

## Critical Regulation of Calcium Signaling and NMDA-type Glutamate Receptor In Developmental Neural Toxicity

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### Abstract

Calcium ( $\text{Ca}^{2+}$ ) is a vital element in the process of neurotransmitter release, and is a common signaling mechanism. Calcium can act in signal transduction after influx resulting from activation of ion channels or as a second messenger.

Glutamate receptors play an important role in the excitatory synaptic action of the central nervous system. Activation of the N-methyl-D-aspartate (NMDA)-type glutamate receptor increases the concentration of ( $\text{Ca}^{2+}$ ) in the cell.  $\text{Ca}^{2+}$  can in turn function as a second messenger in various signaling pathways.

The developing nervous system varies in susceptibility to neurotoxic insults depending on the stage of development. By monitoring alterations in calcium influx, cell signaling processes, expression levels of receptor subtypes and imaging outcomes, it may be possible to completely define the affected system(s) during development.

This review attempts to discuss how the potential application of some sophisticated research approaches, e.g., calcium imaging/ $\text{Ca}^{2+}$  influx and monitoring gene and/or protein expression levels of receptor subtypes, provides a platform in which preclinical research models can inform clinical interventions and vice versa in the developing brain. This review focuses the discussion on representative general anesthetic agents - primarily ketamine - as examples of how the  $\text{Ca}^{2+}$  influx event and specific receptor subunit expression can be used to dissect relevant mechanisms underlying the etiology of the neurotoxicity associated with developmental exposures to anesthetic agents.

**Keywords:** Calcium signal; NMDA receptor; Development; Anesthesia; Neurotoxicity; Mechanism

### Introduction

It has long been known that calcium ions ( $\text{Ca}^{2+}$ ) act as intracellular messengers controlling cell functions in many cellular systems [1,2]. Calcium ( $\text{Ca}^{2+}$ ) ions play an important role in cell signaling. Movement of calcium ions from the extracellular compartment to the intracellular compartment alters membrane depolarization. Therefore, the regulation of  $\text{Ca}^{2+}$  influx is a finely modulated process involving a number of different mechanisms [1,3].

Glutamic acid or glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) [4]. Glutamic acid is a non-essential amino acid, and it is synthesised in neuron mitochondria from glucose and several precursors. After being synthesised, glutamate is released into the cytoplasm where it accumulates in synaptic vesicles through a process dependent on  $\text{Mg}^{2+}$ /ATP. Glutamate interacts with its specific receptors (two types) classified as: metabotropic and ionotropic. Ionotropic receptors are classified according to the affinity of their specific agonists: N-methyl-D-aspartate (NMDA),  $\alpha$ -amino acid-3-hydroxy-5-methyl-4-isoxazole (AMPA) and kainic acid (KA). NMDA receptors are macromolecular structures [5] that are formed by different combinations of subunits, NMDAR1 (NR1), NMDAR2 (NR2) and NMDAR3 (NR3). The NMDA receptor is a specific type of ionotropic glutamate receptor [5-7]. Calcium flux through NMDA receptors is thought to be critical in synaptic plasticity, a cellular mechanism for learning and memory.

Because of the complexity and temporal features of the manifestations of the developing brain, the study of developmental neurotoxicity has great potential for helping to advance our understanding of brain-related biological processes, including neuronal plasticity, degeneration/regeneration, differentiation, toxicity and even therapeutic efficacy [8,9].

It is known that the most frequently used general anesthetics have either NMDA-type glutamate receptor blocking or GABA receptor enhancing properties. The general anesthesia drug products (e.g., ketamine) have been used for many years in pediatric patients without direct clinical evidence of adverse central nervous system sequelae. Data in support of a correlation between surgery and subsequent neuro-physiological changes has accumulated over many years [10-14]. However, at present, causality can not be concluded for either extreme of the life span. Nor have any molecular, cellular, or pathophysiological events linking peri-surgical events to cognitive outcome been indicated in the clinical literature [15]. This review article deals with a calcium-related mechanism to explain the relationship between altered NMDA-type glutamate receptor expression, excessive calcium influx and neural damage that is incurred in prolonged anesthetic (e.g., ketamine) exposure during a sensitive developmental stage. Several advantages for a thorough and systematic characterization of potential ketamine-induced toxicity at genetic, molecular and functional (e.g., calcium imaging) levels will be discussed. These include the relationships between drug-induced neurotoxicity and how sophisticated approaches, such as DNA microarray, *in situ* hybridization and calcium imaging, can be used as tools for dissecting

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out mechanisms underlying pharmacological and toxicological phenomena during development.

## Monitoring the Potential Effect of Anesthetics (e.g., ketamine) on the Regulation in NMDA-Type Glutamate Receptor Expression

A host of mechanistic studies have been completed or are underway which have been helpful in providing rationale for the overall concern over anesthetic and/or sedative-induced neurotoxicity. These studies have been and will be instrumental in teasing apart the causalities, refining hypotheses, developing alternative or protective measures and suggesting clinical strategies for assessing the phenomena in children. Such studies have ranged from cell culture to histopathology, behavioral tests to molecular imaging (*in vivo*) studies – including nonhuman primate [16-21].

There is mounting and convincing preclinical evidence in animal models that anesthetics in common clinical use are neurotoxic to the developing brain; and accumulated data [10-14] indicate the involvement of NMDA-type glutamate receptors in the etiology of neurotoxic effects of anesthetic agents.

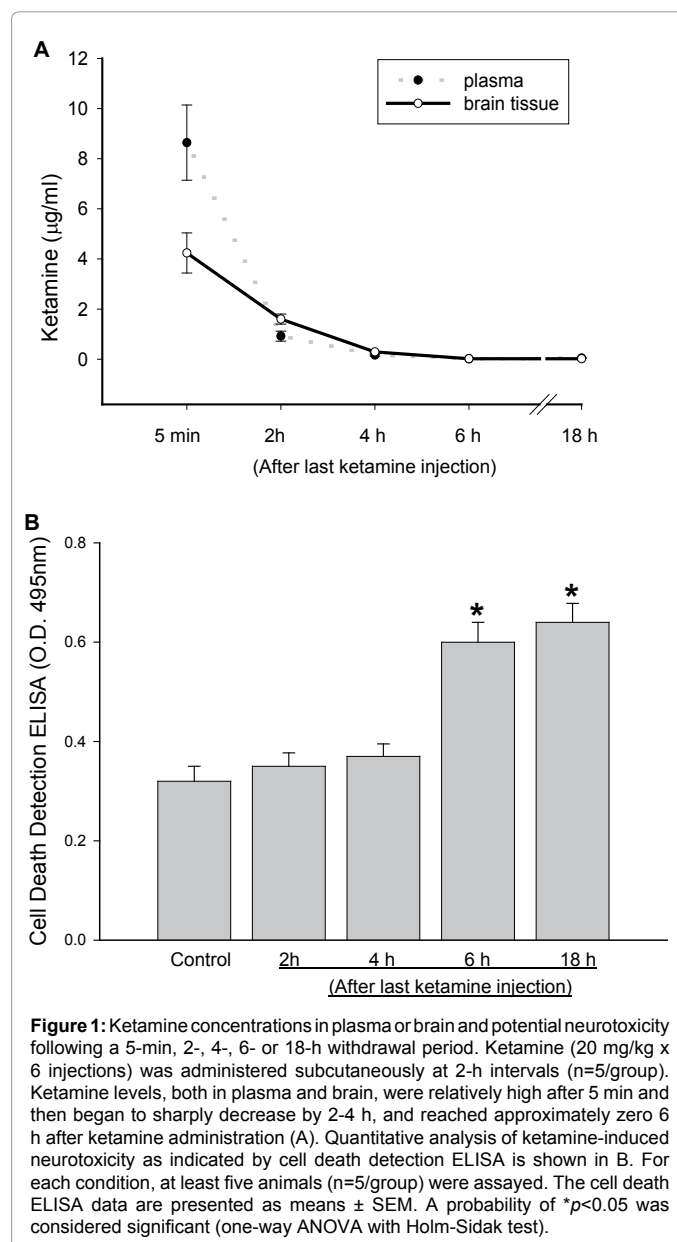
Initially, pharmacokinetic studies [18,22] sought to define the relationship between plasma and brain ketamine levels and the potential neurotoxicity of ketamine. After multiple ketamine injections to rat pups (postnatal day 7), assessment of the effects of plasma and brain (frontal cortex) ketamine levels on fragmented DNA, a potential apoptotic marker, revealed that this marker is not significantly affected at the 2- or 4-h time points compared with controls. With increased withdrawal time (6 hours or longer) plasma and brain ketamine levels are approximately zero, however, neuronal cell death is significantly (~48% increase) increased. These data suggest that enhanced apoptotic cell death, after prolonged ketamine exposure, is not directly associated with the *in situ* blood and brain ketamine levels, but may reflect some indirect or compensatory mechanisms, such as altered NMDA receptor expression (Figure 1).

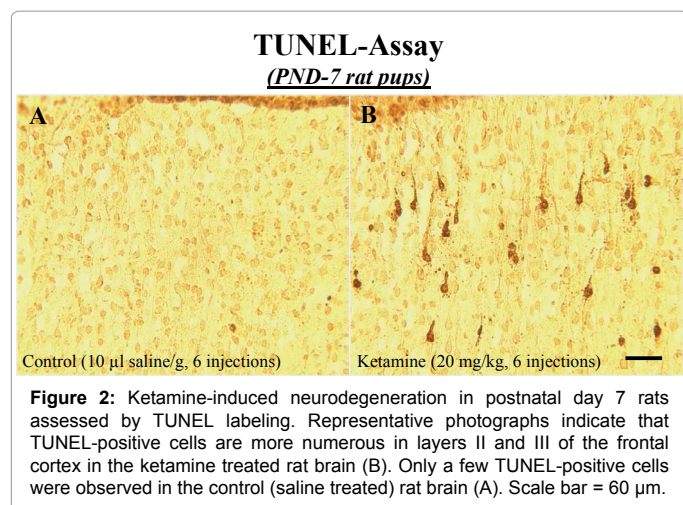
Ketamine has long been known to be a noncompetitive NMDA receptor ion channel blocker. Several lines of evidence [13,14] have indicated a close relationship between the blockade of NMDA receptors and neurodegeneration, but the underlying mechanism involved in such effects remains unknown. It seemed possible that the localization of the most severe brain damage (apoptotic neurons) in the frontal cortex might correspond to alterations in NMDA receptor expression. To understand the molecular pathogenesis of ketamine-induced developmental neurotoxicity, DNA microarray, quantitative (Q)-PCR validation and *in situ* hybridization experiments [23] were carried out to examine the changes in gene expression profiles in the brains of rat pups, which have been shown to display a high susceptibility to ketamine-induced apoptotic neuronal cell death. Brain tissues from frontal cortical levels, where the most severe neuronal damage occurred after prolonged exposure to ketamine, were selected for RNA isolation and microarray analysis. Consistent with the TUNEL assay, microarray data also indicated apoptosis is involved in ketamine neurotoxicity (Figure 2).

Meanwhile, gene expression of the NMDA receptor subunit gene, *Grin1* (*NR1*), was significantly up-regulated in ketamine-treated rat pups as detected in microarray experiments and subsequently confirmed with TaqMan analyses. The NMDA receptor NR1 subunit is widely distributed throughout the brain and is the fundamental subunit necessary for NMDA channel function. At the mRNA level [23,24],

using *in situ* hybridization techniques to monitor the relative densities of NMDA receptor NR1 subunits, a potential parallel relationship between enhanced apoptosis and NMDA receptor expression levels was examined. The *in situ* hybridization data provided direct evidence that prolonged ketamine exposure results in a substantial increase in NR1 subunit mRNA in the developing brain. Both microarray and *in situ* hybridization data support the idea that ketamine-induced pathological change is closely associated with an up-regulation of NMDA receptor.

It is proposed [23] that elevated regulation of *Grin1* (*NR1*) could be accompanied by an altered expression of other glutamate receptor subunits. The data from the microarray analyses revealed an increase in *Grin2a* (*NR2A*; 1.5 fold) and *Grin2c* (*NR2C*; 1.7 fold), but no significant effects were observed in *Grin2b* (*NR2B*) or *Grin2d* (*NR2D*). It should be noted that NMDA-R2 subunits produce functional receptors only when co-expressed with NMDA-R1 [5] and heteromeric complexes





increase the responsiveness to NMDA and yield different functional properties [25]. These findings were consistent with those of previous *in situ* hybridization and immunoblotting data that demonstrated a compensatory up-regulation of NMDA-R1 and NMDA-R2 receptors following prolonged exposure to NMDA receptor antagonists [13,14,18,24] (Table 1).

### Calcium Influx Pathways in Anesthetic (e.g., ketamine)-induced Neuronal Toxicity

NMDA receptor density has been shown to increase in cultured cortical neurons after exposure to the NMDA receptor antagonists D-AP5, CGS-19755, and MK-801, but not after exposure to the AMPA/kainate receptor antagonist CNQX [26]. No significant changes were detected in the gene expression patterns of AMPA or kainate receptors after prolonged ketamine exposure [23]. These findings support previous pharmacological data showing that application of the non-NMDA receptor antagonist, CNQX, or nifedipine (an antagonist of the L-type voltage sensitive calcium channel) did not produce a significant protective effect against ketamine-induced neuronal apoptosis [13,14]. It has been postulated that the continuous exposure of developing brains to anesthetics/continuous blockade of NMDA receptors by NMDA antagonists (e.g., ketamine) causes a compensatory up-regulation of NMDA receptors. This up-regulation makes neurons bearing these receptors more vulnerable, after ketamine withdrawal, to the excitotoxic effects of glutamate, because this up-regulation of NMDA receptors allows for the accumulation of toxic levels of intracellular calcium, even under normal physiological conditions. This hypothesis is also supported by data from previous *in vitro* studies where it was shown that co-administration of antisense oligonucleotides that specifically target NMDA receptor NR1 and NR2A subunit mRNAs were able to block the neuronal damage induced by phencyclidine (PCP; NMDA antagonist) or ketamine [13,14,27].

NMDA receptors are a class of ion channel-forming receptors that are highly permeable to calcium. Cytosolic calcium is an important mediator of neuronal signal transduction, participating in diverse biochemical reactions that elicit changes in synaptic function, metabolic rate, and gene transcription [28-31]. Up-regulation of NMDA receptors can result in an excessive entry of calcium, triggering a series of cytoplasmic and nuclear processes such as loss of mitochondrial membrane potential, which ultimately results in neuronal cell death. Therefore, the interactions between altered ionotropic receptors (e.g., compensatory up-regulation of NMDA receptor) and intracellular

calcium signaling,  $[Ca^{2+}]_i$ , as well as how enhanced  $Ca^{2+}$  flux associated with ketamine exposure influences reactive oxygen species (ROS) generation and subsequent neuronal apoptosis, could appropriately be clarified by monitoring changes in intracellular calcium concentration. Thus, the relationship between anesthetic (ketamine)-induced NMDA receptor dysregulation and signal transduction could systematically be analyzed.

To elucidate the underlying mechanisms associated with ketamine-induced neuronal toxicity, a primary nerve cell culture system was utilized. Neurons harvested from the forebrain of newborn rats were maintained under normal control conditions or exposed to ketamine for 24 hours, followed by a 24-hour withdrawal period [32]. It was demonstrated that ketamine exposure has a significant impact on intracellular  $Ca^{2+}$  homeostasis: the amplitudes of calcium influx caused by activating concentrations of NMDA were significantly increased in neurons from ketamine-exposed cultures (Figure 3).

It has been shown that local and global elevations in neuronal cytosolic calcium are important for a variety of physiological and pathological processes [3]. Calcium imaging techniques were utilized to investigate the potential interactions between NMDA-evoked calcium influx and NMDA receptor activation of mGlu receptors (metabotropic glutamate receptors) in the mediation of calcium signals in cultured neurons. In a recent study, NMDA-elicited increases in intracellular  $Ca^{2+}$  were blocked by perfusing cultures with  $Ca^{2+}$ -free buffer (e.g., in the presence of EGTA), clearly demonstrating that the NMDA-evoked increases in intracellular calcium originated from an extracellular source, rather than from depletion or release of calcium from the endoplasmic reticulum or intracellular calcium store [32].

It should be mentioned that a magnesium-free buffer (perfusion) was used in the calcium imaging study in order to minimize magnesium-blockade of NMDA receptor activation. Since neurons in frontal cortical cultures are known to contain other  $Ca^{2+}$  channels such as voltage-dependent  $Ca^{2+}$  channels and AMPA/kainate receptors that are  $Ca^{2+}$  permeable [33], it was not a surprise to see an even higher intracellular calcium concentration (Figure 3) when the neurons were stimulated by glutamate [32]. Together, these observations provide further support for the hypothesis that enhanced NMDA-type glutamate receptor expression (compensatory un-regulation after prolonged NMDA receptor blockade) promotes the specific signal transduction (e.g., enhanced  $Ca^{2+}$  influx) that plays a critical role in ketamine-induced neurotoxicity.

### Summary

A major emphasis of this review is to elucidate how an appropriate application of genetic/cellular/molecular/biochemical research approaches, including calcium imaging ( $Ca^{2+}$  influx), is crucial for understanding the cellular processes and mechanisms underlying the expression of, and sensitivity to, neurotoxicity. This review discusses several advantages and important issues for using ketamine as an example, and advanced research approaches, e.g., *in situ* hybridization,

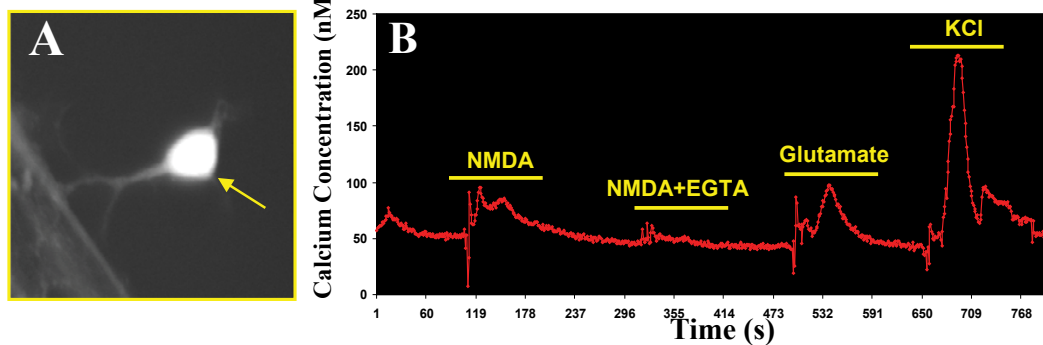
Gene symbols	Fold-change (Q-PCR)	Fold-change (microarray)
<i>Grin1</i> (NR1)	1.8*	1.5*
<i>Grin2a</i> (NR2A)	1.5*	1.2
<i>Grin2b</i> (NR2B)	1.0	0.9
<i>Grin2c</i> (NR2C)	1.7*	1.5*
<i>Grin2d</i> (NR2D)	1.2	1.1

\* $P < 0.05$ , as compared to the control.

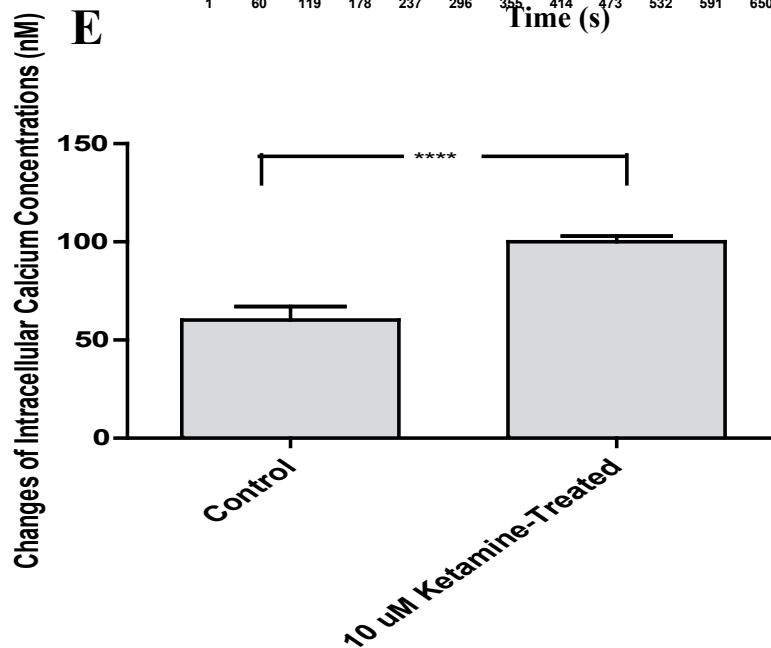
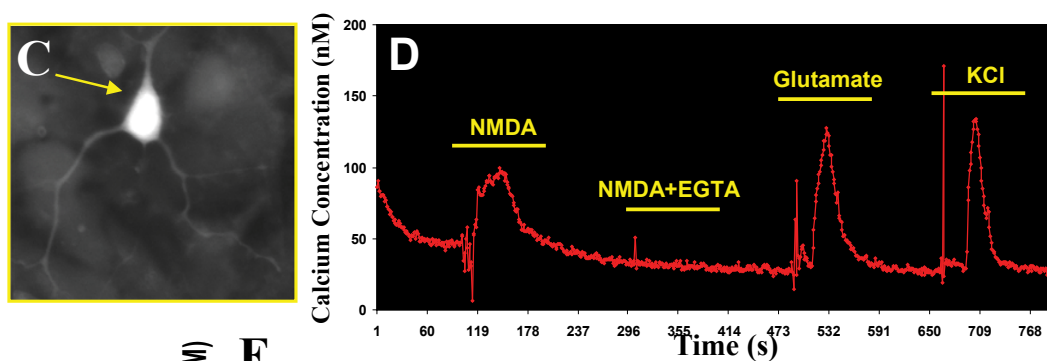
**Table 1:** Selective validation of the microarray results by Q-PCR.

## Changes in $[Ca^{2+}]_i$ in Fura-2-Loaded Neurons

### A representative control neuron



### A representative ketamine-treated neuron



**Figure 3:** Dynamic changes in intracellular calcium concentrations  $[Ca^{2+}]_i$  of a control neuron (A) and a ketamine-exposed neuron (C). Application of NMDA (50  $\mu$ M) or glutamate (25  $\mu$ M) caused an immediate elevation in intracellular free  $Ca^{2+}$  for both control (B) and ketamine-exposed (D) neurons. No NMDA-evoked  $[Ca^{2+}]_i$  rise was observed when the extracellular  $Ca^{2+}$  was chelated and, thus, unavailable for intracellular transport (50  $\mu$ M NMDA + 200  $\mu$ M EGTA in the perfusion buffer). A significant increase in intracellular free calcium  $[Ca^{2+}]_i$  was detected in ketamine-exposed neurons (D and E) compared to control neurons (B and E) after NMDA (50  $\mu$ M) stimulation. Each condition was assessed at least in triplicate and experiments were repeated independently three times. Data are presented as means  $\pm$  S.D.

DNA microarray and calcium imaging, for addressing critical issues related to the toxicity of pediatric anesthetics. The discussed hypothesis indicates a potential specific involvement of NMDA receptor-mediated excitation in ketamine-induced neurotoxicity. Continuous blockade of NMDA receptors by NMDA antagonists, such as ketamine, causes a compensatory up-regulation of the NMDA receptor. This regulation could make cells bearing the receptors more vulnerable, after ketamine washout, to glutamate, because this up-regulation allows for a toxic accumulation of intracellular calcium. Activation of up-regulated NMDA receptors results in intracellular  $Ca^{2+}$  overload that exceeds the buffering capacity of the mitochondria and interferes with electron transport in a manner that results in an elevated production of reactive oxygen species. Thus, NMDA-type glutamate receptor expression levels and the specific signal transduction (e.g.,  $Ca^{2+}$  influx) play a critical role in anesthetic (ketamine)-induced neurotoxicity. Such information will be needed in order to increase the likelihood of the clinical success of our attempts to develop effective rescue and prevention strategies.

## Disclaimer

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