5-1-2016

BUMETANIDE DEMONSTRATES AMELIORATION OF LEARNING AND MEMORY DEFICITS INDUCED BY KETAMINE ADMINISTRATION IN A NEONATAL RAT MODEL

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by

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Presented to the Faculty of the Honors College of
The University of Texas at Arlington in Partial Fulfillment
of the Requirements
for the Degree of

HONORS BACHELOR OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2016

ACKNOWLEDGMENTS

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The research outlined within this paper was supported by NIH R01 grant NS 040723 to Q. Lin and the Ronald E. McNair Post Baccalaureate Achievement Program at UT Arlington to R. Stevens. Additional support was awarded to R. Stevens via the UT Arlington Honors College through an Undergraduate Research Fellowship. A special thank you is given to Saurabh Kokane, M.S. for his assistance and guidance throughout this project and all undergraduate researchers in Dr. Qing Lin's Laboratory. Finally, thanks are given to Dr. Perry Fuchs' and his group for sharing their laboratory space for behavioral testing.

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April 15, 2016

ABSTRACT

BUMETANIDE DEMONSTRATES AMELIORATION OF LEARNING AND MEMORY DEFICITS INDUCED BY KETAMINE ADMINISTRATION IN A NEONATAL RAT MODEL

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The University of Texas at Arlington, 2016

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Ketamine, which is widely used as a pediatric anesthetic, has been reported by our and other groups to demonstrate persistent deficits in learning and memory, and alterations in N-methyl-D-aspartate receptor (NMDAR) functioning. In neonates, γ-aminobutyric acid (GABA) is excitatory upon activation of $GABA_A$ receptors rather than its mature action of neuronal inhibition. This is due to greater Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) and weak K^+-Cl^- co-transporter (KCC2) expression in the neonatal cell membrane. Thus, bumetanide – an NKCC1 inhibitor – may prevent intracellular chloride accumulation by reducing or inhibiting GABA excitation in immature neurons. Therefore, we hypothesized that bumetanide may serve as a neuroprotectant via interfering with this GABA excitatory pathway through inhibiting NKCC1 to minimize ketamine-induced neuroexcitotoxicity.

Seven-day-old rats were administered ketamine subcutaneously, with intracerebroventricular delivery of bumetanide concurrently with ketamine or vehicle. Three weeks following treatment, four groups were tested for spatial learning and memory deficits using the Morris Water Maze. Prolonged latency in learning was noted in the ketamine treated animals with deficits in recall of the target platform location. However, the bumetanide co-treatment group showed a learning rate and recall similar to the control. Thus, these results suggest a new mechanism by which neonatal ketamine-induced learning and memory deficits can be alleviated through reducing hyperactive GABAergic-excitatory neonatal synaptic signaling.

Keywords: γ-aminobutyric acid, Morris Water Maze, neonatal, Na^{+}-K^{+}-$2Cl^{-}$ co-transporter

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CHAPTER 1

INTRODUCTION

Over the past decade, concern over potential neurological damage caused by pediatric anesthetic use has become more notable (Bong, Allen, & Kim, 2013; DiMaggio, Sun, & Li, 2011; Flick et al., 2011; Ing et al., 2012). This is due to animal data and population based retrospective studies of children who have received anesthetics. These studies report a possible relationship between prolonged anesthetic administration during early brain development, and extended or possibly permanent cognitive damage (Jevtovic-Todorovic, 2013; Reddy, 2012; X. Wang, Xu, & Miao, 2014; Wilder et al., 2009).

Ketamine, a non-competitive N-methyl-D-aspartate receptor (NMDAR) antagonist, is commonly used as an anesthetic in pediatric procedures and provides sedation with limited obvious side effects. Due to its increased potency in pediatric patients, ketamine has become attractive to health care providers as an anesthetic and analgesic. Additionally, ketamine can be delivered incrementally to maintain its desired effects without adverse reaction. This is of importance because, in comparison to other pediatric general anesthetics, it does allow for normal respiration and cardiovascular stability (Green & Johnson, 1990). However, studies published by our laboratory and others show that neonatal exposure to ketamine caused deficits in learning and memory, alterations in long-term potentiation (LTP), and NMDAR functioning (Kokane et al., 2014; R. R. Wang et al., 2014; Womack et al., 2013).

Therefore, the goal of our research is to further understand ketamine-induced altered synaptic transmission and intracellular signal transduction as it relates to learning and memory throughout development.

In adults, the primary inhibitory neurotransmitter is γ-aminobutyric acid (GABA). However, in neonates the Cl^- permeable neuronal $GABA_A$ receptors ($GABA_AR$) are excitatory (Ben-Ari, 2002; Owens & Kriegstein, 2002). The mechanism for $GABA_AR$ excitation is due to elevated intracellular levels of Cl^- ions. These ions are specifically transported into the cell via Na^+-K^+-$2Cl^-$ co-transporter (NKCC1). Published research by Clayton and others (1998) has shown that mRNA expression of NKCC1 remains constant into adulthood, however in neonates this cotransporter is elevated during early postnatal days and decreases as early development progresses. In contrast, the mRNA expression of K^+-Cl^- co-transporter (KCC2) has a delayed developmental pattern that functions to cotransport Cl^- outward from within the cell (Clayton et al., 1998). During postnatal development, KCC2 up-regulation is thought to be directly responsible and necessary to generate an electrochemical Cl^- gradient, allowing for a switch from the excitatory behavior of GABA, to a mature inhibitory effect occurring near the second to third week after birth (Rivera et al., 1999; Yamada et al., 2004).

Bumetanide, an inhibitor of NKCC1, has been of great interest to researchers investigating potential epileptic therapeutics specifically for neonates. Barbiturate drugs are primarily used to treat epileptics that are agonist to $GABA_AR$ (Shetty, 2015). In neonates, this leads to Cl^- efflux and hyper-excitation of immature neurons. However, bumetanide inhibition of NKCC1 in these neurons allows for intracellular Cl^- concentrations to decrease (Brandt, Nozadze, Heuchert, Rattka, & Löscher, 2010).

Therefore, when bumetanide is administered prior to KCC2s normal developmental upregulation, and further affecting cellular Cl^- concentrations, neuroprotective benefits should be seen. As a result, previous research in epileptic animal models have shown bumetanide co-treatment with a barbiturate such a phenobarbital, to have decreased GABA excitability in neonates (Dzhala et al., 2005; Dzhala, Brumback, & Staley, 2008).

Relevant to this study, excitatory $GABA_AR$ depolarization has been suggested to increase the susceptibility to neurotoxic injury during neonatal exposure to anesthetics (Patel & Sun, 2009). Thus, it is hypothesized that excitatory GABAergic synaptic transmission has a distinct role in the neuroexcitotoxicity of ketamine's action on glutaminergic neurons, resulting in significant or a potentially permanent impact on learning and memory later in life. Therefore, it is proposed that blockade of NKCC1 during ketamine exposure in the developing brain allows for intracellular Cl^- ions to shift from high to low concentrations rendering GABA inhibitory, and further restricting other intracellular mechanistic downstream effects that $GABA_AR$ mediated excitation may induce. As previously mentioned, because earlier studies have already looked at the effects of ketamine on learning and memory, it was important to replicate this model with modification to test the impact of bumetanide co-treatment during ketamine exposure. To accomplish this, the Morris Water Maze test was performed to test spatial learning and memory rate of formation, and short-term recall.

CHAPTER 2

METHODS

2.1 Animals

All experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Arlington. Sprague-Dawley rat pups (male and female) in age groups of post-natal seven-day-old (PND 7) and three to five week-old were randomly sampled and used in the proposed study. All animals were housed in a 12-12-hour constant light/dark cycle at controlled temperature (22-25°C) and humidity (55-60%). Additionally, animals were allowed free access to food and water.

2.2 Drug Administration

Ketamine hydrochloride was diluted in 0.9% normal saline solution (NS) and was administered subcutaneously (SC). A dose of 20 mg/kg ketamine, six times at two-hour intervals was administered to rat pups at age PND 7. Bumetanide was solvated in a 1:49 (DMSO:0.9% NS) working solution and administered intracerebroventricularly (ICV). ICV injections were given by following an established method with the goal of delivering bumetanide into the right lateral ventricle. Bumetanide was delivered 2 mm into the skull surface by using a 31 gauge, 6 mm in length syringe (BD Ultra-Fine™). The insertion site was 1.5 mm lateral and 2 mm rostral with coordinates measured in respect to the bregma (Han & Holtzman, 2000; Kim, Cho, Nelson, Zipfel, & Han, 2014).

A dose of 0.02 mg/kg bumetanide, 3 times at 4-hour intervals was administered to rat pups at age PND 7. Control animals were administered vehicle injections of either 0.9% NS and/or DMSO working solution at the same time points. Combinations of these drugs were given in order to assess the impact of NKCC1 blockade during ketamine administration. Groups were labeled according to drug administration route as follows: vehicle + vehicle (VEH + VEH, n = 21), vehicle + ketamine (VEH + KET, n = 11), bumetanide + vehicle (BUM + VEH, n = 6), and bumetanide + ketamine (BUM + KET, n = 5).

2.3 Spatial Navigation Training and Water Maze Procedures

Spatial acquisition training and testing was executed utilizing the MWM to measure and compare spatial learning rate, and short-term recall of spatial memory. All training and testing occurred during the age of adolescence (4-5 weeks old). A pool, 160 cm in diameter was filled with water, which was made opaque by addition of nontoxic acrylic paint and kept at room temperature (~25°C). The pool was divided into quadrants NW, NE, SW, and SE. Distinct visual cues were placed on the walls surrounding the MWM to allow for spatial navigation. A hidden platform was placed in one specific target quadrant (NW), subsurface within the pool, and was not moved during the entire training phase. Spatial acquisition software (EthoVision XT by Noldus) was used to monitor all movement within the pool during training and probe trials. Animals were placed into treatment groups. Performing two trials per day, per animal, each group underwent a training phase for five days in the Water Maze. Additionally, each trial did consist of a different start location per animal – for example, Trial-one: North entry, Trial-two: East entry – both in near-equal distance from the hidden platform. This allowed for ten trials per animal over the course of the five-

day training phase. Each trial during the training phase allowed for each animal a maximum of 120 s to find the hidden platform. If the animal were unable to find the platform, they were either guided or placed on the platform for 10 s. Following the training phase, all animals were administered a probe trial test, in which the hidden platform was removed from the pool 24 hours following the fifth training day. Each animal was allowed one probe trial lasting 120 s with a start location differing from the training start locations (e.g. Probe Trial: South entry).

2.4 Quantitative Analysis

Statistical significance in MWM testing over five days of training was analyzed using repeated-measures ANOVA and Bonferroni post hoc analysis if necessary to correct for multiple comparisons via Sigmaplot (Systat Software Inc.). Additionally, MWM probe analysis was tested for significance via Student's t-test utilizing the same statistical software. All groups were tested against a control (VEH + VEH) group with consideration of probability values of less than 0.05 as significant. In instances for which greater accuracy was preferred due to skewed distribution or abnormal variance, a bootstrap statistical model was used (Stine, 1989).

CHAPTER 3

RESULTS

3.1 Training Phase: Time Taken to Learn a Hidden Escape Platform Location

Three weeks following drug administration all groups were trained to find a hidden platform over the course of five days. Learning was measured by latency to the hidden platform with no statistical significance seen in days one through three (Figure 1). Beginning on day four there was a significant decrease in latency to find the platform of the BUM + KET group as compared to the control means (Table 1) with statistical significance, $F(1,34) = 10.249$, $t(33) = 3.20$, $p = .003$. Furthermore, by day five both groups, BUM + KET and VEH + KET showed a significant difference in mean values (Table 1) as compared to the control. The BUM + KET group demonstrated a continued significance in latency to find the platform, $F(1,34) = 6.20$, $t(33) = 2.49$, $p = .018$. The VEH + KET group demonstrated a moderate increase in latency to find the platform as compared to the control, $F(1,44) = 4.14$, $t(43) = 2.04$, $p = .048$.

Furthermore, as seen in Figure 2, a comparison was made between BUM + VEH versus the control to rule out the possibility of bumetanide having an effect on spatial memory. Over the course of the five day training period no significant difference was found.

Table 3.1: Training Phase Comparison of Treatment Groups

	n	*M* (s)	*SD* (s)	*SEM* (s)
VEH + VEH				
Day 1	42	86.01	38.68	8.07
Day 2	42	87.03	43.96	9.17
Day 3	42	71.28	45.34	9.46
Day 4	42	66.71	47.03	9.81
Day 5	42	38.66	33.24	6.93
VEH + KET				
Day 1	22	91.54	37.73	8.04
Day 2	22	74.05	45.28	9.65
Day 3	22	73.85	47.87	10.21
Day 4	22	69.45	47.62	10.15
Day 5	22	61.47	41.68	8.89
BUM + KET				
Day 1	12	76.82	46.05	14.56
Day 2	12	70.13	48.25	13.93
Day 3	12	50.79	42.47	12.26
Day 4	12	20.63	21.85	6.31
Day 5	12	13.28	15.70	4.53
BUM + VEH				
Day 1	10	106.77	26.19	7.56
Day 2	10	102.08	26.49	7.65
Day 3	10	63.80	44.93	12.97
Day 4	10	38.04	34.74	10.03
Day 5	10	37.28	25.88	7.47

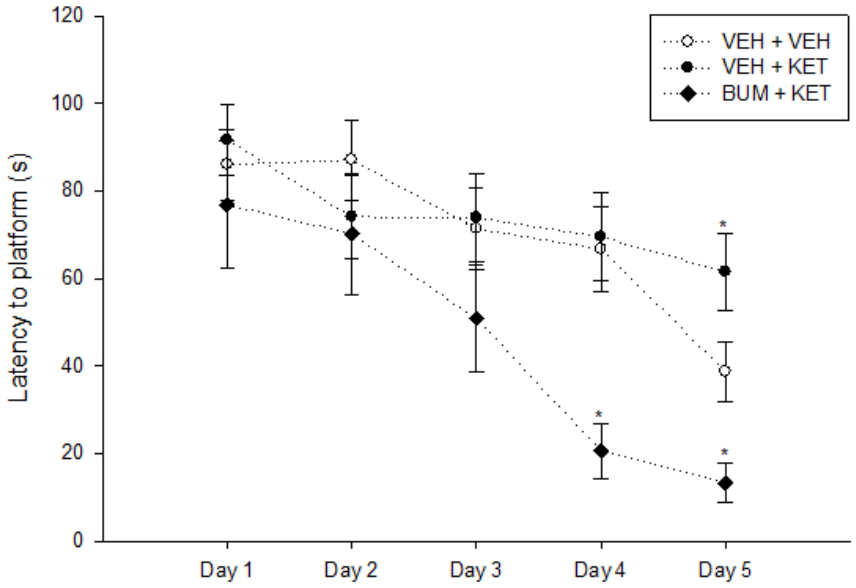

Figure 3.1: Five Days of Training for Navigation to a Hidden Escape Platform within a Morris Water Maze for Comparison across Treatment Groups

Statistical significance was determined by comparison against the control (VEH + VEH) group and labeled with '*' representing $p < 0.05$.

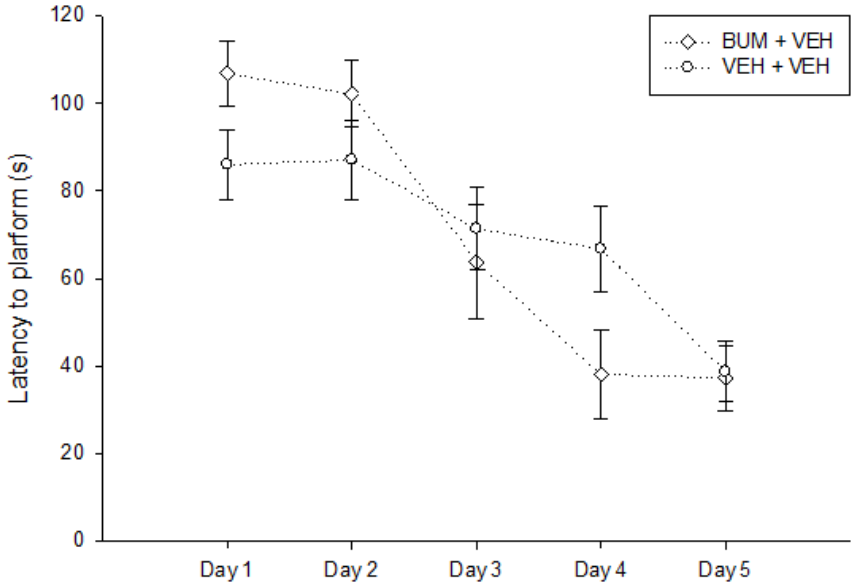

Figure 3.2: Five Days of Training for Navigation to a Hidden Escape Platform within a Morris Water Maze for Comparison across Treatment Groups (cont.)

No statistical differences between the groups were noted.

3.2 Probe Test Phase: Time Spent in Target Quadrant

Twenty-four hours following day five of training a probe trial was conducted. The mean difference among drug treatment groups were compared for the total time spent within the target quadrant against the control (Table 2). As expected the BUM + KET group did not show a significant difference from the control, $t(25) = -4.25$, $p = .555$. Additionally, the BUM + VEH group also did not show significant as compared to the control, $t(24) = .424$, $p = .675$. However, as shown in Figure 3, significance was detected among the VEH + KET group as compared to the control, $t(30) = 2.31$, $p = .028$.

Table 3.2. Probe Test Target Quadrant Duration

Column1	*n*	*M* (s)	*SD* (s)	*SEM* (s)
VEH + VEH	21	59.05	15.90	3.47
VEH + KET	11	46.42	11.84	3.57
BUM + KET	6	63.30	12.92	5.28
BUM + VEH	5	55.98	2.03	0.91

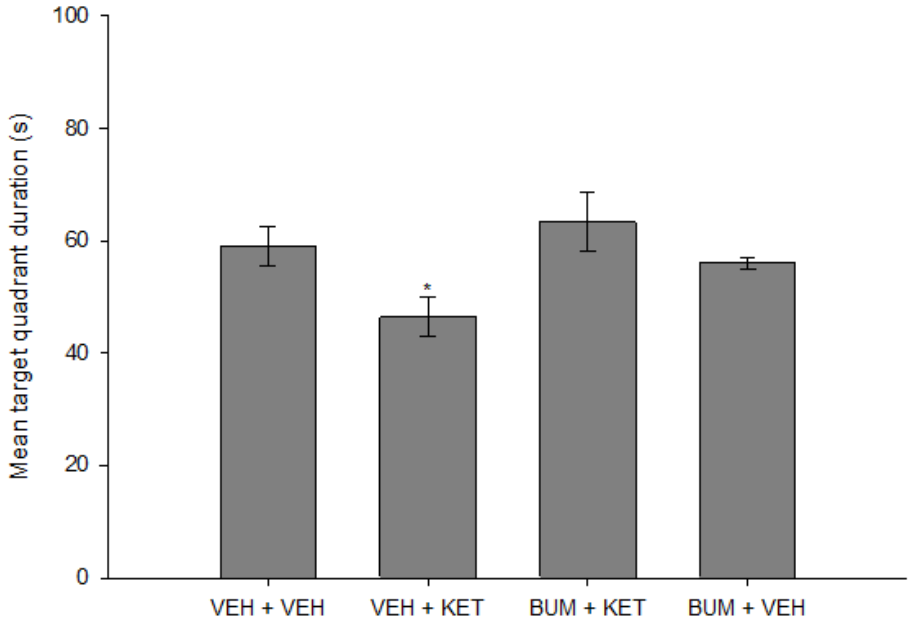

Figure 3.3: Average Time Spent Within the Target Quadrant during a Probe Trial

Statistical significance was determined by comparison against the control (VEH + VEH) group and labeled with '*' representing $p < 0.05$.

3.3 Probe Test Phase: Latency to Locate the Target Quadrant

Data collected from the probe test was analyzed to compare latency to enter the target quadrant upon initial entry into the pool (Table 3, Figure 4). The VEH + KET group showed a very significant increase for time taken to find the target quadrant, $t(29) = 5.56$, $p < .001$. The BUM + KET, and BUM + VEH groups also showed statistical significance. However, both groups demonstrated a decrease in their latency to enter the target quadrant, $t(24) = 3.76$, $p < .001$; $t(23) = 3.44$, $p = .002$, respectively. An ANOVA was conducted on the bumetanide co-treated groups as compared to the control to further investigate this decreased latency, $F(2,30) = 12.26$, $p < .001$. A post hoc t-test was performed to assess the group deviation in both the BUM + KET and BUM + VEH groups as compared to the control; both groups showed statistical significance, $t(27) = 4.00$, $p = .001$; $t(27) = 3.71$, $p = .003$, respectively.

Table 3.3: Probe Test Latency to Target Quadrant

Column1	*n*	*M* (s)	*SD* (s)	*SEM* (s)
VEH + VEH	21	2.26	0.87	0.20
VEH + KET	11	6.91	3.60	1.09
BUM + KET	6	0.87	0.37	0.15
BUM + VEH	5	0.87	0.34	0.15

Note: bootstrap statistical model used.

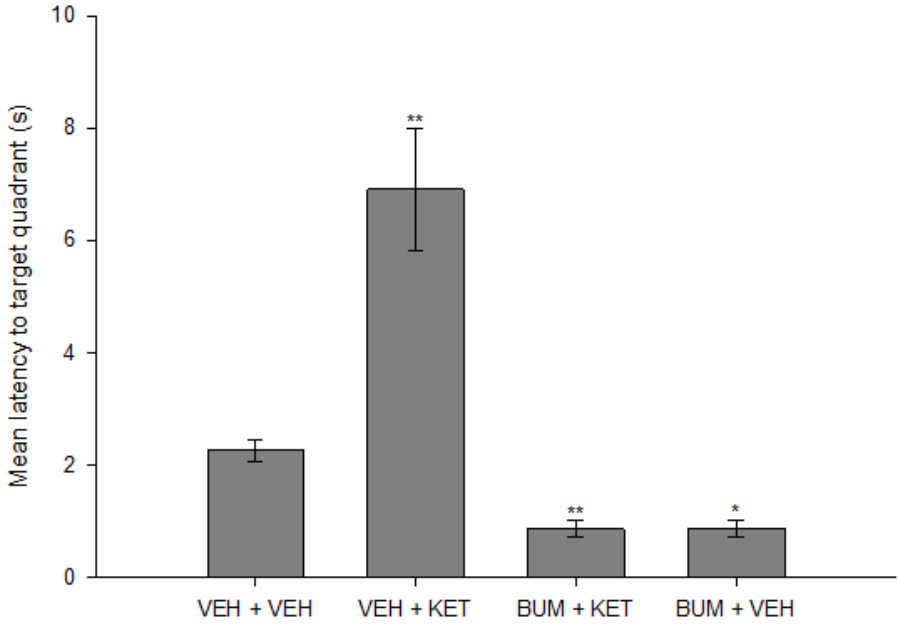

Figure 3.4: Latency to Find the Target Quadrant during a Probe Trial

Statistical significance was determined by comparison against the control (VEH + VEH) group and labeled with '*' and '**' representing $p < 0.05$ and $p < 0.001$, respectively (bootstrap statistical model used).

3.4 Probe Test Phase: Frequency of Escape Platform Location Crossing

Probe test data was analyzed for the frequency each group crossed over the trained escape platform location. Due to varying sample size, the frequency data was arithmetically calculated (Table 4). In determining significance all groups were compared to the control (Figure 5). The VEH + KET group demonstrated a marginally significant decrease in the frequency of escape platform crossing, $F(1,31) = 4.35$, $t(31) = 2.08$, $p = .046$. Additionally, the BUM + KET and BUM + VEH groups showed significance in comparison to the control, $F(2,31) = 8.65$, $p = .001$. Post hoc analysis assessed the group deviation in both the BUM + KET and BUM + VEH groups as compared to the control; both groups showed statistical significance, $t(29) = 3.54$, $p = .004$; $t(29) = 3.71$, $p = .023$, respectively.

Table 3.4: Probe Test Frequency of Escape Platform Crossing

Column1	*n*	*M* (s)	*SD* (s)	*SEM* (s)
VEH + VEH	21	0.78	0.09	0.02
VEH + KET	11	0.71	0.10	0.03
BUM + KET	6	0.92	0.06	0.03
BUM + VEH	5	0.90	0.06	0.03

Note: bootstrap statistical model used.

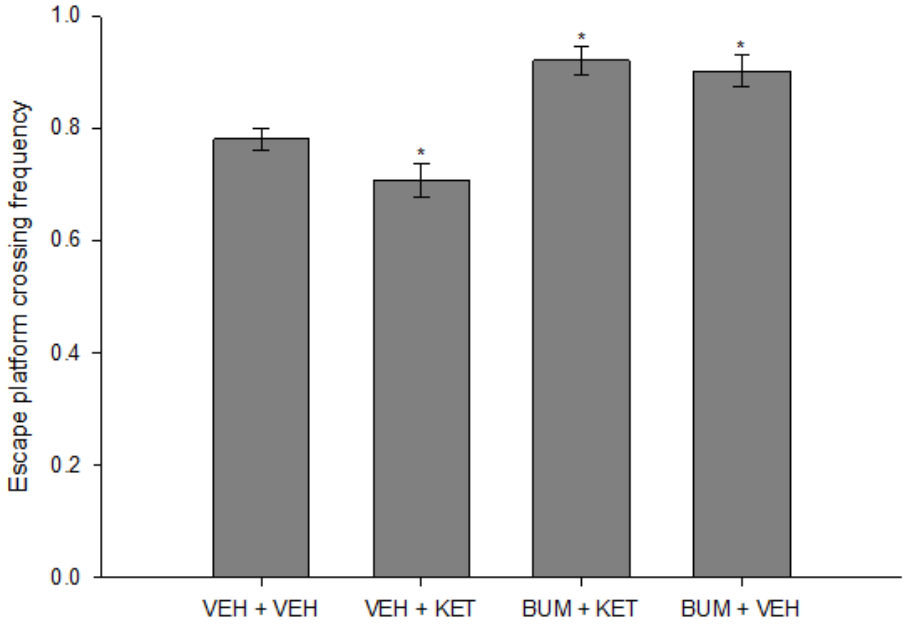

Figure 3.5: Frequency of Crossing the Previously Trained Escape Platform Location during a Probe Trial

Statistical significance was determined by comparison against the control (VEH + VEH) group and labeled with '*' representing $p < 0.05$ (bootstrap statistical model used).

3.5 Probe Test Phase: Animal Velocity

Average animal velocity was calculated for data collected during the probe test (Table 5, Figure 7). This was done in order to rule out the possibility of velocity being a confounding variable leading to deviated latency to target the quadrant and escape platform area crossing (Table 3, 4). No statistical significance was determined in comparing all treatments to the control. The VEH + KET group showed no significance, $F(1,31) = .76$, $p = .392$.

Additionally, the BUM + KET and BUM + VEH group showed no statistical significance, $F(2,31) = .014$, $p = .986$. This behavior was subjectively confirmed via comparison of average tracking patterns during the water maze probe test (Figure 6). Animal behavior as seen in these images is consistent with objective data obtained and quantified seen in Figure 3 through Figure 5.

Table 3.5: Probe Test Velocity Across Groups

Column1	*n*	*M* (cm/s)	*SD* (cm/s)	*SEM* (cm/s)
VEH + VEH	21	21.49	2.86	0.62
VEH + KET	11	22.38	2.55	0.77
BUM + KET	6	21.48	2.54	1.04
BUM + VEH	5	21.26	2.68	1.20

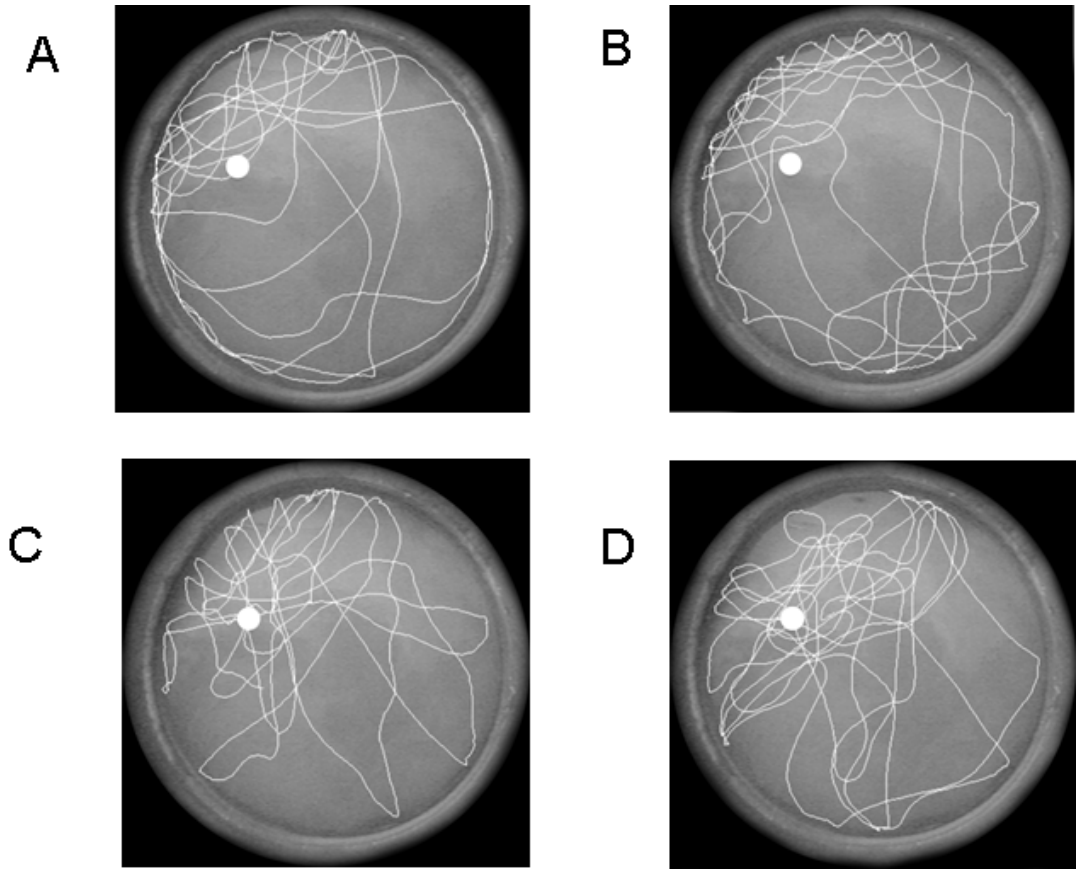

Figure 3.6: Subjective Analysis of the Water Maze Probe Trial Search Strategies and Patterns

Images generated via Noldus Ethovision to visually demonstrate average tracking patterns during the water maze 24 h post-training probe. The location of the hidden escape platform — that was removed during the probe test — is indicated by a white circle. - Comparisons across treatment groups were made by calculating the latency to the target (North-west) quadrant, frequency crossing the escape platform location, and duration in seconds spent within the target quadrant that are seen in Fig. 3-4. Fig. 6A shows the average tracing patterns of VEH+VEH treatment. Fig. 6B shows the average tracing patterns of VEH + KET treatment. Fig. 6C shows the average tracing patterns of BUM + KET treatment. Fig. 6D shows the average tracing patterns of BUM + VEH treatment.

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CHAPTER 4

DISCUSSION

This study investigated the effects of bumetanide on learning and memory when co-administered with ketamine early in life, and to our knowledge is the first of its kind. These effects were empirically measured using a water maze test during the age of adolescence, which were found to be in support of our hypothesis. By blockade of NKCC1 via bumetanide, learning and memory deficiencies were alleviated resulting in memory retention similar to the control. In addition to these mentioned novel aspects of our research, this study was able to replicate previous results for the testing ketamine's effect on spatial learning and memory (Huang, Liu, Jin, Ji, & Dong, 2012; Womack et al., 2013).

Published *in vitro* studies that tested bumetanide's effect on reversing GABA excitation were found to be effective (Dzhala et al., 2005, 2008). However, other groups that have used bumetanide to selectively block NKCC1 *in vivo* experienced difficulties due to increased elimination and potential issues with the drug's ability to cross the Blood-Brain Barrier (BBB) (Brandt et al., 2010; Töllner et al., 2014). Brandt and others (2010) reported intraperitoneal bumetanide administration as having a short half-life, with poor BBB penetration, and rapid elimination in a rat model. Because of this, researchers in this study attempted to overcome these issues by administering bumetanide via continuous intravascular (IV) infusion. Because of these known limitations with using bumetanide to block NKCC1 *in vivo* and the level of difficulty in successfully administering IV drugs in neonatal rats, we decided to administer bumetanide ICV as explained in our methodology.

The behavior demonstrated by the BUM + KET group as compared to the control during Days 4 and 5 of the training phase sparked some interest during analysis. Although not statistically significant, there was an average increase in memory retention during the probe trial in addition to an increased rate of learning during the training phase (Table 1 and Table 2). To further investigate this demonstrated memory retention, latency to find the target (Northwest) quadrant was quantified (Table 3, Figure 4). It was determined that the BUM + KET group outperformed the control by reaching the target quadrant more rapidly. Furthermore, the frequency of how often the animals crossed the previous location of the hidden escape platform was measured (Table 4, Figure 5). Again, the BUM + KET group demonstrated an increase in memory retention as compared to the control in this test. Lastly, it was our concern that these results may have been confounding with the possibility of these experimental animals having a varied level of activity among treatment groups. This concern was accounted for by quantification of animal velocity during the probe trial phase (Table 5, Figure 7). As reported, there were no significant differences among the groups travel velocity. These findings suggest that bumetanide treatment when given neonatally might enhance synaptic plasticity at some level. However, this report cannot say definitively what might be occurring within the brain, and therefore further studies should be completed to investigate this phenomenon.

It is imperative we come to understand disorders related to anesthetic-induced brain damage, and it is equally important to investigate therapeutic interventions that help in attenuating or alleviating their negative effects. Within the past couple of decades, as interest has increased regarding the neurotoxic impact of anesthetics on the developing brain, some limitations have been identified; more specifically, the connection between

human retrospective cohort studies, and their implications for animal research (Reddy, 2012). Over developmental stages, the differences between a neonatal animal and the human child varies between species specific events of neuronal maturation, resulting in some ambiguity for research design applications (Workman, Charvet, Clancy, Darlington, & Finlay, 2013).

Within this study, the administration of ketamine at age PND 7 was acceptable in order to replicate previous studies. This age, which is related to whole brain development, is arguably equivalent to a human prenatal/neonatal stages when calculating an approximate developmental window (Workman et al., 2013). However, by establishing a behavioral connection between deficiencies of early ketamine exposure and neuroprotective mechanisms that mitigate said deficits, the ability presents itself to make inferences on the underlying mechanisms involved. Therefore, future studies should investigate the behavioral and neurochemical mechanisms related to ketamine induced learning deficits at other developmental stages in relation to NKCC1 mediated indirect neuroprotective effects.

In conclusion, we were able to replicate and confirm that ketamine does show a negative impact on learning and memory that can be measured to last into adolescence after neonatal exposure. Additionally, we were able to apply previous knowledge gained from animal research related to other diseases to selectively block NKCC1 via bumetanide during ketamine exposure, which demonstrated a neuroprotective effect in correcting and somewhat improving spatial memory retention. As mentioned, future studies may want to investigate later neonatal days of development based on whole brain or specific brain areas and their different patterns of maturation as it relates to human pediatrics when

administered ketamine. In addition, other routes or methods of bumetanide administration might be considered due to technical difficulties or potential issues in attempting to increase the dose for dose-response studies. Also, bumetanide's ability to selectively block NKCC1, resulting in neonatal neuroprotection when co-administered with ketamine, needs to be investigated further to understand the developmental mechanisms involved and how they influence learning and memory later in life. Finally, it is our hope that this study has advanced our understanding of the long-term implications of neonatal ketamine exposure. Furthering this understanding may hold significance in adjusting anesthetic duration protocols and in the development of potential neuroprotective strategies that can minimize or eliminate neurotoxic injury from multiple or prolonged anesthetic procedures when used in pediatric patients.

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BIOGRAPHICAL INFORMATION

Ryan Allen Stevens is a man of purpose, who consistently pursues excellence in all that he sets out to accomplish. He is someone who holds humanitarianism to high esteem, and has publicly stated his belief that, “we can make the world a better place by positively affecting the lives of others.” He has served in the United States Army as a Combat Medic from 2009 to 2015, rising to the position of Platoon Sergeant. From 2013-2015 Ryan continued his military service in the Army Reserve, where he enjoyed mentoring and leading those newly enlisted into military service. After returning home from a combat deployment in Afghanistan, Ryan decided to further his education and career. In 2013, Ryan was admitted to UT Arlington where he began studying the biological sciences as a pre-medical student. During his time at UT Arlington, Ryan has continued to rise among his peers in academics, scientific research, and continued service. Ryan intends to gain admittance to an accredited medical program in the United States and become a physician. With a heart of service and an undying thirst for knowledge, Ryan seeks to encompass these characteristics as a future leader in healthcare and biomedicine.