THE EFFECTS OF KETAMINE EXPOSURE ON NMDA RECEPTOR-MEDIATED SYNAPTIC TRANSMISSION IN THE DEVELOPING BRAIN – A MECHANISTIC STUDY OF KETAMINE INDUCED NEUROAPOPTOSIS

by

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Abstract

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Ketamine is a widely used pediatric anesthetic because of its high potency in pediatric patients. Side-effects produced by ketamine anesthesia in adults are not seen in children after ketamine use. However, several preclinical studies have shown that prolonged or repeated exposure to ketamine resulted in widespread death of neurons (neuroapoptosis) in almost all areas of the neonatal brain (Brambrink et al, 2012; Hayashi et al, 2002; Ikonomidou et al, 1999; Scallet et al, 2004; Zou et al, 2009a, b). Increasing evidence shows that this neuroapoptosis affects prenatal and neonatal brain adversely, specifically in brain areas involved in learning and memory, leading to persistent deficits in learning and cognition (Ikonomidou et al, 1999; Paule et al, 2011). Therefore, understanding the molecular mechanism underlying ketamine-induced neuroapoptosis in the neonatal brain becomes important in order to identify

potential therapeutic agents that would prevent neuroapoptosis and/or long-term cognitive deficits. In this study, we demonstrated that repeated ketamine administration (6 injections of 20 mg/kg dose given over 12 h time period) in neonatal (Post-natal Day 5-7; PND 5-7) Sprague-Dawley (SD) rats induced progressive increase in N-methyl-D-aspartate receptor (NMDAR)-mediated miniature excitatory post synaptic potentials (mEPSCs) in the anterior cingulate cortex (ACC) for up to 6 hours after the last ketamine dose. Specifically, there was a significant increase in the mEPSCs mediated by GluN2B-containing NMDARs in neurons of ACC. Taken together; these results indicated that ketamine exposure enhanced synaptic transmission mediated by GluN2B-containing NMDARs in the neonatal brain during ketamine washout period. This was significant as it showed for the first time that ketamine had subunit-specific effects on NMDARs, potentially implicating the involvement of these subunits in increased vulnerability of immature neurons of the neonatal brain to ketamine-induced neuroapoptosis.

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Chapter 1

Introduction

With the advancement in medicine and increase in the use of surgical procedures, the importance of anesthetics has become inevitable. Pediatric surgical procedures typically require specialized anesthetics that are different from those used in adults. However, several preclinical studies have demonstrated that the repeated or prolonged use of anesthetics has a strong potential to cause long lasting neurotoxic effects on the developing brain (reviewed in Jevtovich-Todorovich, 2013). Ketamine is no exception to this effect.

Ketamine as a pediatric anesthetic

Ketamine is a widely used pediatric anesthetic due to its potency and ability to provide rapid anesthesia in children. Because it does not follow the typical stages of dose-dependent anesthesia, its anesthetic effects do not compromise cardiorespiratory functioning when the subject is under anesthesia. Along with anesthesia, it is capable of providing complete analgesia which allows for the performance of painful procedures and surgeries easily. Ketamine can be administered via several routes but the most effective is intravenous or intramuscular (Marland et al, 2013; See Table 1.1). Due to high potency, ease of administration and rapid induction of anesthesia, it can be used to calm uncooperative and shocked children. It is used for minimally painful procedures like minor laceration repair as well as painful procedures such as fracture reduction, bone marrow aspiration and pediatric dental procedures. It is also used in pediatric plastic surgery, oral surgery, neurosurgery, cardiac surgery,

ophthalmic surgery, gastrointestinal procedures and for diagnostic and interventional cardiac procedures in children and neonates (Dong and Anand, 2013). It is used along with other anesthetic agents, like propofol, bupivacaine, ropivacaine, clonidine, fentanyl, sevoflurane, etc., for increasing the rate of anesthesia induction, getting a stronger analgesic effect and maintaining spontaneous respiration and hemodynamic stability (Mion and Villevieille, 2013). Its efficacy as an anesthetic and analgesic is more potent in children than in adults. It also does not show severe psychotomimetic side-effects in children that are commonly observed in adults treated with ketamine-induced anesthesia. Since it provides strong analgesia in children, it eliminates the need to use post-operative non-opioid analgesics. Incremental dosages of ketamine can be administered to maintain longer dissociative states because of its high therapeutic index (reviewed by Green and Johnson, 1990). Thus, its use as a general anesthetic in long pediatric surgeries is increasing. Therefore, ketamine is widely used in the emergency department of hospitals, operating room and dental clinics for sedation and anesthesia while performing procedures on children.

Table 1-1 Route of administration, bioavailability and anesthetic dose of ketamine (Adapted from Marland et al, 2013)

Route of Administration	Bioavailability	Anesthetic starting dose in Children
Intravenous	100%	1-2 mg/kg
Intramuscular	93%	8-10 mg/kg
Intraossesus	100%	1-2 mg/kg
Nasal	45-50%	3-9 mg/kg
Rectal	25-30%	8-15 mg/kg

Ketamine-induced neuroapoptosis

Although ketamine's usefulness as a pediatric anesthetic is unquestionable, a number of preclinical studies have raised potential concerns of ketamine's use in causing neuroapoptosis (Brambrink et al, 2012; Ikonomidou et al, 1999; Hayashi et al, 2002; Scallet et al, 2004; Zou et al, 2009a, b). Repeated or long-term exposure of ketamine, specifically during crucial stages of brain development known as the brain growth spurt period, has been shown to cause widespread neuroapoptosis in several cortical as well as sub-cortical areas of the brain (See Table 1.2; Ikonomidou et al, 1999). What is more interesting is that this neuroapoptosis is a delayed event. Maximum cell death occurs several hours after the administration of last dose of ketamine (Brambrink et al, 2012; Zou et al, 2009a, b). This phenomenon occurs over several different dosing regimens (See

Table 1.3). Single doses and repeated sub-anesthetic doses do not seem to induce neuroapoptosis. Adults appear to be resistant to this neuroapoptosis while fetal and neonatal stages are particularly vulnerable to neurotoxic effects of prolonged or repeated ketamine exposure (Brambrink et al, 2012). Ikonomidou et al. in 1999 observed that substantial cell death occurred in several important areas of the neonatal brain including the hippocampus, thalamus, hypothalamus, frontal and parietal cortices, retrosplenial cortex and cingulate cortex due to ketamine exposure. They postulated that these effects may have long-term detrimental consequences on learning, memory and cognitive processes. Later research showed that there are indeed persistent deficits in learning and memory observed during adulthood in non-human primates that were exposed to ketamine during neonatal ages (Paule et al, 2011). Wang et al. (2014) also showed impaired long-term potentiation (LTP) in rodent animal model that was administered anesthetic doses of ketamine repeatedly. Thus, age-dependent and dose-dependent neurotoxic effects of ketamine specifically on the neonatal brain became a potential cause of concern for the use of ketamine as a pediatric anesthetic.

Table 1-2 Ketamine-induced neuroapoptosis in neonatal brain regions

(Adapted from Ikonomidou et al, 1999)

Brain region	Numerical density of degenerating neurons (means/mm^3 ± SEM)		
	Vehicle	Ketamine	FC
Hippocampus CA1	1,863 ± 242	6,649 ± 1,161	3.6
Dentate gyrus	1,045 ± 170	3,181 ± 860	3.0
Subiculum	1,168 ± 172	22,572 ± 4,194	19.3
Ventromedial Hypothalamus	1,209 ± 14	3,576 ± 452	3.0
Laterodorsal thalamus	402 ± 67	12,297 ± 2,142	30.9
Mediodorsal thalamus	768 ± 20	3,914 ± 726	5.1
Ventral thalamus	1,003 ± 66	3,886 ± 882	3.9
Frontal cortex L2	3,398 ± 395	25,696 ± 2,175	7.6
Parietal cortex L2	2,421 ± 602	34,030 ± 6,796	14.1
Cingulate cortex L2	3,369 ± 445	18,736 ± 3,178	5.6
Cingulate cortex L4	196 ± 44	3,378 ± 674	17.2
Retrosplenial cortex L2	2,089 ± 159	19,710 ± 1,771	9.4
Retrosplenial cortex L4	476 ± 115	4,309 ± 474	9.1

Table 1-3 Effects of ketamine exposure in various animal models

Study	Ketamine dosing regimen	Animal age	Effects
Hayashi et al 2002	7 doses of 25 mg/kg	PND 7 old rats	Increased neuroapoptosis
	Single dose of 25,50 and 75 mg/kg	PND 7 old rats	No neuroapoptosis
Fredricksson et al 2004	Single dose of 50 mg/kg	PND 10 old mice	Increased neuroapoptosis, abnormal behavior, impaired learning acquisition and memory retention during adult hood
Rudin et al 2005	Single doses of 5-40 mg/kg	PND 7 old mice	Increased neuroapoptosis
Young et al 2005	Single dose of 20-40 mg/kg	PND 7 old mice	Increased neuroapoptosis
	Single dose of 10 mg/kg	PND 7 old mice	No neuroapoptosis
Zou et al 2009a	9 h and 24 h infusion of 20-50 mg/kg	PND 5-6 rhesus monkey	Increased neuroapoptosis
Zou et al 2009b	6 doses of 20 mg/kg	PND 7 old rats	Increased Caspase-3 and Fluoro-Jade C positive cells increased
Soriano et al 2010	5 doses of 20 mg/kg	PND 7 old rats	Decrease in cell cycle signaling molecules and increased neuroapoptosis
Paule et al 2011	24 h infusion of 20-50 mg/kg	PND 5-6 rhesus monkey	Persistent and progressive cognitive deficits

Table 1—3—*Continued*

Study	Ketamine dosing regimen	Animal age	Effects
Brambrink et al 2012	5 h infusion of 20-50 mg/kg	E.120 rhesus monkey	Increased neuroapoptosis
		PND 6 rhesus monkey	
Huang et al 2012	Single doses of 25, 50, 75 mg/kg for 3 consecutive days	PND 7 old rats	Increased neuroapoptosis and behavioral deficits seen only at 75 mg/kg dose

NMDARs and ketamine pharmacology

NMDARs belong to the family of ionotropic glutamate receptors and are widely expressed throughout the nervous system. They are heterotretrameric in structure comprising of four subunits. Seven different types of NMDAR subunits have been identified; namely GluN1, GluN2 (A-D) and GluN3 (A, B). Various combinations of these subunits give rise to different subtypes of NMDARs that vary in pharmacological and biological properties. Almost all of the NMDARs comprise of two GluN1 subunits which form the ion channel pore. The remaining two subunits of the NMDAR tetramer are made up of different combinations of GluN2 or GluN3 subunits. Most of the NMDARs have two GluN2 subunits (typically two GluN2B subunits or one GluN2B and one GluN2A subunit) along with the GluN1 subunits. It has been observed that the expression of GluN2 subunits varies across development. The composition of the GluN2 subunits also varies according to different brain areas and neuronal populations (Paoletti, 2011). In this study, we have exploited this functional and developmental variability of the NMDARs to determine ketamine's differential effects on immature neurons.

Pharmacologically, ketamine is a non-competitive antagonist of NMDAR. It binds at a transmembrane site within the ion channel pore of the NMDAR thus blocking the flow of sodium and calcium ions into the neuron (Marland et al, 2013). Apart from being an NMDAR antagonist, ketamine is a weak agonist of dopaminergic D2 receptors (Kapur and Seeman 2002). It also interacts with muscarinic acetylcholine receptors to decrease release of γ-Amino butyric acid

(GABA) in immature GABAergic neurons (Vutskits et al 2006, 2007). Along with that, it also interacts with opioid receptors causing analgesic effects (Lahtinen et al. 2004). Ketamine exists as two isomers. S (+)-ketamine is the most potent form and is twice as strong as the racemic mixture and four times as strong as the R (-)-ketamine isomer. Most commonly used form of ketamine is a hydrochlorate solution of the racemic mixture containing benzothonium chloride or chlorbutanol as preservatives (Mion and Villeieille, 2013).

Molecular mechanism of ketamine-induced neuroapoptosis

Ketamine induces its most important function of anesthesia by blocking the NMDARs. However, concern regarding its use has increased in the minds of pediatric medical practitioners due to increasing preclinical evidence demonstrating ketamine's ability to induce widespread neuroapoptosis specifically in neonates and especially during the brain growth spurt period (Ikonomidou et al, 1999). Understanding the molecular mechanism that caused this neuroapoptotic effect thus became necessary. The most well accepted mechanism proposed to explain this neuroapoptosis came from the study conducted by Liu et al in 2013. According to this mechanism, ketamine-induced neuroapoptosis involves up regulation of NMDARs, increased calcium influx and elevated reactive oxygen species. Briefly: NMDAR block by ketamine prevents calcium from entering into the cell. The neuron's internal calcium sensing mechanism detects this lack of calcium and responds to it by up regulating NMDAR expression. Following ketamine washout, the NMDARs regain their function and start bringing calcium into the neuron. However, due to increased

levels of NMDARs, the amount of calcium influx into the neuron is increased which results in a calcium overload that exceeds the buffering capacity of the neuron's internal calcium regulators (i.e. mitochondria and endoplasmic reticulum). The neuron gets saturated with calcium and enters a state of excitotoxicity. Prolonged excitotoxicity ultimate initiates apoptotic pathways and leads to the death of the neuron (Liu et al, 2013; Wang et al, 2006).

The above mentioned mechanism has been well accepted based on different studies that have demonstrated specific aspects of this mechanism (Liu et al, 2013; Wang et al, 2006; Zou et al, 2009a, b). However, there is a lack of evidence that explains the specificity of this mechanism to occur only in neonates during a particular developmental stage. Since ketamine-induced neuroapotosis is a delayed event, evidence of temporal changes in the activity of the up regulated NMDARs during and after ketamine washout also does not exist. These vacuoles of information inspired our group to study the specific pharmacological and biological properties of the NMDARs present in neonates during the brain growth spurt period. Understanding these specific properties of the NMDARs would provide insight into increased vulnerability of immature neurons to ketamine.

Preliminary data:

<u>EPSCs mediated by NMDARs undergo time-dependent enhancements selectively in immature but not mature neurons during ketamine washout</u>.

Preliminary unpublished data from our group has identified that prolonged ketamine exposure induced time-dependent changes in NMDAR activity

following ketamine exposure and that these changes were specific for immature neurons. We recorded evoked excitatory post synaptic currents (eEPSCs) mediated by NMDARs from the anterior cingulate cortex (ACC) of neonatal (PND 5-7) and adolescent (3-5 weeks old) SD rats at different time points after ketamine washout.

A repeated ketamine exposure rat model (Zou et al, 2009b) was used for this study. Briefly, PND 5-7 and 3-5 weeks old rats were administered 20 mg/kg of ketamine (hereafter referred to as experimental group) or 20 µL of saline (hereafter referred to as Control group), sub-cutaneously, six times, at 2 hour intervals. A 2 hour time window between injections was selected because the half-life of ketamine ranges between 1 to 1.5 hours. The rats were sacrificed at 3, 8 and 12 hours after the last ketamine dose (See Table 1.4).

Table 1-4 Experimental setup for recording NMDAR-mediated eEPSCs

Groups	Treatment	Time of sacrifice after last dose	Age group
Control	Saline 20 µL/dose	12 hours (C 12h)	Neonatal
			Adolescent
Experimental	Ketamine 20 mg/kg/dose	3 hours (K 3h)	Neonatal
			Adolescent
		8 hours (K 8h)	Neonatal
			Adolescent
		12 hours (K 12h)	Neonatal
			Adolescent

Whole brain was isolated from these animals and sectioned to obtain coronal slices of the forebrain containing ACC. Slices were maintained in oxygenated artificial cerebrospinal fluid (aCSF) at all times to maintain viability of the neurons. NMDAR-mediated eEPSCs were recorded from pyramidal neurons in layers 2 and 3 of the ACC. *In vitro* whole cell patch clamp recordings were used to measure NMDAR-mediated eEPSCs. NMDA receptor-mediated eEPSCs were pharmacologically isolated by adding, 6-cyano-7-nitroquinoxaline (CNQX - 10 µM; Tocris) which is a competitive non-NMDA glutamate receptor antagonist and bicuculline (10 µM; Tocris) which is a competitive γ-Amino butyric acid (GABA) A receptor antagonist, to the external solution. All currents were recorded under voltage clamp mode (holding membrane potential +40 mV) of Axon 200B amplifier connected to Digidata 1440A manipulator (Molecular

devices). eEPSCs were induced by delivering single electrical pulse (0.25-0.5 mA, 0.3 ms) to layer 5 of the ACC using Master 10 stimulator system. The stimulus intensity used to evoke eEPSCs was adjusted to produce 50% of maximal response as determined for each neuron recorded. eEPSCs from both adolescent and neonatal rats were recorded at 3 time points (3, 8 and 12 h) after last ketamine dose. We selected the adolescent age group for comparison because the NMDAR composition is stabilized at this developmental stage and is also different from that observed at earlier developmental stages. Data was recorded using Axon pCLAMP 10.3 and analyzed offline using Clampfit 10.0 software (Molecular devices). eEPSCs from control group were recorded only at 12 h time point. The amplitude of eEPSCs recorded from control groups was used to determine percent change in amplitude of eEPSCs recorded from experimental group. A 2 (Neonatal vs adolescent) X 3 (Time) repeated measures analysis of variance with post hocs employing Bonferroni correction was conducted to determine statistically significant group differences.

Within the experimental group, NMDAR-mediated eEPSCs showed a significant time-dependent increase in amplitude, selectively in neurons of neonatal rats when compared to the amplitude of eEPSCs recorded from neurons of adolescent rats. This increase in amplitude of eEPCSs was significant for up to 12 hours following ketamine washout. The sustained increases in NMDA receptor-mediated eEPSCs, specifically in immature neurons of neonatal rats, indicated increased NMDAR-mediated synaptic activity at the postsynaptic terminals for up to 12 hours after ketamine washout. The increase in amplitude of

eEPSCs was restricted to only the experimental group. Control group did not show increased NMDAR-mediated synaptic activity. Thus, ketamine exposure produced significant time-dependent changes in NMDAR-mediated eEPSCs of immature neurons but not mature neurons (See Fig 1-1). This indicated that ketamine exhibited differential effects on NMDARs of immature neurons as compared to mature neurons. Based on this finding, all further studies were conducted only on immature neurons of PND 5-7 old SD rats.

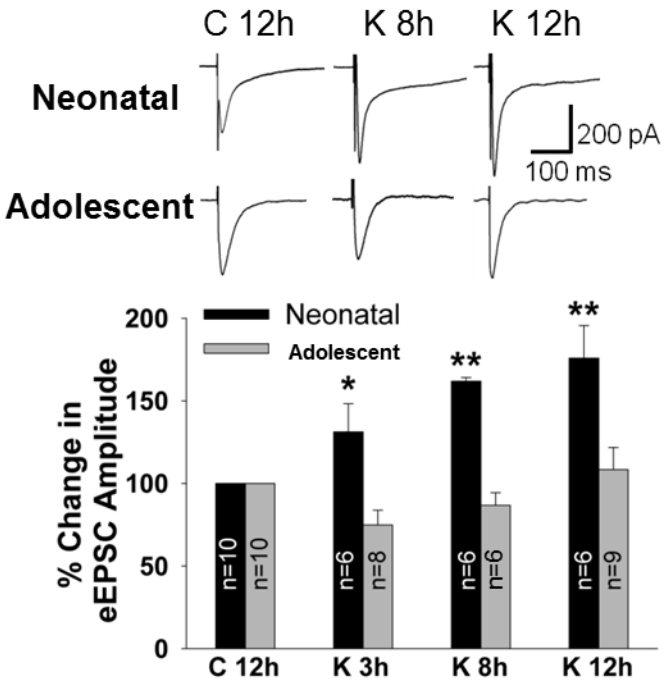

Figure 1-1 A time-dependent enhancement of NMDA receptor-mediated eEPSCs Enhanced whole-cell NMDAR-mediated currents occurred specifically in immature neurons of the neonatal, but not mature neurons of adolescent rats following ketamine washout. NMDAR-mediated eEPSCs were recorded at 3, 8 and 12 h (K 3-12h) for the experimental group (ketamine exposed). NMDAR-

mediated eEPSCs were recorded at 12 h only for the control group (C 12h). Amplitude of NMDAR-mediated eEPSCs from neurons of both, neonatal and adolescent rats was set as controls (100%) for assessing statistical significance. Data are presented as mean ± S.E. values. Probability of *$p < 0.05$ & **$p < 0.01$ was considered significant.

Rationale and Hypotheses

Jin et al. in 2013 demonstrated, *in vitro,* that neonatal NMDARs respond differently to the blocking effects of the ketamine. Specifically, blocking effects of ketamine were stronger for neonatal NMDARs as compared to adult NMDARs.

It is well studied that the NMDAR is under developmental regulation. The subunit composition of NMDARs changes across development. At prenatal and neonatal stages, GluN2B-containing NMDARs dominate the excitatory synapses of the immature brain and as development progresses, subunit composition of NMDARs, changes from GluN2B-containing to GluN2A-containing. This switch in expression pattern is particularly evident in the ACC (Paoletti, 2011).

Due to this difference in the composition of neonatal NMDARs, we hypothesized that the immature neurons would be highly vulnerable to ketamine's neurotoxic effects. Specifically, we anticipated that ketamine would trigger apoptotic cascades especially in the immature neurons via changes in the expression and activity of the GluN2B-containing NMDARs. This anticipation stemmed from the fact that GluN2B-containing NMDARs have been associated with glutamate excitotoxicity both *in vitro* and *in vivo* (Chen et al, 2012, vonEngelhardt et al, 2007, Liu et al, 2007). Thus, we expected to see

characteristic vulnerability of immature neurons to ketamine predominantly in the ACC due to typical subunit composition of the NMDA receptors.

Hypothesis 1

Repeated ketamine exposure of PND 5-7 old neonatal rats will cause time-dependent increase in NMDAR-mediated synaptic transmission in the pyramidal neurons of the ACC. Specifically; NMDAR activity will peak between 5 hours to 7 hours post last ketamine exposure.

Hypothesis 2

Increased NMDAR activity will be specific for GluN2B-containing NMDARs in neonatal neurons.

Chapter 2

Materials and methods

Animals

All animal procedures were carried out according the protocols approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington. All animals were obtained from the animal breeding colony at the University of Texas at Arlington. Neonatal rats (Sprague-Dawley, male and female) in the age group of PND 5-7 were randomly sampled and used for all experiments. All animals were housed in a 12/12-hour constant light/dark cycle at controlled temperature (22-25°C) and humidity (55-60%). Food and water was provided *ad libitum*.

Drug Administration

Animal-grade ketamine hydrochloride solution (100 mg/mL; AmTech) was diluted in saline to get a working solution (10 mg/mL). A dose of 20 mg/kg ketamine was administered six times, sub-cutaneously, at two-hour intervals to rat pups at age PND 5-7 (Wang et al, 2014). All further procedures were conducted after appropriate washout period post ketamine exposure. The washout period was set at different time points between 1 to 8 hours after the last dose of ketamine was administered (See Fig. 2.1 and 2.2 for detailed time-line of drug administration).

In vitro whole-cell patch-clamp electrophysiology

Electrophysiological recordings were conducted on cortical neurons (pyramidal cells of layers 2 and 3) of the ACC in acutely isolated coronal

forebrain slices. The slice sampling, recording procedures, and data acquisition have been described in detail and are similar with those in previously published work by our group (Jin et al, 2013; Wang et al, 2014). Briefly:

Slice sampling

After decapitation under deep anesthesia (sodium pentobarbital, 50 mg/kg, i.p.), the whole brain was carefully isolated and quickly transferred to an ice-cold (0-4°C) bath of artificial cerebrospinal fluid (aCSF; composition in mM: NaCl 124, KCl 3.3, KH2PO4 1.2, CaCl2 2H2O 2.5, MgSO4 2.4, NaHCO3 26, and glucose 10) bubbled with a 95% O2/5% CO2 gas mixture (pH 7.3-7.4). Brain slices (~350 μm) were sectioned using a vibrating tissue slicer (DTK-1000), and then immediately transferred to a chamber with oxygenated (95% O2 and 5% CO2) aCSF maintained at 32°C. Slices were incubated for at least 90 minutes for recovery. A single brain slice was then held down in the recording chamber, while being immersed in oxygenated aCSF which was perfused at a flow rate of 2.2-2.6 ml/min using a fast perfusion system (WPI). Patch electrodes, of borosilicate glass, were prepared using a horizontal electrode puller (P-87, Sutter) to produce tip openings of 1-2 μm (2-4 MΩ bath resistance). These electrodes were filled with an internal solution containing (in mM): Cs2SO4, 110; CaCl2, 0.5; MgCl2, 2; EGTA, 5; HEPES, 5; tetraethylammonium-Cl, 5; with pH adjusted to 7.2-7.4 by CsOH, and an osmolarity of 290-320 mOsm. Pyramidal neurons, located across layers 2 and 3 in the forebrain cortex and visualized with an infrared video microscope, were recorded. Miniature excitatory post synaptic currents (mEPSCs) were recorded under voltage-clamp mode at a holding

membrane potential of +40 mV with an Axon 200B amplifier (Molecular Devices) connected to a Digidata interface (Digidata 1440A, Molecular Devices).

Experiment 1

Time-dependent changes in NMDAR-mediated synaptic transmission were recorded 2, 4, 6 and 8 hours after last ketamine dose. NMDAR-mediated mEPSCs were pharmacologically isolated by bath application of CNQX (10 μM, competitive antagonist non-NMDA glutamate receptors; Tocris) and bicuculline (10 μM, competitive inhibitor of $GABA_A$-receptor; Sigma/Aldrich) (Zhao et al, 2009). TTX (1 μM; Abcam) was also added to block sodium channel activity. Approximately 500 events (~10-12 minutes) were recorded from each neuron. All ion channel inhibitors were bath applied 5 minutes before recording began.

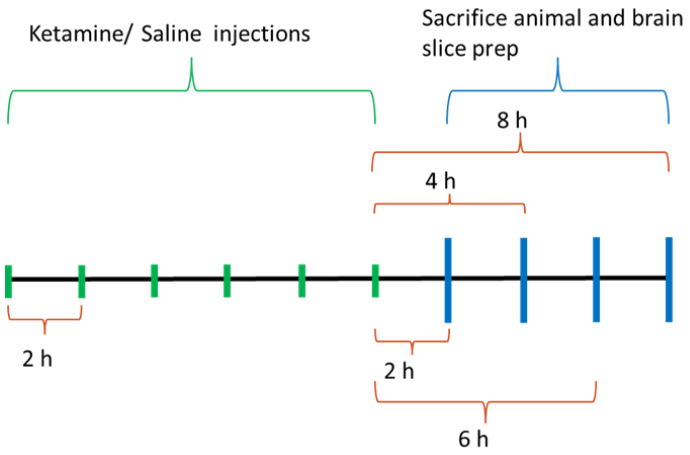

Figure 2-1 Time-line of experimental paradigm for Experiment 1

Ketamine was administered consecutively six times on the same day at 20 mg/kg dose every 2 hours (indicated by green marks) for the experimental group. Saline (20 µL) was administered to the control group in the same way. 2 (first blue mark), 4 (second blue mark), 6 (third blue mark) and 8 hours (fourth blue mark) after the last ketamine dose, animals were sacrificed and whole brain was isolated, sectioned and used for whole-cell patch clamp recordings.

Experiment 2

NMDAR-mediated mEPSCs were recorded at 6 hours after the last ketamine dose. Subunit-specific antagonists were used to determine composition of the up-regulated NMDARs. Ro25-6981, GluN2B-specific antagonist (1 µM; Tocris; Fischer et al, 1997) was bath applied in addition to the other inhibitors (TTX, CNQX and bicuculline) to selectively block mEPSCs mediated by GluN2B-containing NMDARs. Similarly, to selectively block mEPSCs mediated by

GluN2A-containing NMDARs, TCN 201 (10 μM; Tocris; Edman et al, 2010) was bath applied along with TTX, CNQX and bicuculline. Approximately 500 events (~10-12 minutes) were recorded from each neuron. All ion channel inhibitors were bath applied 5 minutes before recording began.

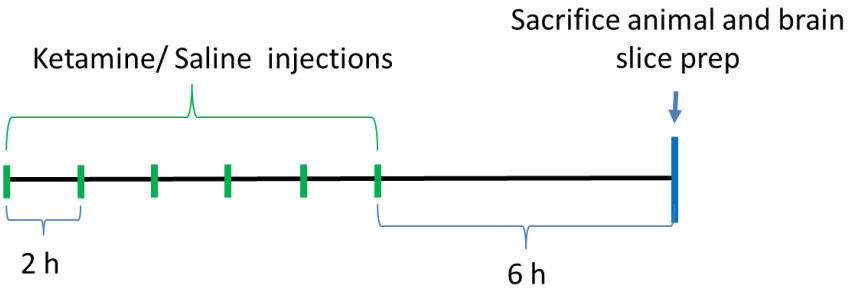

Figure 2-2 Time-line of experimental paradigm for Experiments 2 and 3 Ketamine was administered consecutively six times on the same day at 20 mg/kg dose every 2 hours (indicated by green marks) for the experimental group. Saline (20 μL) was administered to the control group in the same way. 6 hours (blue mark) after the last ketamine dose, animals were sacrificed and whole brain was isolated, sectioned and used for whole-cell patch clamp recordings.

Experiment 3

To pharmacologically confirm contribution of GluN2B-containing NMDARs towards increased synaptic transmission, Ro25-6981 (Tocris) was bath applied to the brain slice at increasing concentrations (0.01, 0.1 and 1 μM) along with TTX, CNQX and bicuculline. This was done to selectively isolate and then block mEPSCs mediated by GluN2B-containing NMDARs. Robust dose-dependent

decrease in NMDAR-mediated mEPSCs would indicate involvement of GluN2B-containing NMDARs in ketamine-induced increased synaptic transmission of NMDARs. NMDAR-mediated mEPSCs were recorded at 6 hours after the last ketamine dose. Approximately 500 events (~10-12 minutes) were recorded from each neuron. All ion channel inhibitors were bath applied 5 minutes before recording began.

Data recording

All live electrical activity data was digitized online using pCLAMP 10.3 and analyzed offline using Mini Analysis Program (Synaptosoft). For detection of NMDAR-mediated mEPSCs, threshold amplitude was set at four times greater than baseline (30 pA) and threshold area was set at 200.

Data Analysis

Amplitude (in pA) and Frequency (in Hz) of NMDAR-mediated mEPSCs was determined for all recordings of both the groups (experimental – ketamine exposed; control – saline exposed). For experiment 1, percent change in amplitude and frequency of NMDAR-mediated mEPSCs for the experimental group was determined by using amplitude and frequency of NMDAR-mediated mEPSCs of the control group. Statistical significance was determined for this percent change in amplitude and frequency of NMDAR-mediated mEPSCs. For experiment 2 and 3, raw scores of amplitude and frequency were used for statistical analyses.

All data analyses were conducted using Sigma plot software (Systat software Inc.). For experiment 1, 2(ketamine vs saline) X 4(Time) repeated

measures analysis of variance was conducted. For experiment 2 and 3, one-way analysis of variance was conducted to determine between-groups differences. Post hoc analyses using Bonferroni correction were conducted for all experiments to determine individual group level differences. Probability levels (*p*-values) of less than 0.05 were considered statistically significant.

Chapter 3

Results

NMDAR-mediated mEPSCs increased progressively

Amplitude and frequency of NMDAR-mediated mEPSCs, recorded from pyramidal cells of layers 2 and 3 of the ACC, of neonates that were exposed to ketamine showed significant time-dependent increase when compared to control group. There was a main effect of time on amplitude $F(3, 1125) = 30.57$, $p < 0.001$ and frequency $F(3, 1124) = 36.87$, $p < 0.001$ of NMDAR-mediated mEPSCs for the experimental group, such that as time increased both amplitude and frequency of NMDAR-mediated mEPSCs increased for 2, 4 and 6 hours while both amplitude and frequency decreased significantly at 8 hour time point. Control group did not show a main effect of time on either amplitude or frequency. Percent change in amplitude and frequency of NMDAR-mediated mEPSCs was then determined to see group level changes at different time points. Beginning 2 hours after the last ketamine dose, both amplitude and frequency of NMDAR-mediated mEPSCs progressively increased. The increase in amplitude and frequency of mEPSCs peaked at 6 hours after the last ketamine dose (See Table 3.1 and Fig 3.1 and 3.2). These results supplemented the changes seen in the evoked currents (Section 1.5), indicating that ketamine administration induced increased activity of the up-regulated NMDARs at the post synaptic neurons for up to 6 hours following ketamine washout.

In summary, repeated ketamine exposure induced progressive increase in the synaptic transmission mediated by NMDARs. This enhanced synaptic transmission began 2 hours after the last ketamine dose and lasted for up to 8 hours after the last dose. Peak activity of NMDARs was seen at 6 hours after the last ketamine dose.

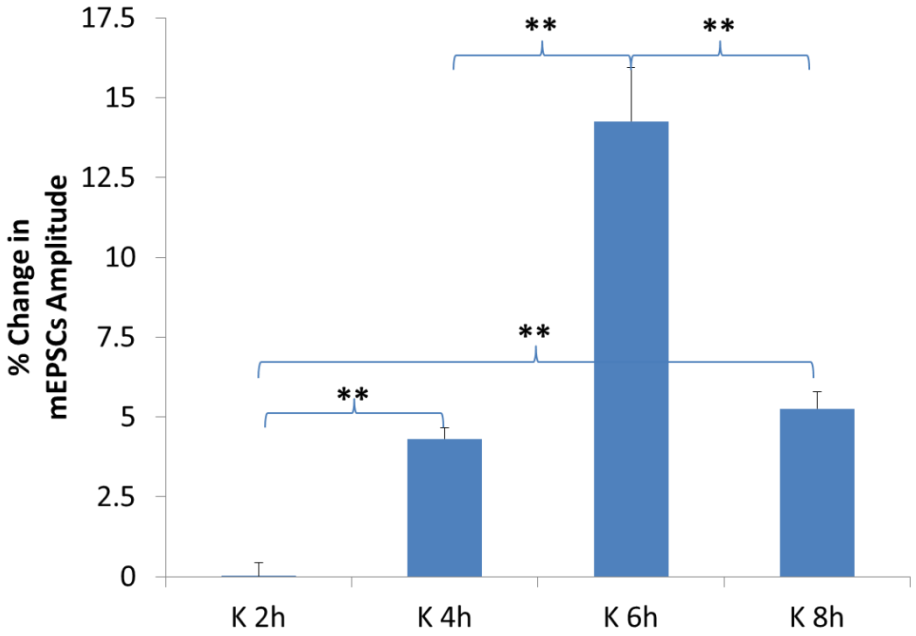

Figure 3-1 Relative change in amplitude of NMDAR-mediated mEPSCs

Amplitude of NMDAR-mediated mEPSCs was measured at 2, 4, 6 and 8 hours after the last ketamine dose. Percent change in amplitude of NMDAR-mediated mEPSCs of ketamine exposed neurons in comparison with control was plotted. Amplitude of mEPSCs increased progressively with time beginning at 2 hours and lasting until 8 hours. Peak increase in amplitude is observed at 6 hours after the last ketamine dose. n = 10 neurons recorded from 4 animals. **$p < 0.01$.

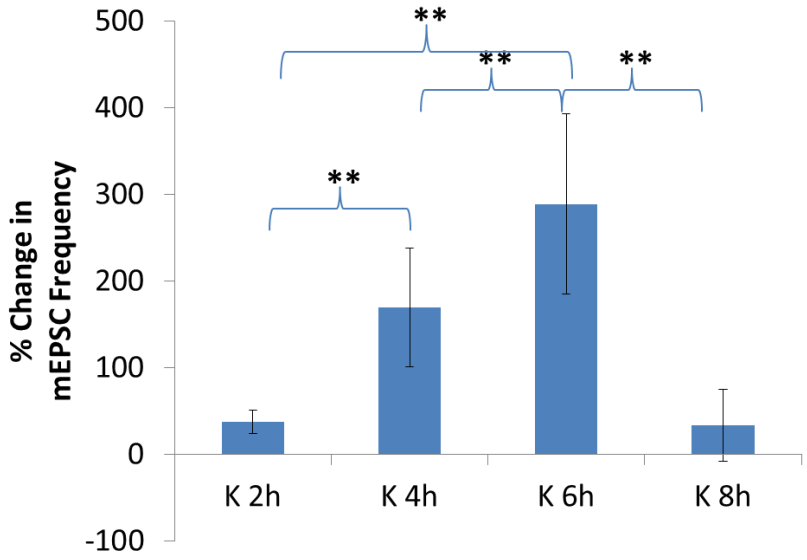

Figure 3-2 Relative change in frequency of NMDAR-mediated mEPSCs

Percent change in frequency of NMDAR-mediated mEPSCs was measured at 2, 4, 6 and 8 hours after last ketamine dose. Frequency changes also follow similar trends as observed with the amplitude changes except that at 8 hours after the last dose, frequency of mEPSCs goes back to basal levels (i.e. of 2 hour time point).*n* = 10 neurons recorded from 4 animals for all groups. ***p* < 0.01.

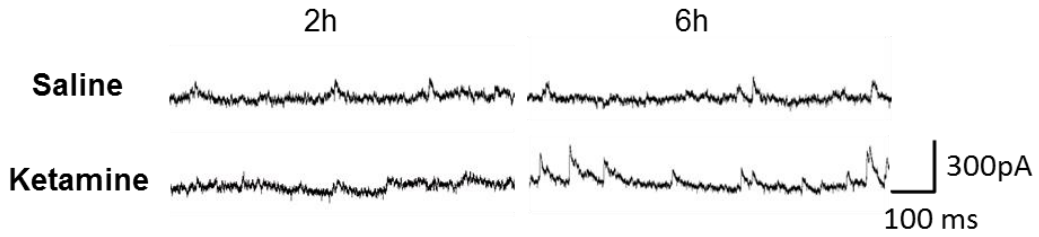

Figure 3-3 Representative traces of NMDAR-mediated mEPSCs

Figure shows representative trace patterns of NMDAR-mediated mEPSCs from experimental and control groups at 2 hours and 6 hours after last ketamine dose.

Table 3-1 Means and SEM of % change in amplitude and frequency of NMDAR-mediated mEPSCs across different time points

	K 2 h	K 4 h	K 6 h	K 8 h
% Change in Amplitude	0.02	4.32	14.25	5.26
S.E.M.	0.41	0.35	1.70	0.51
% Change in Frequency	37.74	169.31	288.79	33.49
S.E.M.	13.55	68.15	104.35	41.35

Blocking GluN2B subunits and not GluN2A subunits reduced NMDAR-mediated enhanced synaptic transmission

Subunit-specific antagonists, Ro25-6981 (GluN2B-specific antagonist) and TCN 201 (GluN2A-specific antagonist) were bath applied to the brain slices *in vitro.* Significant changes in both amplitude, $F(3, 3992) = 322.13$, $p < 0.001$

and frequency, $F(3, 3992) = 9.92$, $p < 0.001$ of NMDAR-mediated mEPSCs were recorded. NMDAR-mediated mEPSCs were recorded from four groups: 1. Control only (saline), 2. Experimental only (ketamine), 3. Experimental with Ro25-6981 and 4. Experimental with TCN 201. Bath application of, Ro25-6981 significantly decreased NMDAR-mediated synaptic transmission. Both amplitude and frequency (See Fig 3.7 and 3.8) of NMDAR-mediated mEPSCs were reduced to control levels after application of 1 μM of Ro25-6981. In contrast, application of TCN 201 (10 μM), reduced the amplitude and frequency of NMDAR-mediated mEPSCs but not as strongly as that when Ro25-6981 was applied to the neurons. All recordings were done at 6 hours after the last ketamine dose, when NMDAR-mediated synaptic transmission was at its peak. These data indicated that majority of the upregulated NMDARs were of GluN2B-subtype.

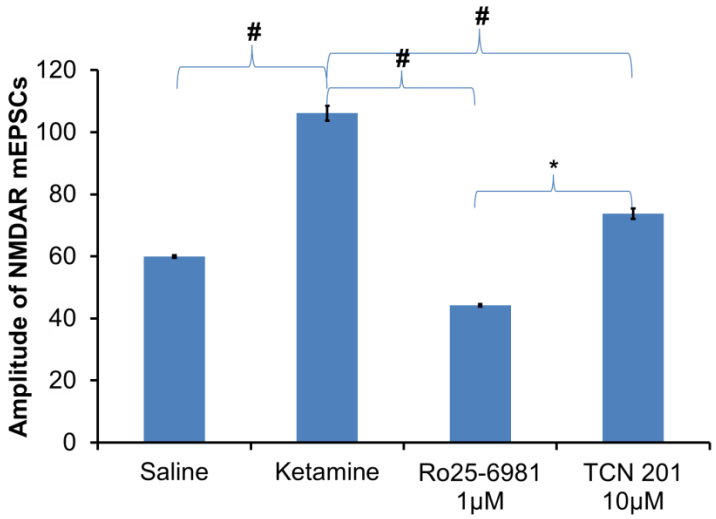

Figure 3-4 Comparing contribution of GluN2A v/s GluN2B to amplitude of NMDAR-mediated mEPSCs

Ketamine exposure increased amplitude of NMDAR-mediated mEPSCs when compared to saline controls. Application of Ro25-6981 decreased amplitude of mEPSCs significantly in comparison to experimental only. TCN 201 also lowered amplitude as compared to experimental only group. However, the extent to which Ro25-6981 was able to lower the amplitude is significantly greater than that of TCN 201. n = 10 neurons from 4 animals per group. $^{*}p < 0.05$, $^{\#}p < 0.01$

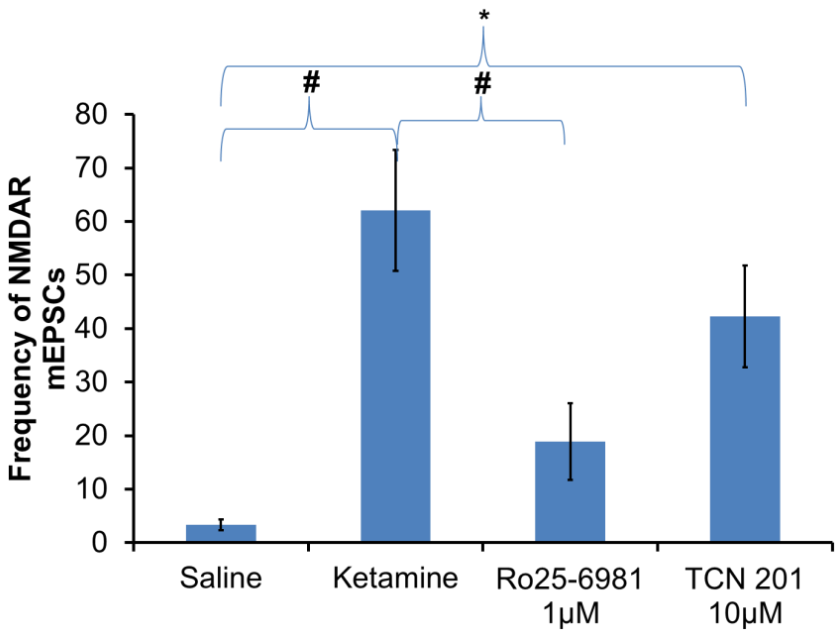

Figure 3-5 Comparing contribution of GluN2A v/s GluN2B to frequency of NMDAR-mediated mEPSCs

Ketamine treatment increased frequency of NMDAR-mediated mEPSCs when compared to saline controls. Application of Ro25-6981 decreased frequency of mEPSCs significantly. Although TCN 201 also lowered frequency, it was not significantly lower than ketamine alone group. n = 9 neurons from 4 animals per group. $^{*}p < 0.05$, $^{\#}p < 0.01$

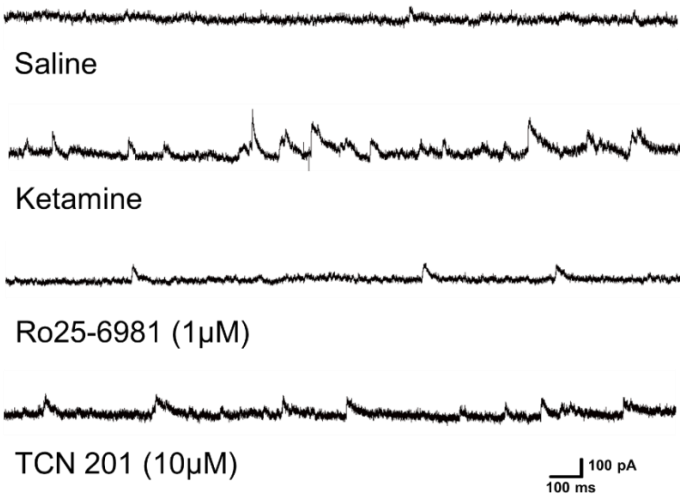

Figure 3-6 Representative traces of NMDAR-mediated mEPSCs after Ro25-6981 or TCN 201 treatment

NMDAR-mediated mEPCs are depicted under different conditions. Saline trace shows least number of mEPSCs followed by ketamine + Ro25-6981 (1 μM). Ketamine + TCN 201 (10 μM) have mEPSCs with lower amplitude but equal frequency as compared to only ketamine.

Table 3-2 Means and SEM of amplitude and frequency of NMDAR-mediated mEPSCs across different conditions

Condition	Saline	Ketamine	Ketamine + Ro25-6981	Ketamine + TCN 201
Amplitude (± SEM)	59.93 (± 0.35)	106.12 (± 2.37)	44.21 (± 0.39)	73.79 (± 1.66)
Frequency (± SEM)	3.23 (± 1.01)	62.09 (± 11.31)	18.92 (± 7.14)	42.31 (± 9.51)

Ro25-6981 decreased NMDAR-mediated enhanced synaptic transmission in a dose-dependent manner.

Bath application of increasing doses of Ro25-6981 progressively decreased both amplitude $F(4, 4990) = 344.09$, $p < 0.001$ and frequency $F(4, 4990) = 7.37$, $p < 0.001$ of NMDAR-mediated mEPSCs. NMDAR-mediated mEPSCs were recorded from five groups: 1. Control only (saline), 2. Experimental only (ketamine), 3. Experimental with 0.01 μM Ro25-6981, 4. Experimental with 0.1 μM Ro25-6981 and 5. Experimental with 1 μM Ro25-6981. Post-hoc analysis revealed that when 0.01 μM of Ro25-6981 was applied, it significantly reduced the amplitude of NMDAR-mediated mEPSCs as compared to experimental only condition without Ro25-6981. 0.1 μM application of Ro25-6981 enhanced the reduction in amplitude further. Bath application of 1 μM of Ro25-6981 reduced both amplitude and frequency of NMDAR-mediated mEPSCs. Frequency of mEPSCs decreased significantly only when 1 μM of Ro25-6981 was applied (See Fig 3.3 and 3.4; Table 3.2). Together, this data indicates that amplitude of NMDAR-mediated mEPSCs were progressively inhibited by Ro25-6981 in a dose-dependent manner. However, frequency of mEPSCs was inhibited by the highest dose. Lower doses of Ro25-6981 did not have significant inhibition on the frequency of mEPSCs. This further confirmed that inhibition of NMDAR-mediated mEPSCs by Ro25-6981 was because of its pharmacological effect on these receptors and not due to its effect at the presynaptic terminal.

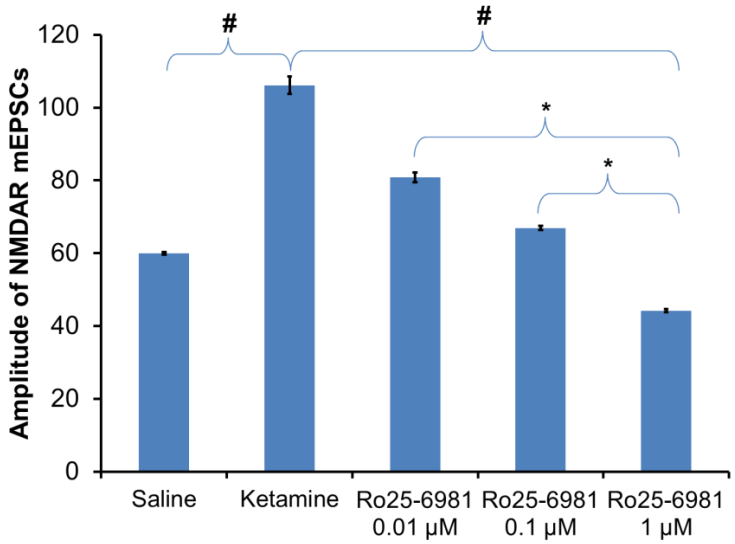

Figure 3-7 Ro25-6981 administration dose-dependently reduced the amplitude of NMDAR-mediated mEPSCs

Increasing doses of Ro25-6981 (Ketamine = 0, 0.01, 0.1 and 1 μM) significantly decreased the amplitude of NMDAR-mediated mEPSCs in a dose-dependent manner. Experimental only group (ketamine) did not have any Ro25-6981 added to it, hence its represents 0 μM of Ro25-6981. n = 7 neurons from 3 animals for all groups. *$p < 0.05$, $^{\#}p < 0.01$

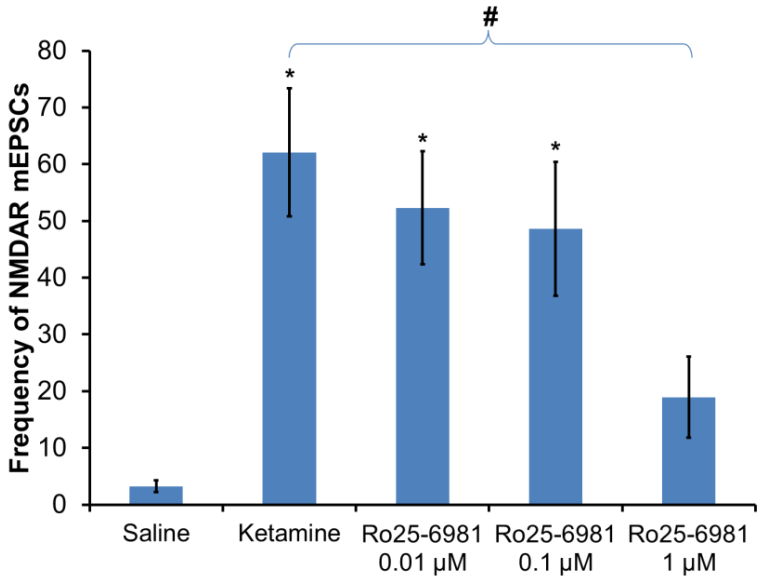

Figure 3-8 Ro25-6981 administration dose-dependently decreased the frequency of NMDAR-mediated mEPSCs

All doses of Ro25-6981 (Ketamine = 0, 0.01 and 0.1 μM) did not decrease frequency of NMDAR-mediated mEPSCs. However, frequency of mEPSCs was significantly greater than that of saline control. 1 μM application of Ro25-6981, significantly lowered the frequency of mEPSCs to control levels. n = 7 neurons from 3 animals for all groups. $^{*}p < 0.05$, $^{\#}p < 0.01$

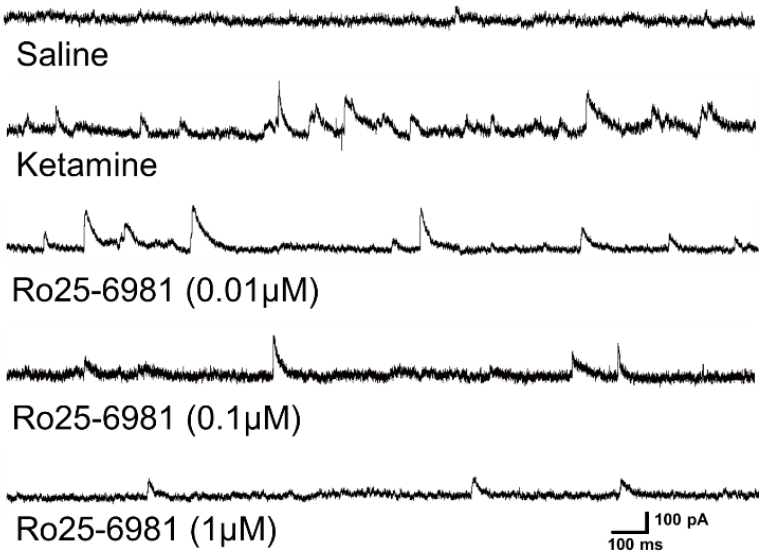

Figure 3-9 Representative traces of NMDAR-mediated mEPSCs under different doses of Ro25-6981

NMDAR-mediated mEPCs are depicted under each condition. Saline trace shows least number of mEPSCs followed by Ro25-6981 (1 μM). As concentration of Ro25-6981 goes down, amplitude as well as frequency of NMDAR-mediated mEPSCs goes on increasing.

Table 3-3 Means and SEM of amplitude and frequency of NMDAR-mediated mEPSCs across different Ro25-6981 doses

Condition	Saline	Ketamine 0 μM Ro25-6981	Ketamine + 0.01 μM Ro25-6981	Ketamine + 0.1 μM Ro25-6981	Ketamine + 1 μM Ro25-6981
Amplitude (± SEM)	59.93 (± 0.35)	106.12 (± 2.37)	80.85 (± 1.33)	66.91 (± 0.56)	44.21 (± 0.39)
Frequency (± SEM)	3.23 (± 1.01)	62.09 (± 11.31)	52.30 (± 9.97)	48.60 (± 11.79)	18.92 (± 7.14)

Chapter 4

Discussions

In this study, we successfully demonstrated that prolonged and/or repeated ketamine administration led to increased NMDAR activity. The model that was selected for studying these effects has been used previously by several other groups to demonstrate neuroapoptotic effects of ketamine on the developing brain (reviewed in Yan and Jiang, 2014). Our data showed that repeated ketamine administration caused time-dependent increase in the synaptic activity of NMDARs after ketamine washout. Beginning at 2 hours after the last ketamine dose, NMDAR-mediated synaptic activity progressively increased and peaked at 6 hours, after which it began to decline again by 8 hours. Peak increase in NMDAR-mediated synaptic activity coincided with the time when neuroapoptosis was seen in several different preclinical animal models of prolonged and/or repeated ketamine administration (reviewed in Yan and Jiang, 2014). Taken together, it can be concluded that increased NMDAR activity should be strongly associated with ketamine-induced neuroapoptosis. We further demonstrated that majority of the NMDARs which exhibit increased synaptic activity contain the GluN2B subunit. We determined this pharmacologically by using GluN2B-specific antagonist (Ro25-6981). We specifically chose the 6 hour time point after the last ketamine dose to identify if the increased NMDAR-mediated synaptic activity was mediated by a specific subtype of the NMDAR. Application of Ro25-6981 selectively blocked the increased synaptic activity mediated by NMDARs. Conversely, when we applied

GluN2A-specific antagonist (TCN 201), we did not observe as strong an inhibitory response in the NMDAR-mediated synaptic activity as we saw with GluN2B antagonism. From this we concluded that majority of the NMDARs, which exhibit increased synaptic activity after ketamine exposure, contain the GluN2B subunit. Therefore, if we associated increased NMDAR-mediated synaptic activity to ketamine-induced neuroapoptosis, we cannot ignore the involvement of GluN2B-subunits in this neuroapoptotic effect.

Heightened synaptic plasticity is a key feature of the developing brain. Specifically, during periods of intense brain development (i.e. brain growth spurt period; beginning around PND 5-7 in neonatal rats), maintenance of homeostatic mechanisms regulating synaptic plasticity is extremely important. This makes the developing brain highly susceptible to the negative environmental influences. Several studies have proved beyond doubt the important role played by NMDARs in synaptic plasticity (Hayashi et al., 2000; Pi et al., 2010; Lisman et al., 2012). They have come up as key mediators of synaptogenesis, dendritic arborization as well as synaptic pruning (Yan and Jiang, 2014). Hence, if during this crucial period of development, NMDAR-mediated synaptic transmission is perturbed, it can have potent negative effects on the survival and functioning of developing neurons. NMDARs are also key mediators of intracellular calcium during this period (Fan et al, 2014). Since majority of the cellular functions depend on an efficient calcium signaling system, most of the cellular energy is directed towards maintaining optimum intracellular calcium levels. Also, intracellular calcium sensing mechanisms are highly fine-tuned to detect changes

in calcium signaling. This makes the cell extremely sensitive to changes in intracellular calcium levels. Under these circumstances, if ketamine stopped NMDARs from bringing calcium into the developing neuron, the neuron will respond more strongly to its inhibitory effect and take immediate measures to counteract this deficit by up-regulating NMDARs. Once ketamine is eliminated from the system, the up-regulated NMDARs would get activated. This would explain the time-dependent increase in NMDAR activity after ketamine washout that was seen in our time-course experiments.

Another peculiar feature of the NMDAR-subunit composition during this period of development is that most of the NMDARs are GluN1/GluN2B heterotetramers except in the diencephalon where GluN2D replaces GluN2B. The GluN1 subunit is constitutively expressed across all developmental stages and in all parts of the brain; however, the expression of GluN2B subunit is developmentally regulated. During the first two weeks after birth, the expression of GluN2B subunit changes drastically and is slowly replaced by GluN2A, until in adults, maximum NMDARs are of GluN1/GluN2A subtype. GluN2B expression peaks around PND 7-10 in almost all areas of the brain and after this period becomes more restricted to the forebrain areas during adulthood (Paoletti, 2011). These changes in the subunit composition of the NMDARs affect its sensitivity to pharmacological modulators (Paoletti, 2011). Thus, the reason why neonates show a heightened response to prolonged ketamine exposure as compared to adolescents (as seen in our preliminary studies) could be because of this developmental diversity. Since, this developmental period is characterized by

GluN1/GluN2B-containing NMDARs, it is within reason to assume that the ketamine block of NMDARs would cause the neuron to up-regulate GluN2B-containing NMADRs specifically. Hence, we see that most of the NMDARs that show increased synaptic activity are of the GluN2B-subtype.

Opposing roles of NMDARs have remained elusive to the scientific community. At one end, NMDARs are involved in synaptogenesis, while on the other they are the key modulators of glutamate excitotoxicity. This dual and opposite role makes them key players in ketamine-induced neurotoxicity in the developing brain. While they are extremely important during the brain growth spurt period, they are also extremely dangerous since it is not completely understood when NMDARs switch from their neurogenic to neurotoxic effects. We believe that the differential subunit composition of the NMDAR plays a role, to an extent, in its opposing functions. Specifically, the GluN2B-subunit containing NMDARs are the ones that would have strongest potential to induce neurotoxicity. Several studies in stroke and hypoxia-induced neural pathophysiology have conclusively shown the involvement of GluN2B subunit in glutamatergic excitotoxicity in adults (Knox et al, 2013; Shu et al, 2014). In neonates, however, such conclusive evidence is lacking. Nevertheless, due to the fact that prolonged ketamine exposure induced an increased expression of GluN2B-containing NMDARs at the same time when ketamine-induced neuroapoptosis occurred raises the question if GluN2B subunit would mediate this effect. Thus, future directions for this study would be to dissect the

contribution of overactive GluN2B-containing NMDARs in the apoptotic process that is associated with prolonged ketamine exposure.

A number of studies have shown that after prolonged and/or repeated ketamine exposure, neonatal neurons showed increased levels of either GluN1 mRNA or increased expression of GluN1 subunit itself (Wang et al, 2005; Zou et al, 2009b). Microarray analysis also indicated an increased gene expression of *GRIN1* (Liu et al, 2011). We argue that in-spite of up-regulated GluN1 expression, its contribution to the enhanced vulnerability of immature neurons to ketamine's neurotoxic effects should be minor. This is because GluN1 does not show differential expression patterns across different developmental ages (Paoletti, 2011) and hence does not warrant for any kind of altered functioning.

All of our experiments were carried out in the pyramidal neurons of layers 2 and 3 of the ACC. The reason for our selection of this area was because it is one of the primary areas of the forebrain where high levels of ketamine-induced neuroapoptosis occurred (Ikonomidou, 1999). Previous work from our group has shown that prolonged ketamine-treatment during neonatal stages led to long-lasting (extending to adolescence) deficits in the induction of long-term potentiation (LTP) in the ACC (Wang et al, 2014). Long-term deficits in spatial learning and memory, a consequence of neonatal ketamine exposure, have also been observed by Womack et al (2014). Since, ACC plays an important role in spatial learning and memory consolidation (Goshen et al, 2011; Einarsson and Nader, 2012), these deficits can be attributed to high levels of ketamine-induced neurotoxicity in the ACC during neonatal stages (Ikonomidou et al, 1999). It is

also the primary area of the brain where GluN2B-containing NMDARs remain constitutively expressed throughout the lifespan (Paoletti, 2011). This would help us to continue testing our working hypothesis of the involvement of GluN2B-subunits in mediating ketamine-induced neuroapoptosis at higher developmental stages all the way into adulthood.

In summary, our study demonstrates that repeated ketamine exposure in neonates led to enhanced activation of GluN2B-containing NMDARs. This enhancement in NMDAR-mediated synaptic transmission was a delayed event and occurred after ketamine washout. Nevertheless, the time course of the NMDAR-mediated enhanced synaptic transmission coincided with the time course of ketamine-induced neuroapoptosis. Since, GluN2B subunits have been implicated in glutamatergic excitotoxicity in several stroke and hypoxia-induced pathophysiological studies (Knox et al, 2013; Shu et al, 2014), we proposed that ketamine's ability to selectively cause enhancement in the activity of GluN2B-containing NMDARs makes the neonatal brain vulnerable to ketamine-induced neuroapoptosis.

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Biographical Information

Saurabh S. Kokane was born in 1988 and completed his Bachelor's in Biotechnology from the University of Pune, India in 2009. Thereafter he went to Sheffield, UK to pursue his Master's in Molecular medicine with Neuroscience honors. After successfully completing his Master's degree in 2011, he secured a prestigious junior research fellowship at Tata Institute of fundamental research (TIFR), Mumbai, India. While working at TIFR, he was involved in studying epigenetic mechanisms regulating homeostasis and metabolism. His primary area of research interest has always been neuroscience. He has been fascinated with the neuronal communication that occurs throughout the central nervous system which eventually gives rise to different behaviors. Combining his expertise in molecular biology with neuroscience, he wants to address the molecular mechanisms that govern inter-neuronal communication in normal and abnormal behavior.

Since joining the Graduate program in Health psychology/ Neuroscience at the University of Texas at Arlington, Saurabh's outlook towards neuroscience research increased to new level. In the future he wants to continue focusing on the mechanisms associated with pain and depression behaviors.