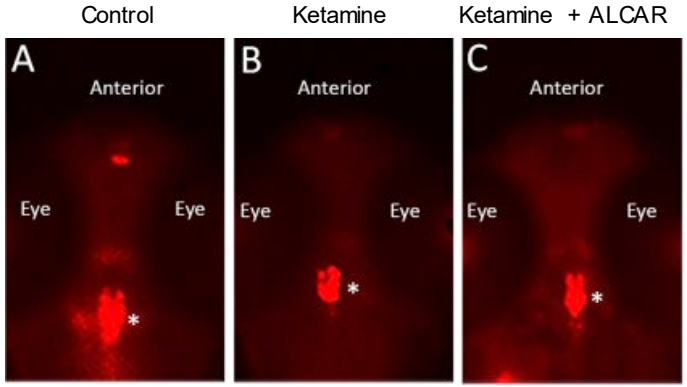
hb9-GFP Tg embryos with motor neurons expressing GFP





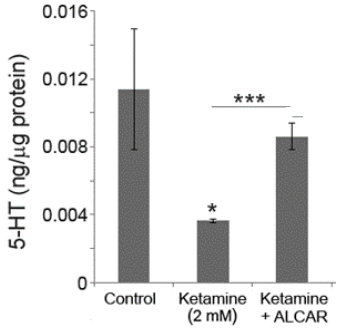
Neurotoxicol Teratol., 2013

Whole embryo 5-HT (serotonin) immunohistochemistry



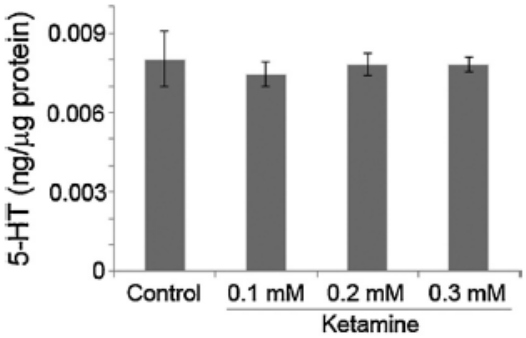
72 hpf embryos

HPLC for serotonin levels



Neurosci Lett., 2015

No effects of subanesthetic doses of ketamine on serotonin levels



Functional studies on drug-drug interactions to delineate mechanism of action

Verapamil: a calcium channel blocker prescribed for cardiac dysrhythmias in all age groups including pediatric patients.

Cyclosporine A (CsA): an immunosuppressant used to prevent organ rejection after organ transplantation.

Ketamine: an anesthetic

When used concomitantly, ketamine and calcium-channel blockers should be titrated carefully to avoid excessive cardiovascular depression (https://www.pdr.net/drug-summary/Verelan-verapamil-hydrochloride-960)

Ketamine and CsA is not safe for organ transplant recipients (develop seizure).

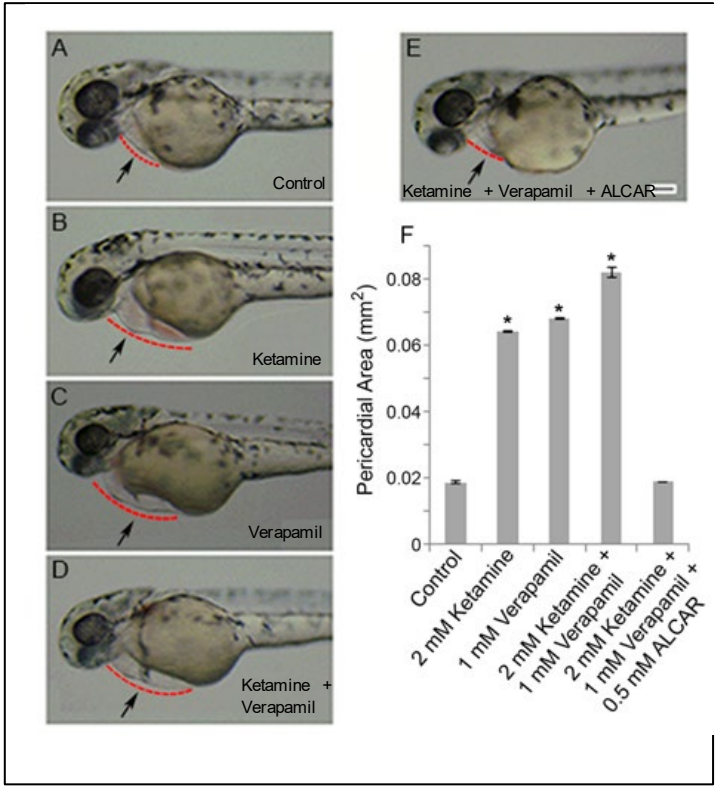
CsA and ketamine co-treatment can not be recommended.

Agarwal et al. (2005) Anesth Analg.
Sato et al. (2006) Anesthesiol.
Subramaniam and Sakai (2016) Anesthesia & Preop Care for Organ Transplant.

Nifedipine: a calcium channel blocker that treats pediatric and adult hypertension.

Ketamine-induced intraoperative hypertension is controlled by nifedipine. Koh et al. (1993) Masui.

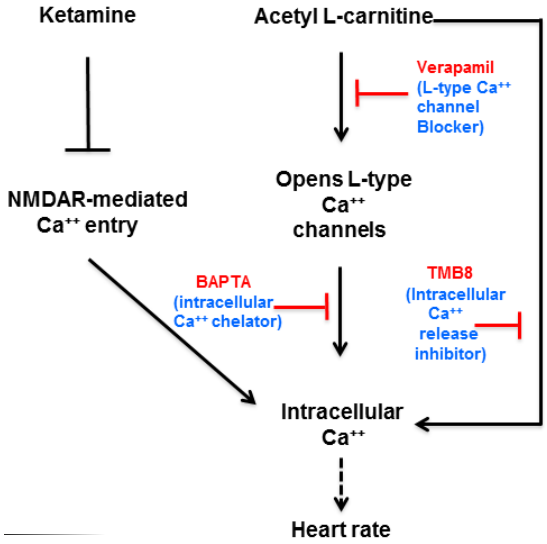
ALCAR prevents ketamine- and verapamil-induced cardiotoxicity

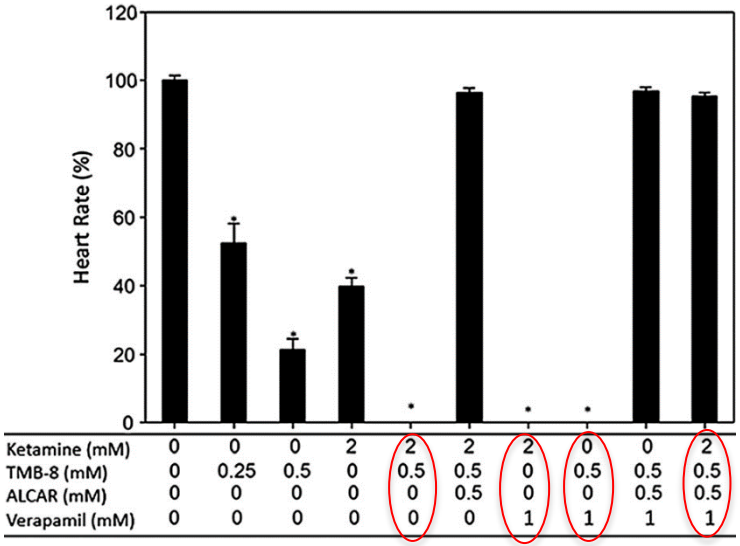


2 dpf embryo
2 h treatment

J Appl Toxicol., 2017

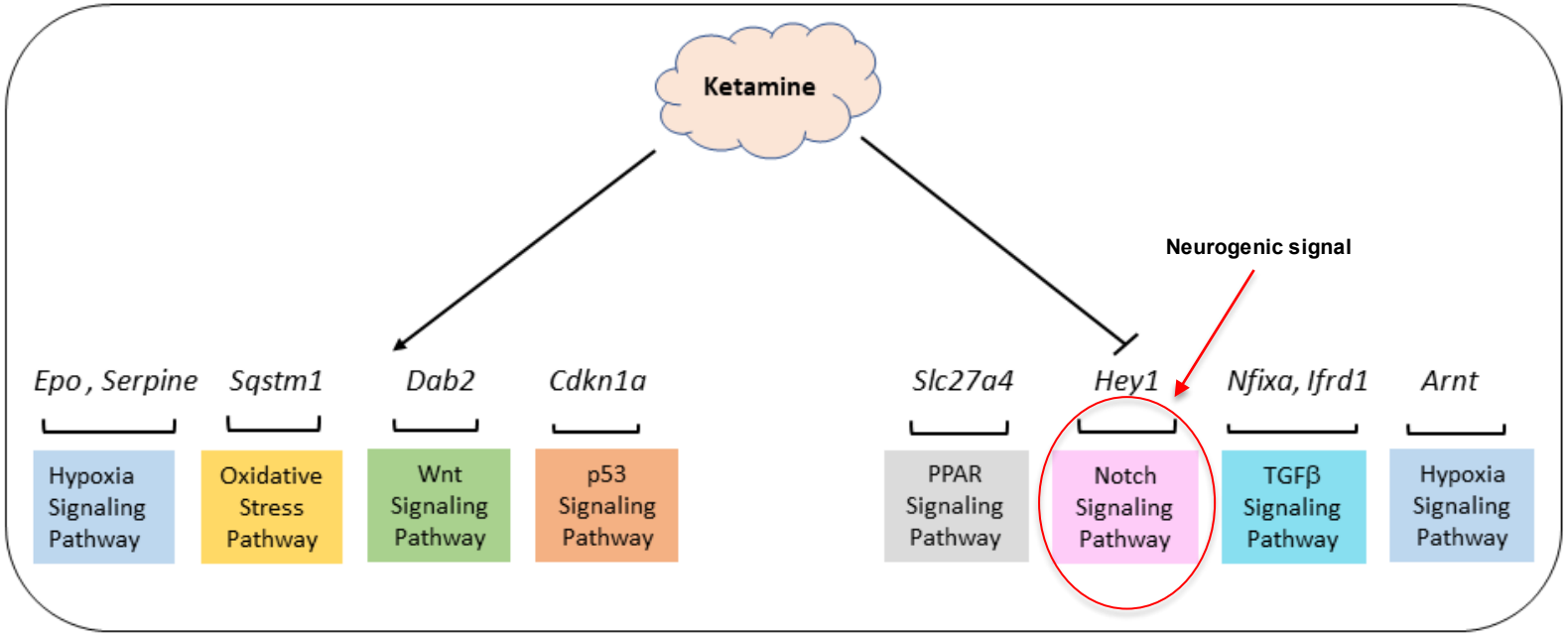
ALCAR counteracts the effects of Ca^{++} inhibitors on heart rate





J Appl Toxicol., 2017

PCR Array (84 genes)



J Appl Toxicol., 2021

In specific regions of the brain, repeated subanesthetic doses of ketamine induces neurogenesis in adult mice (Clarke et al., 2017, Neuropharmacology) and a single dose induces increased number of functional neurons in rats (Soumier et al., 2016, eNeuro).

Microarray
(Control vs. Ketamine-treated Embryos)

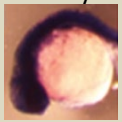
Upregulated: 118 genes

Downregulated: 148 genes

Mib1 Mutation

Notch signaling

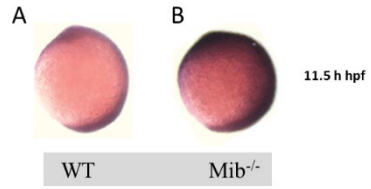
mindbomb embryo



- Premature differentiation of early-born neurons (11.5 – 24 h).
- Depletion of neuronal progenitors
- Loss of late-born neuron generation
- Inhibition of dopaminergic, serotonergic, and motor neuron development

Fold Change		DOWN REGULATED
-2.19	stx12	syntaxin 12
-2.29	adrb3b	adrenergic receptor, beta 3b
-3.00	map1lc3b	microtubule-associated protein 1 light chain 3 beta
-2.06	abcg1	ATP-binding cassette, sub-family G (WHITE), member 1
-2.26	scin	scinderin
-2.51	ncam1b	neural cell adhesion molecule 1b
-3.12	brn1.2	brain POU domain gene 1.2
-2.39	atp5a1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
-2.67	nrxn2a	neurexin 2a
-3.03	pdcd4a	programmed cell death 4a
-2.56	caspb	caspase b
-3.03	nf2a	neurofibromin 2a (merlin)
-3.12	mib	mind bomb
-2.53	synj1	synaptojanin 1
-2.17	nrxn3a	neurexin 3a

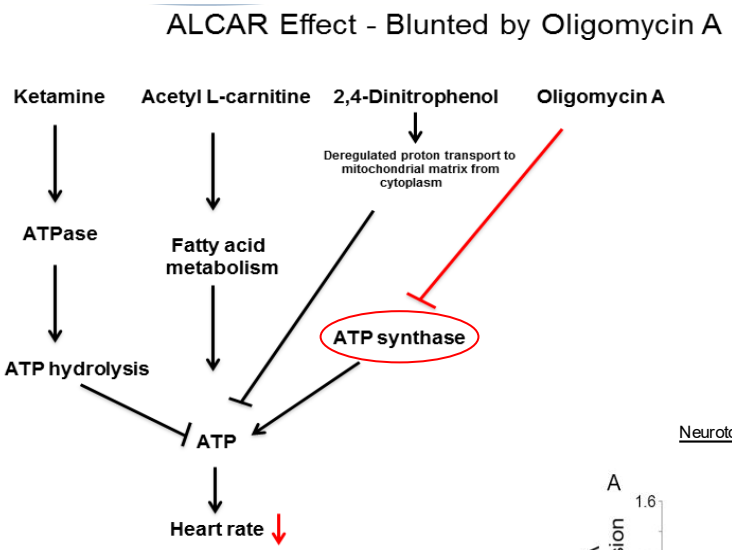
an E3 ubiquitin ligase that processes Notch ligands for internalization

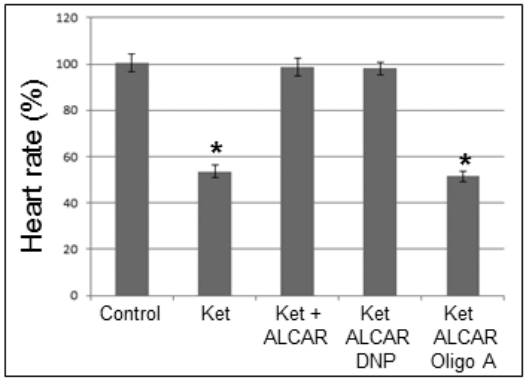


ISH with HuC (an RNA-binding protein) that is expressed in the neuronal precursor cells.

Folia Biol (Prague), 2018, 64: 35-40

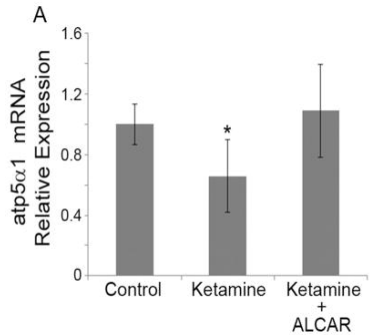
ALCAR's beneficial effects on ketamine toxicity is mediated by ATP synthase

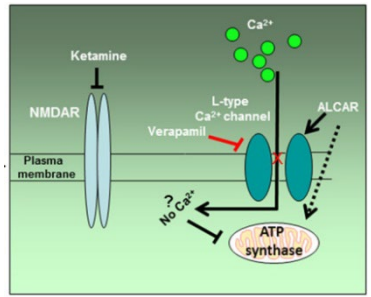




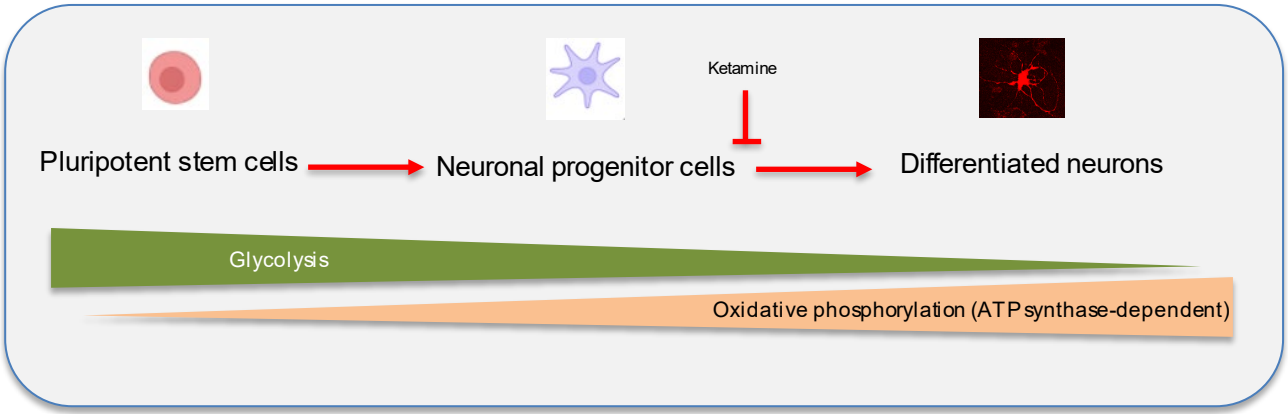
2 h exposure

Neurotoxicol Teratol., 2018

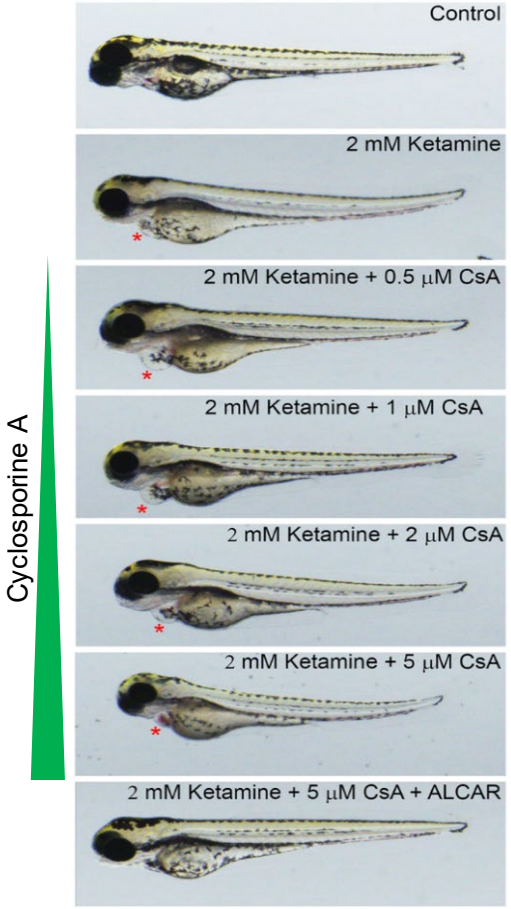




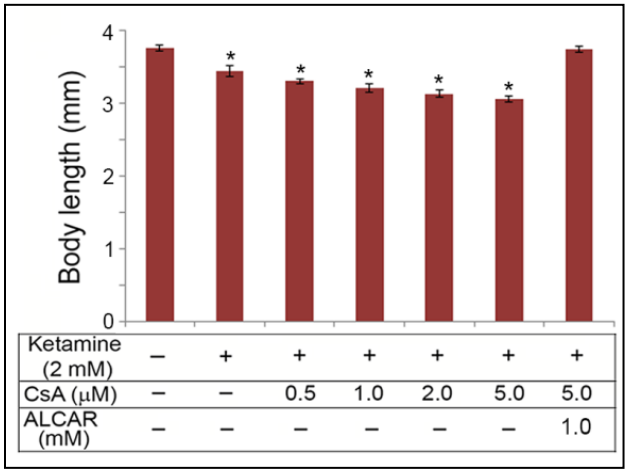
J Appl Toxicol., 2017


Ketamine
Pluripotent stem cells
Neuronal progenitor cells
Differentiated neurons
Glycolysis
Oxidative phosphorylation (ATP synthase-dependent)

Cyclosporine (CsA) exacerbates ketamine toxicity: ALCAR prevents CsA/ketamine combination toxicity

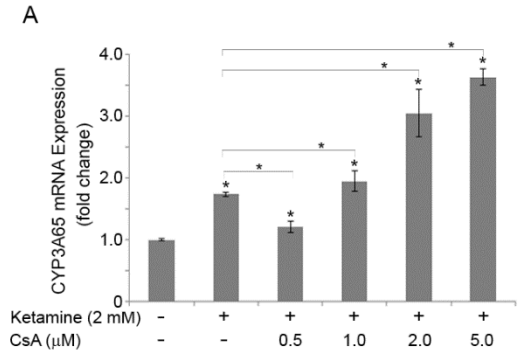


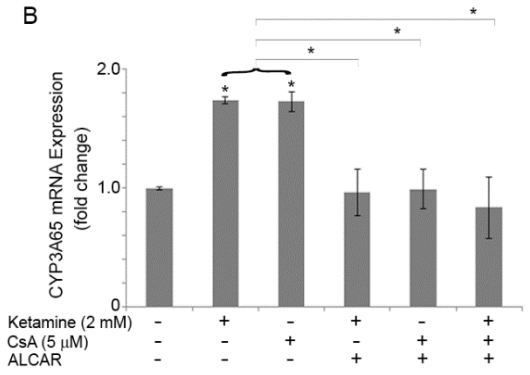
* Pericardial sac edema



Status of drug metabolism may not be a factor in ketamine + CsA toxicity

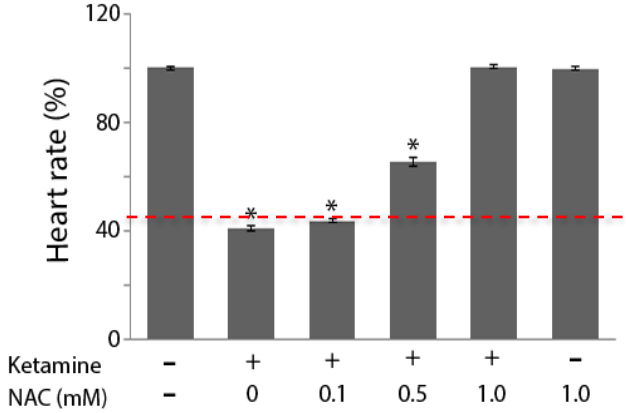
Zebrafish CYP3A65 is an ortholog of mammalian CYP3A4.





J Appl Toxicol., 2017

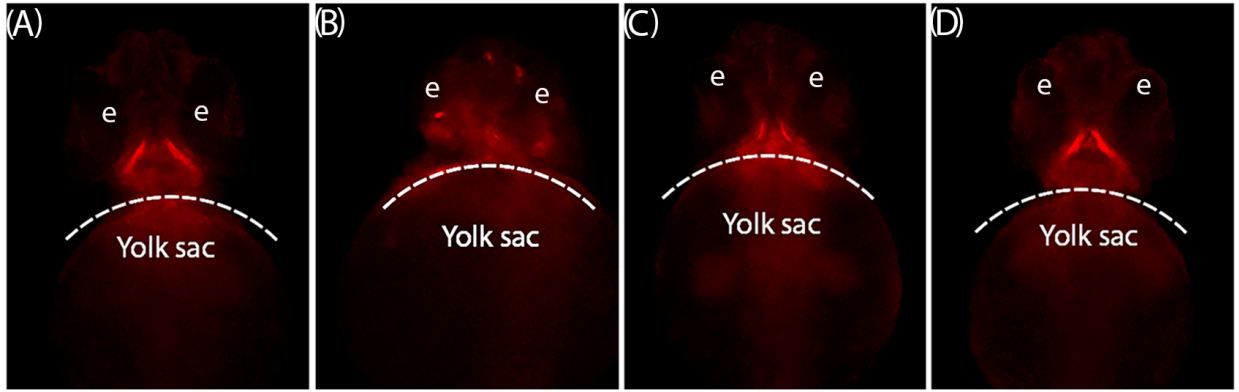
N-acetylcysteine (NAC) prevents ketamine-induced adverse effects on development, heart rate and serotonergic neurons

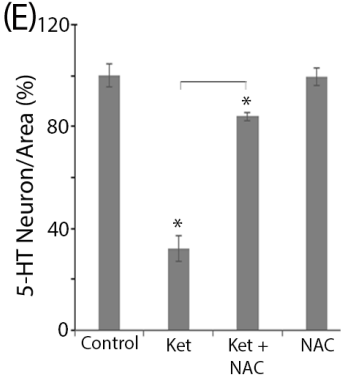


48 hpf embryos

Whole-embryo immunohistochemistry

5-HT neurons

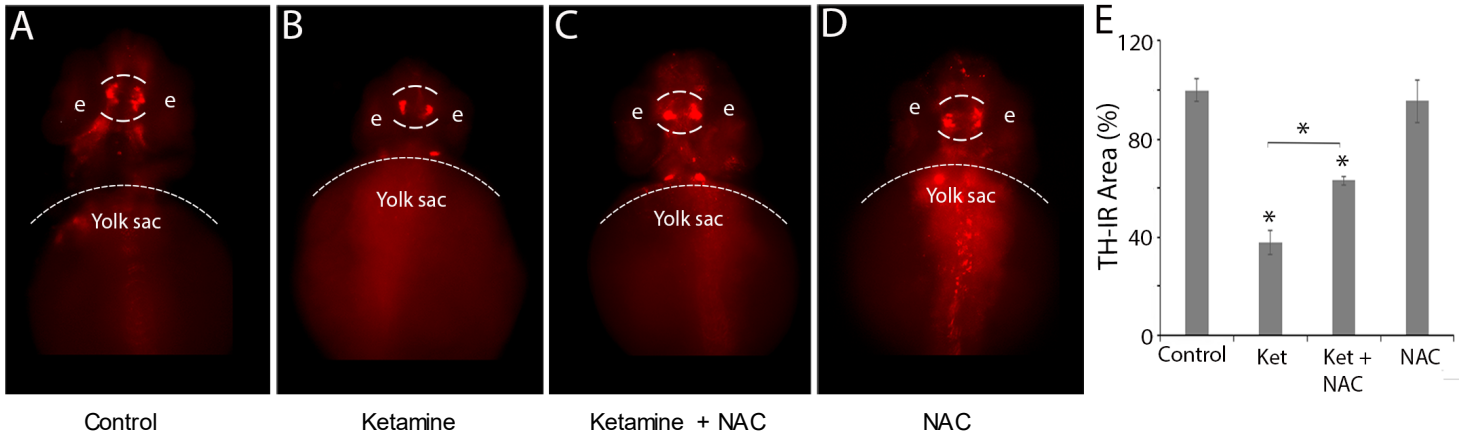




Neurosci Lett., 2018

NAC prevents ketamine-induced adverse effects on tyrosine hydroxylase-positive (TH) neurons

Whole-embryo immunohistochemistry

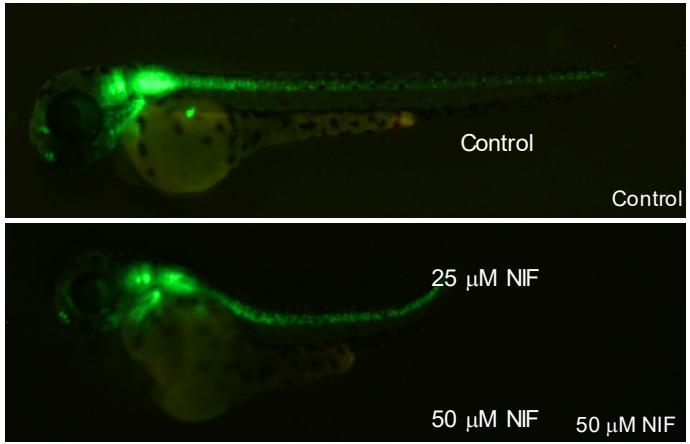


Neurosci Lett., 2018

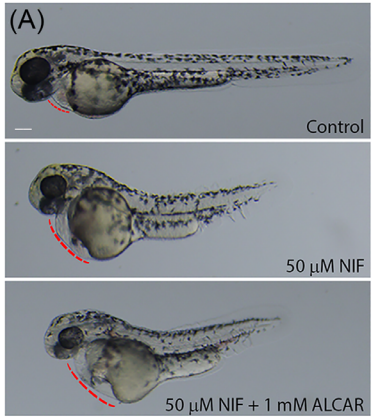
NAC's has beneficial effects on ketamine toxicity (behavioral change) in mice (Phensy et al., 2017a, 2017b).

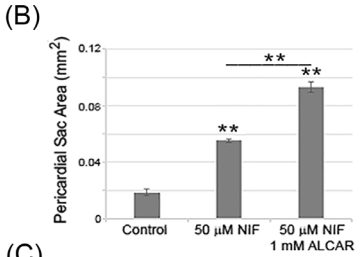
ALCAR exacerbates nifedipine (NIF) toxicity in vivo

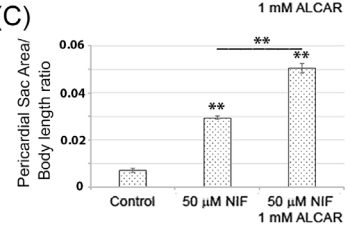
hb9-GFP Tg Embryos



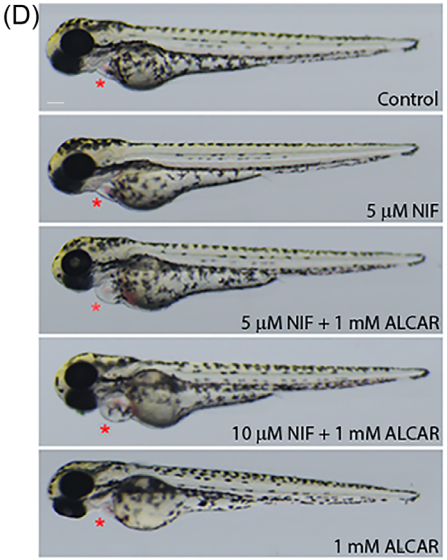
2 dpf

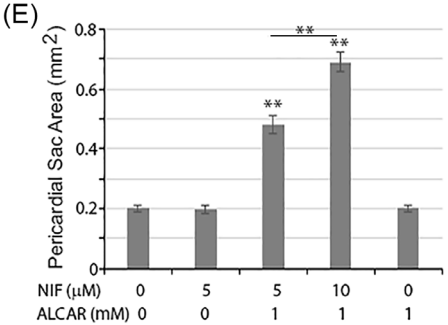






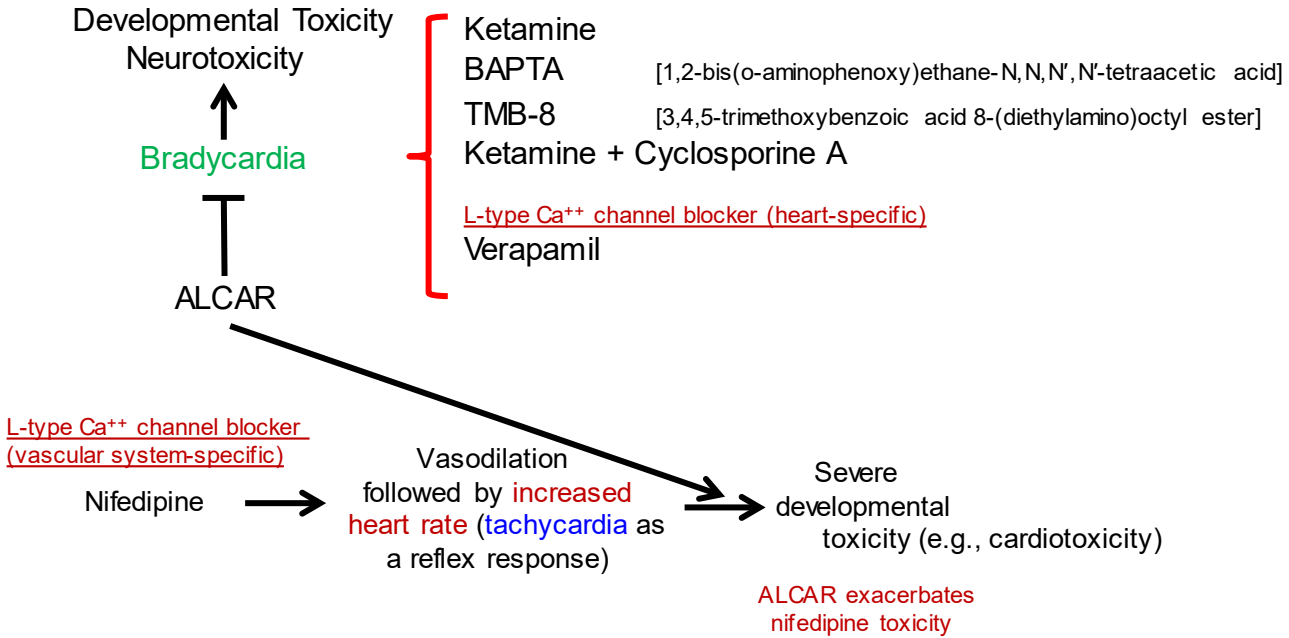
3 dpf





J Appl Toxicol., 2020

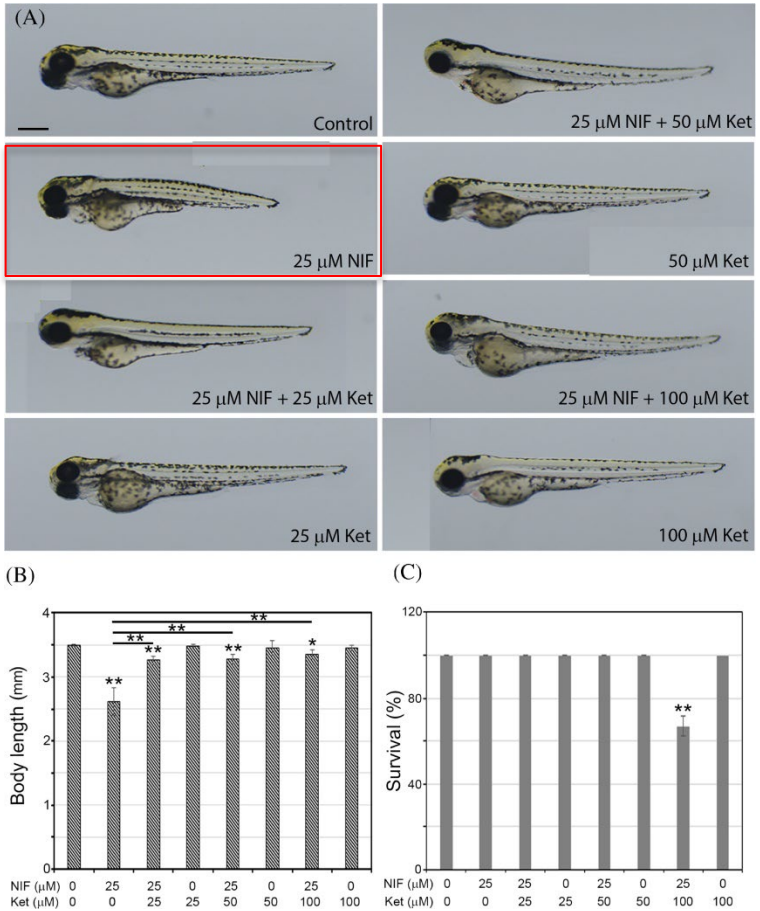
Why ALCAR does not prevent toxicities of all Ca^{++} antagonists?



Amlodipine (an L-type calcium channel blocker) toxicity (bradycardia) in a patient/L-carnitine treatment successful

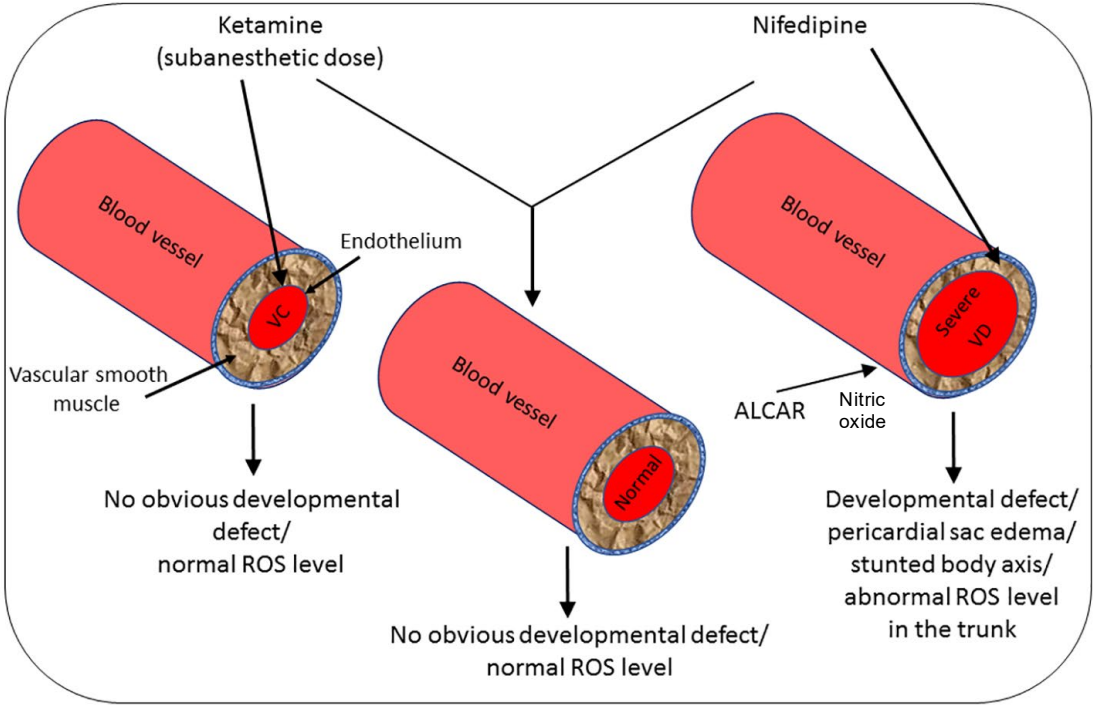
(St-Onge et al., 2013; J Med Toxicol)

Subanesthetic doses of ketamine alleviate NIF toxicity



J Appl Toxicol., 2020

Schematic presentation of potential mechanism to explain the effects of nifedipine, ketamine and ALCAR



J Appl Toxicol., 2020

While ketamine doses used in psychiatry are lower than those used in anesthesia, there are published instances of early termination of psychiatric ketamine infusions due to elevations in blood pressure and heart rate. Vankawala et al., Front. Psychiatry, 24 March 2021.

Ketamine Neurotoxicity and the State-of-the-Heart

- Ketamine's effect on the nervous system may be pleiotropic (NMDA receptor antagonism, L-type calcium channel modulation, etc.), heart rate being one of the contributors.

- Ketamine toxicities (neurotoxicity and developmental toxicity) appear to be consequences of altered hemodynamics.

Pediatric Anesthesia

Brain injury in children with congenital heart disease

Michael J. H. Scallan FRCA

First published: 08 May 2003 | https://doi.org/10.1046/j.1460-9592.2003.00996.x | Citations: 21

M.J.H. Scallan, Department of Anaesthesia, Royal Brompton Hospital, Sydney Street, London SW3 6NP, UK (email: m.scallan@rbh.nthames.nhs.uk)

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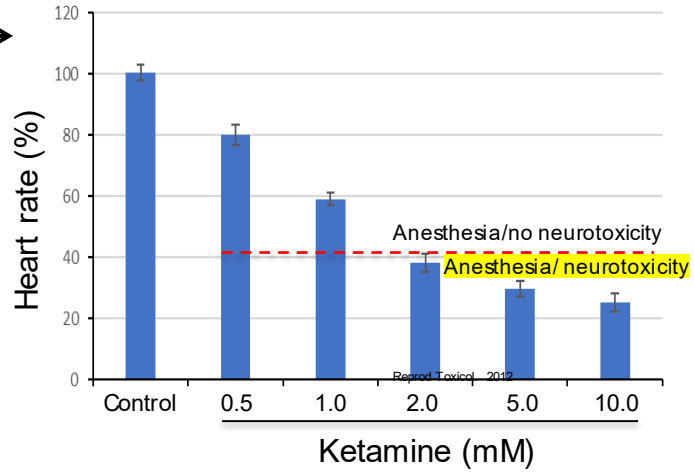
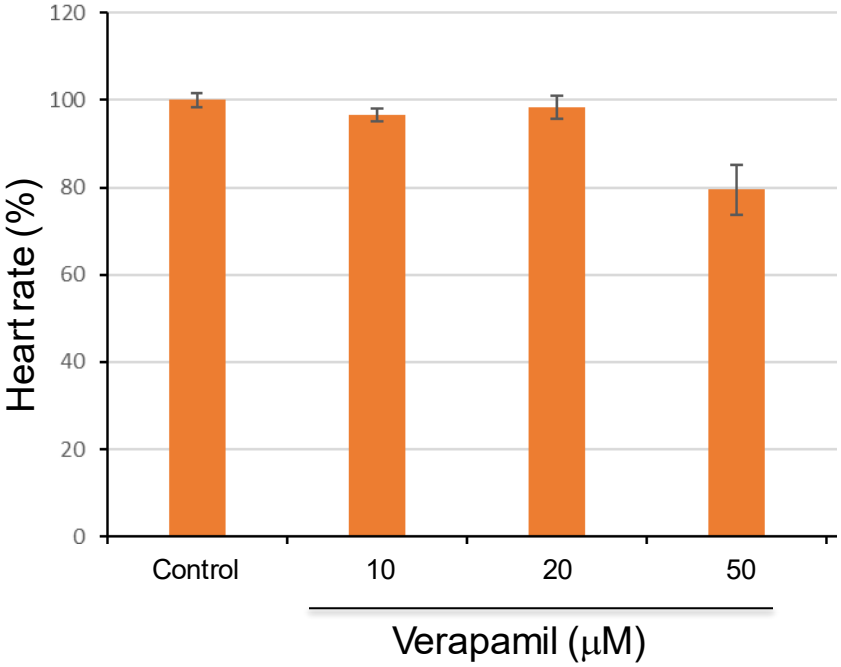
Summary

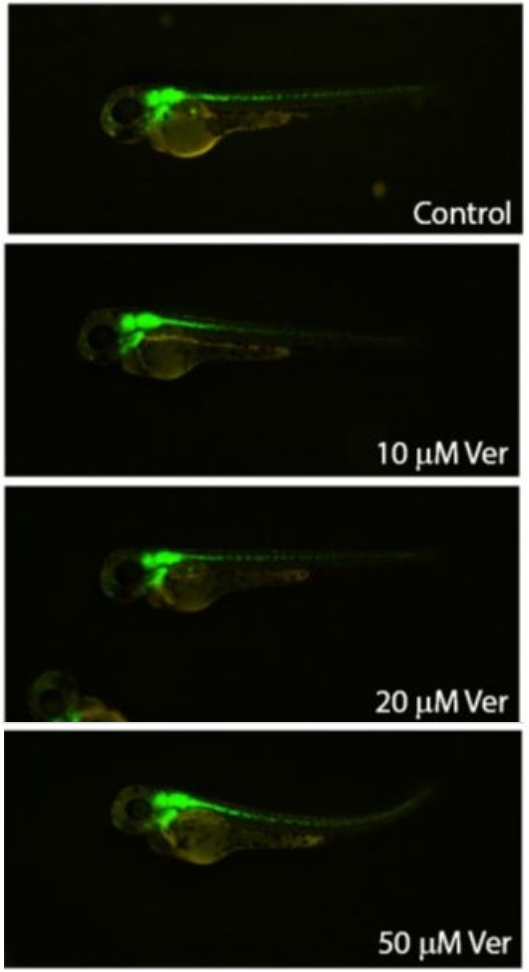
The incidence of neurodevelopmental impairment in children with congenital heart disease is high. Its aetiology is multiple and complex. Prevention and treatment must start during the preoperative period and continue through the intra- and postoperative periods. Research has resulted in a clearer understanding of the relationship between congenital heart disease and the brain, and of the effects of cardiopulmonary bypass, hypothermia and circulatory arrest. This has led to modifications in management which may improve neurological outcome in the future.

Zebrafish embryos: 20 h exposure



Verapamil: Heart rate vs. motor neuron development





Heart rate and neurodevelopment are linked

frontiers in Neuroscience

Front Neurosci. 2019; 13: 411.
Published online 2019 Apr 26. doi: 10.3389/fnins.2019.00411
PMCID: PMC6499022
PMID: 31105521

The Association Between Heart Rate Variability and Neurocognitive and Socio-Emotional Development in Nepalese Infants

Torvald F. Ask,[1,2,*] Suman Ranjitkar,[3] Manjeswori Ulak,[3] Ram K. Chandyo,[3,4] Mari Hysing,[5] Tor A. Strand,[2,6,*] Ingrid Kvestad,[7] Laxman Shrestha,[3] Marita Andreassen,[8] Ricardo G. Lugo,[8] Jaya S. Shilpakar,[3] Merina Shrestha,[3] and Stefan Sütterlin[9,10]

Springer Link

Review | Published: 27 November 2020

Heart rate variability as possible marker of brain damage in neonates with hypoxic ischemic encephalopathy: a systematic review

Iliana Bersani, Fiammetta Piersigilli, Diego Gazzolo, Francesca Campi, Immacolata Savarese, Andrea Dotta, Pietro Paolo Tamborrino, Cinzia Auriti & Corrado Di Mambro

European Journal of Pediatrics **180**, 1335–1345 (2021) | Cite this article

nature > journal of perinatology > original article > article

Open Access | Published: 25 June 2009

Heart rate characteristics and neurodevelopmental outcome in very low birth weight infants

K Addison, M P Griffin, J R Moorman, D E Lake & T M O'Shea

Journal of Perinatology **29**, 750–756 (2009) | Cite this article

Conclusion:

Among VLBW infants, the cumulative frequency of abnormal HRCs, which can be assessed non-invasively in the neonatal intensive care unit, is associated with an increased risk of adverse neurodevelopmental outcome.

Conclusions

- Ketamine induces neurotoxicity in the zebrafish embryos at a dose 4 x that of the anesthetic dose, possibly due to bradycardia.
- For effects of some drugs on the nervous system to be fully assessed, developmental cardiotoxicity may be taken into consideration.
- Drug-drug interaction studies reveal a better understanding of potential mechanisms of neurotoxicity.
- For assessment of neurotoxicity at the cellular and molecular level in intact whole organisms (non-invasive), zebrafish embryos are an ideal vertebrate model system.
- The data from zebrafish embryo studies can bridge the gap between non-clinical and clinical findings.

Acknowledgements

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Merle Paule, PhD

Thank you!