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Synaptic Plasticity in GABAergic Inhibtion of VTA Neurons

Associated with Alcohol Dependence

Jennifer Kei Mabey

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science in Neuroscience

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May 2014

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ABSTRACT

Synaptic Plasticity in GABAergic Inhibtion of VTA Neurons
Associated with Alcohol Dependence

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Master of Science in Neuroscience

Past research has demonstrated that the motivational effects of opiates causes a change in ventral tegmental area (VTA) γ-amino butyric acid (GABA) subtype A receptor [GABA(A)R] complexes in opiate-dependent animals, which switch from a GABA-induced hyperpolarization of VTA GABA neurons to a GABA-induced depolarization. Previously shown in naïve animals, superfusion of ethanol (IC50 = 30 mM) and the GABA(A)R agonist muscimol (IC50 = 100 nM) decreased VTA GABA neuron firing rate in a dose-dependent manner.

The aim of this study was to evaluate VTA GABA neuron excitability, GABA synaptic transmission to VTA GABA neurons, and a potential switch in GABA(A)R functionality produced by alcohol dependence. To accomplish these studies, we used standard whole-cell, perforated patch, and attached-cell mode electrophysiological techniques to evaluate chronic ethanol effects on VTA GABA neurons in CD-1 GAD GFP mice, which enable the visual identification of GABA neurons in the slice preparation. In order to more conclusively demonstrate synaptic plasticity in VTA neurons associated with alcohol dependence, three studies were proposed to elucidate the mechanism underlying the switch in GABA synaptic function with dependence. First, we evaluated the effects of withdrawal from chronic ethanol exposure on muscimol-induced inhibition of VTA GABA neuron firing rate. Second, we evaluated the effects of withdrawal from chronic ethanol exposure on GABA(A)R-mediated synaptic responses in VTA GABA neurons by looking at eIPSCs, and corresponding changes in VTA DA neuron firing rate. Third, we evaluated chloride reversal potentials in VTA GABA neurons using perforated patch recordings in VTA GABA neurons.

Through these studies, we found that there was less sensitivity to muscimol in animals treated with ethanol versus air-exposed controls. However, it is yet to be shown more conclusively if VTA GABA neurons undergo a switch in GABA(A)R function with chronic ethanol.

Keywords: ethanol, muscimol, VTA, GABA, GABA(A)R switch

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TABLE OF CONTENTS

TITLE PAGE i

ABSTRACT ii

ACKNOWLEDGMENTS iii

TABLE OF CONTENTS iv

LIST OF FIGURES vi

INTRODUCTION 1

The Economic and Social Costs of Alcohol Abuse 1

The Mesolimbic Dopamine System 1

GABA(A)R Functionality Switch 3

RATIONALE AND HYPOTHESES 7

METHODS 8

Animal Subjects 8

Preparation of Brain Slices 8

Characterization of Neuron Types 9

Whole-cell Recordings in vitro 9

Cell-attached, Voltage-clamp Recording of Spike Activity in Brain Slices 10

Perforated-patch Electrophysiology 10

Drug Preparation and Administration 11

Statistical Analyses 11

RESULTS 12

Effects of Withdrawal on Muscimol-Induced Inhibition of Firing Rate 12

Effects of Withdrawal on GABA(A)R-Mediated Synaptic Responses 14

Effects of Withdrawal on VTA DA Neuron Firing Rate 15

GABA(A)Rs on VTA GABA Neurons Switch their Function via a Change in Chloride Gradient 16

Effects of Chronic Ethanol on Chloride Reversal Potentials 17

DISCUSSION 18

REFERENCES 21

CURRICULUM VITAE 25

LIST OF FIGURES

Figure 1: Theoretical Framework for Proposed Studies. 3

Figure 2: Proposed Model for Functional Switch of GABA(A)Rs on VTA GABA Neurons. 4

Figure 3: Depletion of TrkB Receptors Prevents Switch in GABA(A)R Function. 5

Figure 4: Furosemide Reduces Muscimol Inhibition of VTA GABA Neurons. 5

Figure 5: Chronic Ethanol Injection Reduces Muscimol Inhibition. 13

Figure 6: Chronic Ethanol Vapor Reduces Muscimol Inhibition. 14

Figure 7: Effects of Ethanol on eIPSCs in VTA GABA Neurons. 15

Figure 8: Effects on Ethanol on VTA DA Firing Rate. 16

INTRODUCTION

The Economic and Social Costs of Alcohol Abuse

Alcoholism is a chronic relapsing disorder that has enormous impact on society. Excessive alcohol use is the third leading lifestyle-related cause of death in the United States (Mokdad, Marks et al. 2004). Indeed, approximately 85,000 deaths each year are attributable to excessive alcohol use (Mokdad, Marks et al. 2004). In 2006 alone, the estimated cost of excessive drinking was $223.5 billion (Bouchery, Harwood et al. 2011). Of these $223.5 billion, the cost of alcohol-attributable crime was $73.3 billion and the cost to government was $94.2 billion (Bouchery, Harwood et al. 2011). While there is a great societal and economic need to cure alcoholism, there is still little neuromechanistic understanding of this pervasive disease. For this project, we aim to better understand the neural basis of alcohol use and the pathological progression to alcohol dependence. By understanding the mechanism by which alcohol disrupts normal brain function, treatments and therapies can be developed in order to ameliorate alcohol's devastating effects.

The Mesolimbic Dopamine System

The regulation of reward and pleasure in the brain is thought to take place in the nucleus accumbens (NAc) of the mesolimbic dopamine (DA) system. The mesolimbic DA system originates in the midbrain ventral tegmental area (VTA) and DA and γ-amino butyric acid (GABA) neurons project to the NAc. Dopamine release is enhanced in the NAc and other limbic structures by drugs including alcohol (Pierce and Kumaresan 2006). Indeed, DA release in the mesolimbic system has been shown to exhibit a scalar index of reward (Wise 2008). While the release of DA in the NAc leads to symptoms of addiction, it has been suggested that the changes in DA are mediated by VTA GABA neurons (Nugent and Kauer 2008). VTA GABA neurons

provide an inhibitory input to DA neurons to regulate their activity level (Tepper, Paladini et al. 1998). Optogenetic studies have shown that selective activation of VTA GABA neurons influences conditioned placed aversion (Tan, Yvon et al. 2012) and can manipulate reward consumption (van Zessen, Phillips et al. 2012). Evidence for GABA's role in reward has been demonstrated by multiple labs, as alcohol can alter GABA activity (Yin and French 2000, Erhardt, Schwieler et al. 2002, Mansvelder, Keath et al. 2002) to influence DA activity (Brodie and Appel 1998). This suggests that GABA is a major regulator of DA neurotransmission and therefore plays a critical role in the rewarding properties of drugs such as ethanol. While current dogma states that the reinforcing properties of alcohol are primarily mediated by its actions on N-methyl-D-aspartate receptors (NMDARs) in the VTA, alcohol dependence accrues from repeated use and associated neuroadaptations of VTA GABA subtype A receptor [GABA(A)R] complexes. However, what is not known is what specific neurons in the VTA have these adaptable substrates. It has been shown that VTA GABA neurons are sensitive to ethanol at behaviorally-relevant concentrations, gain tolerance to ethanol inhibition of their firing rate, and become hyperexcitable during withdrawal to chronic ethanol (Gallegos, Criado et al. 1999). Figure 1 portrays the synaptic hodology of VTA GABA neurons, including the probable receptor involved in ethanol mechanisms.

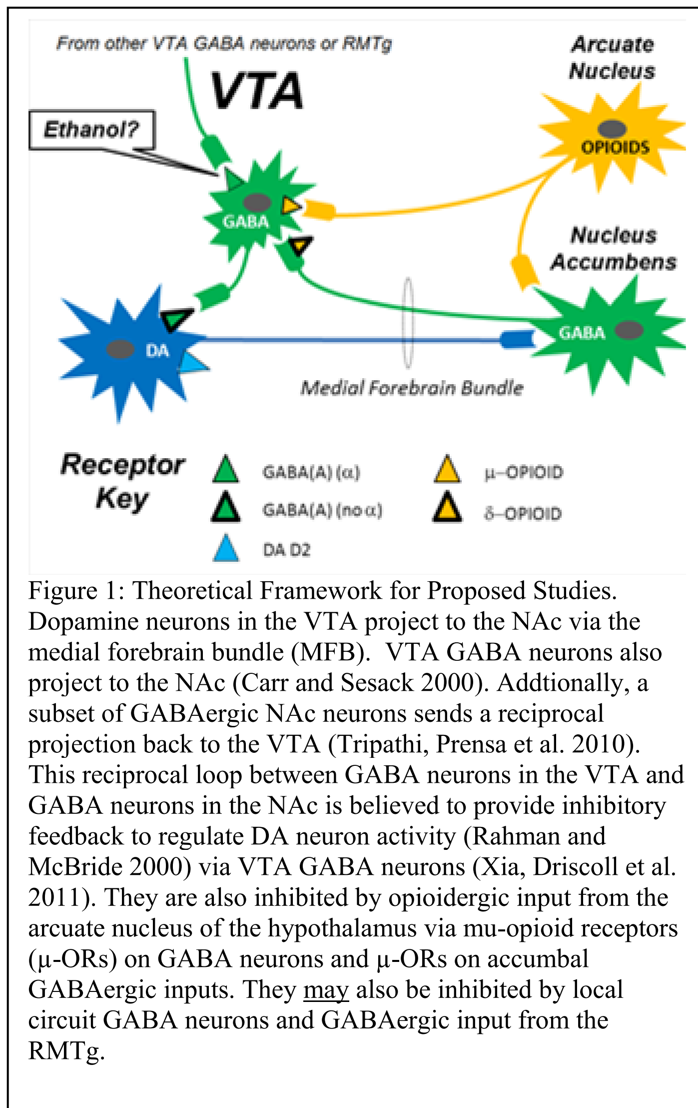

Figure 1: Theoretical Framework for Proposed Studies. Dopamine neurons in the VTA project to the NAc via the medial forebrain bundle (MFB). VTA GABA neurons also project to the NAc (Carr and Sesack 2000). Addtionally, a subset of GABAergic NAc neurons sends a reciprocal projection back to the VTA (Tripathi, Prensa et al. 2010). This reciprocal loop between GABA neurons in the VTA and GABA neurons in the NAc is believed to provide inhibitory feedback to regulate DA neuron activity (Rahman and McBride 2000) via VTA GABA neurons (Xia, Driscoll et al. 2011). They are also inhibited by opioidergic input from the arcuate nucleus of the hypothalamus via mu-opioid receptors (μ-ORs) on GABA neurons and μ-ORs on accumbal GABAergic inputs. They may also be inhibited by local circuit GABA neurons and GABAergic input from the RMTg.

GABA(A)R Functionality Switch

Considerable evidence suggests that activation of GABA(A)R complexes can produce depolarization in lieu of its more traditional hyperpolarizing response (Coull et al. 2003; Hubner et al. 2001; Kaila et al. 1993; Rivera et al. 1999; Staley et al. 1995). One hypothesis to explain this concerns the neuronal potassium-chloride co-transporter isoform 2 (KCC2), which removes intracellular chloride (Cl^-) and thereby maintains an inward-directed hyperpolarizing Cl^- flow (Rivera et al. 2002; Thompson et al. 1988; Viitanen et al. 2010). Consequently, blockade of KCC2 should result in a build-up of intracellular Cl^- and a reduction in Cl^- ion influx after long-term GABA(A) receptor activation, allowing other ion flows [such as a depolarizing bicarbonate ($HCO3^-$) efflux] to dominate (Coull et al. 2003; Kaila et al. 1993; Rivera et al. 1999; Staley et al. 1995; Sun et al. 2012; Sun and Alkon 2001). Figure 2 illustrates the key molecular substrates proposed to mediate the switch in functionality of the GABA(A)R on VTA GABA neurons. This model is based mostly on the work of the Steffensen's lab recent collaboration with Derek van der Kooy's lab studying opiates.

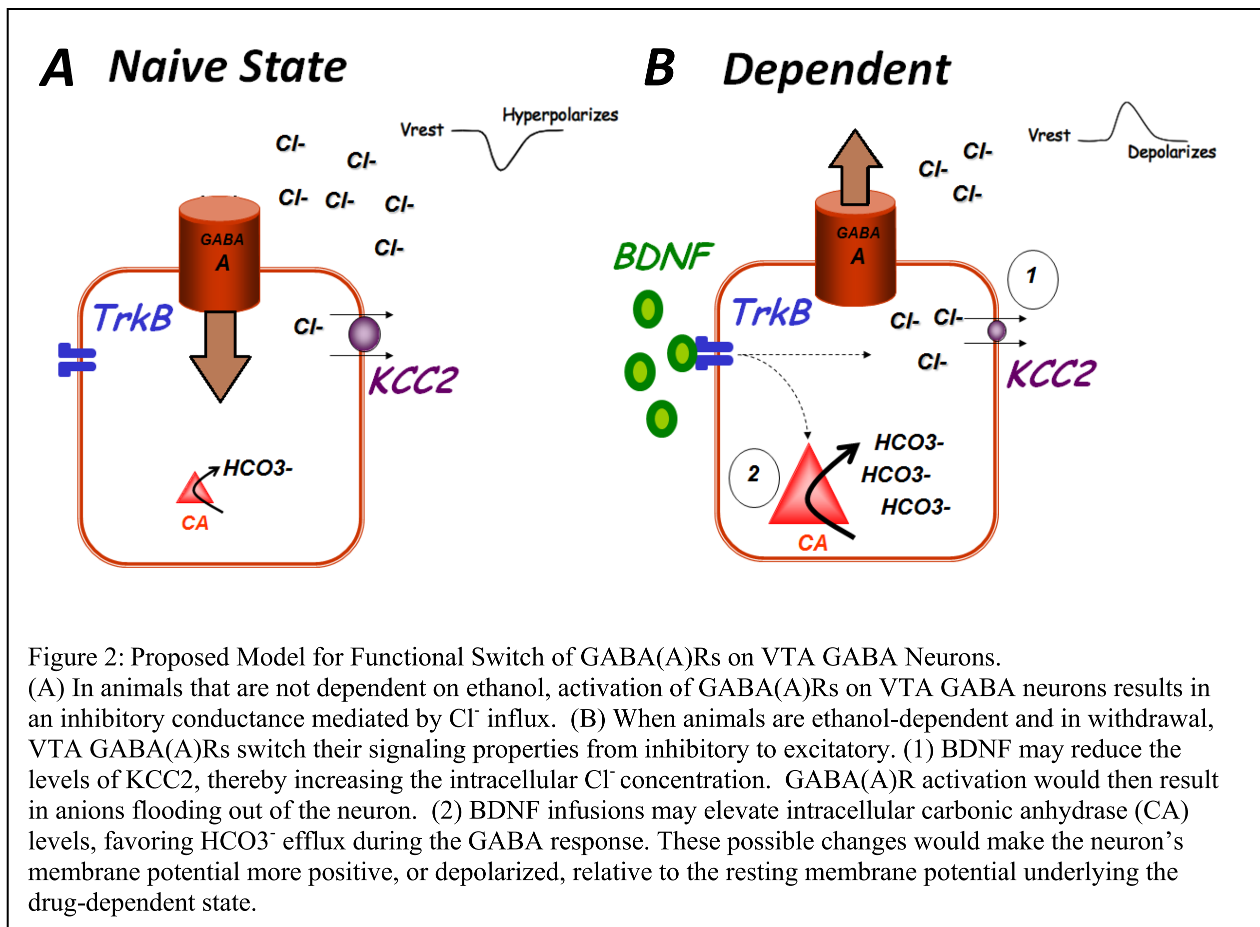

Figure 2: Proposed Model for Functional Switch of GABA(A)Rs on VTA GABA Neurons.
(A) In animals that are not dependent on ethanol, activation of GABA(A)Rs on VTA GABA neurons results in an inhibitory conductance mediated by Cl^- influx. (B) When animals are ethanol-dependent and in withdrawal, VTA GABA(A)Rs switch their signaling properties from inhibitory to excitatory. (1) BDNF may reduce the levels of KCC2, thereby increasing the intracellular Cl^- concentration. GABA(A)R activation would then result in anions flooding out of the neuron. (2) BDNF infusions may elevate intracellular carbonic anhydrase (CA) levels, favoring $HCO3^-$ efflux during the GABA response. These possible changes would make the neuron's membrane potential more positive, or depolarized, relative to the resting membrane potential underlying the drug-dependent state.

Dr. Steffensen's recent collaboration with van der Kooy's lab (Vargas-Perez, Kee et al. 2009) demonstrated that this switch in GABA(A)Rs on VTA GABA neurons associated with chronic opiate administration results from brain-derived neurotrophic factor (BDNF) activation of high-affinity tyrosine kinase B (TrkB) receptor, which is expressed in VTA GABA neurons (Numan, Lane-Ladd et al. 1998). Additionally, depletion of TrkB receptors in the VTA with siRNA-expressing lentiviral vectors (LV) prevented the switch in GABA(A)R function in VTA GABA neurons produced by opiate dependence (Vargas-Perez 2014) and blocking the KCC2 cotransporter with furosemide reduced muscimol inhibition of VTA GABA neurons but not VTA DA neurons (Ting-A-Kee, Vargas-Perez et al. 2013), providing further evidence for the functional switch of GABA(A)Rs and the key substrates mediating such a switch (Figure 3 and Figure 4).

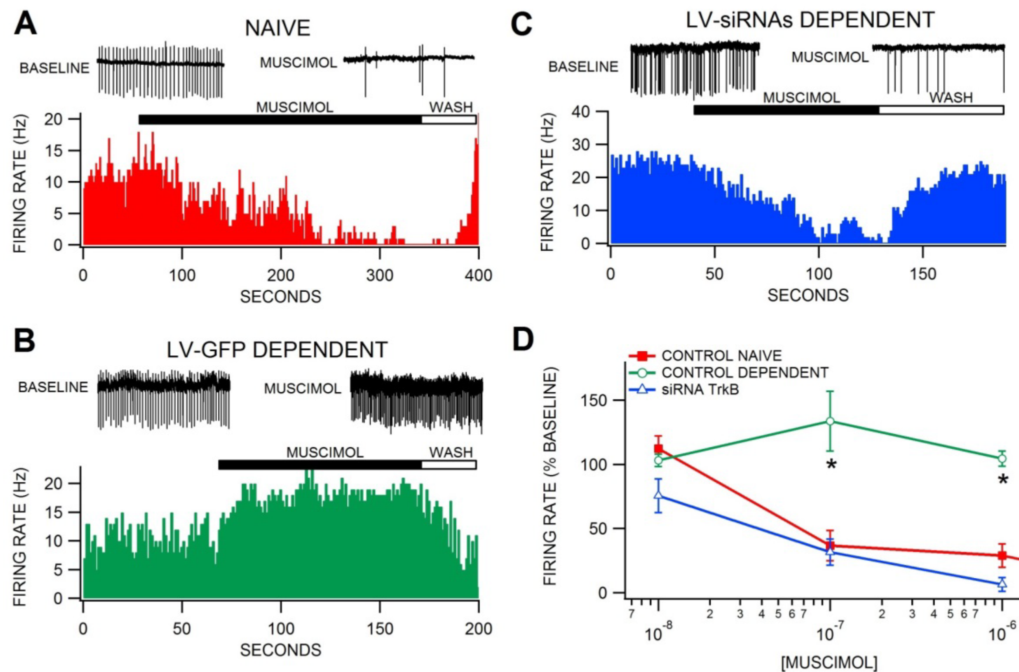

Figure 3: Depletion of TrkB Receptors Prevents Switch in GABA(A)R Function.
(A) The ratemeter shows a representative VTA GABA neuron recorded in a naive mouse with a baseline firing rate of approximately 10 Hz before and after application of 100 nM muscimol, which markedly inhibited the firing rate of this VTA GABA neuron. (B) The ratemeter shows a representative VTA GABA neuron recorded in a LV-GFP opiate-dependent mouse with a baseline-firing rate of approximately 10 Hz before and after 100 nM muscimol, which enhanced the firing rate of this neuron. (C) The ratemeter shows a representative VTA GABA neuron in a LV-siRNAs (TrkB) opiate-dependent mouse with a baseline firing rate of approximately 20 Hz before and after 100 nM muscimol, which markedly inhibited the firing rate of this VTA GABA neuron. (D) Comparison of muscimol (0.01 – 1.0 uM) effects on VTA GABA neuron firing rate in naive and 16-h morphine withdrawal LV-GFP and LV-siRNAs mice. Muscimol significantly inhibited firing rates in naive and LV-siRNAs mice, but not in LV-GFP mice.

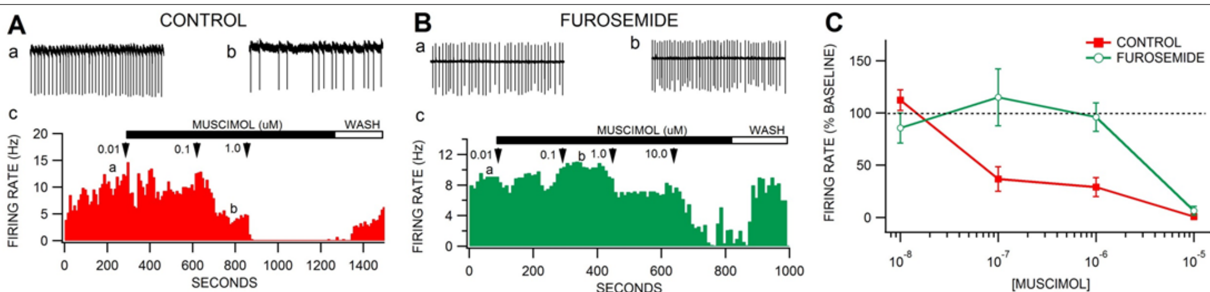

Figure 4: Furosemide Reduces Muscimol Inhibition of VTA GABA Neurons.
(A) The ratemeter shows the firing rate of a VTA GABA neuron (traces in a,b recorded at times indicated on graph), which was spiking at approximately 10 Hz, before and after application of 0.01 – 10.0 µM muscimol, which markedly inhibited the firing rate of this VTA GABA neuron. All insets are representative 5 sec traces of GABA neuron spike activity recorded before and after 100 nM muscimol. (B) The ratemeter shows the firing rate of a GABA neuron, which was approximately 8 Hz, before and after application of 0.01 – 10.0 µM muscimol. Only 10 µM muscimol inhibited the firing rate of this neuron in the presence of furosemide. (C) Muscimol significantly inhibited the firing rate of VTA GABA neurons, which was significantly reduced by furosemide.

Many labs, including ours, have shown that ethanol withdrawal produces adaptations in VTA neurons (Gallegos, Criado et al. 1999, Brodie 2002, Hopf, Martin et al. 2007) and GABA(A)R subunit composition (Charlton, Sweetnam et al. 1997, Cagetti, Liang et al. 2003), and it is reasonable to assume that these changes are important for precipitating this switch in the neurobiological substrates mediating ethanol reinforcement. Functionally, the switch is caused by a change in the ion conductance properties of the GABA(A)Rs themselves (Staley, Soldo et al. 1995, Stein and Nicoll 2003, Laviolette, Gallegos et al. 2004). Thus, our goal is to determine the effects of chronic (dependent condition) ethanol exposure on the function of GABA(A)Rs on VTA GABA neurons.

RATIONALE AND HYPOTHESES

As previously mentioned, GABAergic synaptic transmission is still regarded as one of the main factors underlying the intoxicating, rewarding, and withdrawal-related effects of ethanol (Bonci and Williams 1996, Bonci and Williams 1997). GABAergic projections to the VTA come from several regions including the NAc and the ventral pallidum. However, the primary inhibitory regulation of DA neurons is by GABAergic interneurons within the VTA (Johnson and North 1992, Steffensen, Svingos et al. 1998). The predominant GABA(A)Rs expressed in the VTA are α1−6, β1, β3 and γ2 (Okada, Matsushita et al. 2004). Levels of α1 are decreased by chronic ethanol treatment (Ortiz, Fitzgerald et al. 1995, Charlton, Sweetnam et al. 1997, Papadeas, Grobin et al. 2001). Inhibition of VTA GABA neurons is mediated by α1 subunit-containing GABA(A)Rs that are selectively expressed in GABA neurons, but not in DA neurons, in the VTA. The neurochemical and electrophysiological characterization of the VTA GABA neurons that regulate DA neurotransmission is essential to unravel the complex interactions between neuronal elements underlying the neuroadaptations associated with alcohol dependence.

Thus, the focus of this study is to evaluate plasticity in GABAergic inhibition following chronic ethanol exposure. Our objectives were four-fold: 1) To identify an adaptation of GABA(A)R function in VTA GABA neurons with chronic ethanol; 2) To observe an adaptation of GABAergic synaptic transmission in VTA GABA neurons with chronic ethanol; 3) To observe an adaptation of GABAergic inhibition on VTA DA neurons; and 4) To determine if GABA(A)Rs on VTA GABA neurons switch their function via a change in chloride gradient. By addressing these aims with various experimental procedures, we expected to help evaluate the role of GABA(A)Rs on VTA GABA neurons in mediating the alcohol withdrawal syndrome.

METHODS

Animal Subjects

In all of the experiments male glutamate-decarboxylase-67 (GAD-67)-green fluorescent protein (GFP) knock-in CD-1 (white albino) mice (Tamamaki, Yanagawa et al. 2003) were used. GAD GFP mice were used in order to visualize VTA GABA neurons in the slice preparation. Mice were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals were treated in strict accordance with the Brigham Young University Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. The BYU IACUC has reviewed and approved the procedures detailed herein. Once weaned at PND 21, all mice were housed in maximum groups of four and given ad libitum access to solid food and water and placed on a reverse light/dark cycle with lights ON from 8 PM to 8 AM.

Animals were exposed to chronic intermittent ethanol in the alcohol vapor chambers where BALs could be determined. They were exposed to 200 mg% BAL for eight hours (1000-1800 hrs) during their dark cycle (i.e., reverse cycle light) each day for at least 14 days. In a separate group, animals were treated with twice-daily intraperitoneal (IP) injection of 3 g/kg ethanol. Regardless of treatment approach, withdrawal from chronic ethanol was studied 12-24 hours following withdrawal, which is considered to be the peak of the alcohol withdrawal syndrome in rodents.

Preparation of Brain Slices

All brain slice preparations were performed in P18-45 day old C57BL/6 and GAD-GFP knock-in mice. Mice were anesthetized with isoflurane (5%) and then administered ketamine (60 mg/kg) intraperitoneally prior to decapitation. The brain was then extracted from the cranium,

cropped, glued onto a cutting stage, and then sectioned in ice-cold cutting solution (in mM: 220 Sucrose, 3 KCl, 1.25 NaH_2PO_4, 25 NaH_2CO_3, 12 $MgSO_4$, 10 Glucose, 0.2 $CaCl_2$, and 0.4 Ketamine) and bubbled with 95% O_2 / 5% CO_2. Targeting the VTA, horizontal slices (210 µM thick) were placed in an incubation chamber containing artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$) perfused with 95% O_2 / 5% CO_2 for at least 30 minutes. After 30 minutes, brain slices were then placed in a recording tissue chamber with ACSF continuously flowing at physiological temperatures (36 °C).

Characterization of Neuron Types

GABA neurons were studied in GAD-GFP knock-in mice. In GAD-GFP knock-in mice, VTA GABA neurons were identified by a characteristic glow under fluorescence illumination and by using a GABA spike command waveform [spikes at 200 Hz for 500msec; (Steffensen, Taylor et al. 2008)], as GABA neurons will follow the command waveform. Neurons that did not fluoresce and/or exhibited a non-cation specific inward rectifying current (I_h) with low input resistance, were assumed to be DA neurons (Johnson and North 1992, Allison, Ohran et al. 2006, Margolis, Lock et al. 2006, Allison, Wilcox et al. 2011, Steffensen, Bradley et al. 2011).

Whole-cell Recordings in vitro

Electrodes were pulled from borosilicate glass capillary tubes and filled with a KCl pipette solutions [in mM: 128 KCl, 20 NaCl, 0.3 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.25 Na-GTP (pH 7.3)] for eIPSC studies. Pipettes having tip resistances of 2.5 - 5MΩ, and series resistances typically ranging from 7 to 15 MΩ were used. Voltage clamp recordings were filtered at 2 kHz while current-drive spikes were filtered at 10 kHz with an Axon Instruments Multiclamp 700B amplifier and digitized at 5 to 20 kHz respectively using an

Axon 1440A digitizer. Axon Instruments pClamp ver10, Mini Analysis (Synaptsoft: Decatur, GA), and Igor Pro (Wavemetrics: Oswego, OR) software packages were utilized for data collection and analysis. Evoked IPSCs (eIPSCs) were recorded in the presence of 50μM APV and 30μM CNQX or 3mM kynurenic acid to block NMDA and AMPA mediated GLU receptor synaptic currents. Using a paired-pulse stimulus, the stimulation was adjusted to a half-max level in order to allow measurements of changes that increase or decrease eIPSC levels.

Cell-attached, Voltage-clamp Recording of Spike Activity in Brain Slices

Electrodes pulled from borosilicate glass capillaries and then filled with 150mM NaCl (2.5-5MΩ) were used for cell-attached DA studies. Positive pressure was applied to the electrode when approaching the neuron. By applying suction to the electrode, a seal (10MΩ – 1GΩ) was created between the cell membrane and the recording pipette. Spontaneous spike activity was then recorded in voltage-clamp mode with an Axon Instruments Multiclamp 700B amplifier and sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. Neurons were voltage-clamped at 0 mV throughout the experiment. A stable baseline recording of firing activity was obtained for 5-10 min before adding any substances.

Perforated-patch Electrophysiology

To determine the E_{Cl^-}, peforated patch electrophysiology recordings were performed on GABA and dopamine neurons in the ventral tegmental area (VTA) of the rat midbrain. A gramicidin stock solution was made fresh daily (5mg/ml in dimethylsulfoxide). The stock solution was sonicated for ~30 min, then continuously vortexed at a low speed for the duration of the recording session. The tips of the microelectrodes, with resistances between 4 and 8 MΩ, were prefilled with the pipette solution containing (in mM): 135 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 Na-EGTA adjusted to pH 7.2 with KOH and then backfilled with the same pipette

solution containing 10 µg/ml of gramicidin. The access resistance was used to monitor the progression of the perforation, which was considered to be complete when it became < 80 MΩ. In voltage-clamp mode, inhibitory currents were elicited by stimulation and then recorded with an Axon Instruments Multiclamp 700B amplifier and sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. Each cell was held at the following potentials as stimulation was administered: -100 mV, -80 mV, -60 mV, -40 mV, -20 mV, 0 mV.

Drug Preparation and Administration

CNQX (30 µM; Abcam) and APV (50 µM; Abcam) were dissolved in distilled water and frozen until used. Kynurenic acid (3 mM; Sigma-Aldrich) was made fresh in distilled water and sonicated to get into solution. Muscimol (10 nM – 10 µM; Sigma-Aldrich) was made fresh and dissolved in distilled water. While measuring eIPSCs, kynurenic acid or the combination of APV and CNQX were added to the ACSF perfused to the brain slice to block currents from AMPA and NMDA. Afterwards, ethanol (5 – 50 mM) was administered to the ACSF to observe its effect on eIPSCs in chronic alcohol and chronic air exposed animals.

Statistical Analyses

All results are presented as raw mean values and percent control ± SEM. Results between groups were compared using a two-way ANOVA and a simple main effects analysis as required. Statistical significance required ≥ 95% level of confidence ($p \leq 0.05$). Analysis software included Microsoft Excel, SPSS Statistics, and Igor Pro (Wavemetrics, Oswego, OR). Significance levels are indicated on graphs with asterisks *,**,*** and correspond to significance levels $p < 0.05$, 0.01 and 0.001, respectively. Figures were constructed with Igor Pro software.

RESULTS

Effects of Withdrawal on Muscimol-Induced Inhibition of Firing Rate

We evaluated the effects of the GABA(A)R agonist muscimol on VTA GABA neuron firing rate in GAD GFP mice treated with twice-daily IP injections of 3 g/kg ethanol for 2 wk. It took 2 wk of chronic intermittent exposure to produce alcohol dependency (Gallegos, Criado et al. 1999). After 2 wk of chronic intermittent exposure to alcohol, we evaluated muscimol effects on VTA GABA neuron firing rate in GAD GFP mice in horizontal brain slices and compared them to their saline-injected controls. For firing rate studies, neurons were recorded in cell-attached voltage-clamp mode. The Steffensen lab has used this model for two previous publications involving GABA(A)R switching with opiate dependence (Ting-A-Kee, Vargas-Perez et al. 2013, Vargas-Perez 2014). Figure 5 illustrates that there was less sensitivity to muscimol in animals treated with ethanol versus saline-injected controls. A two-way ANOVA (treatment vs. muscimol concentration) revealed non-significant main effects of treatment (saline vs. ethanol injection) [$F(1, 45) = 3.189$, $p = .081$], significant muscimol concentration [$F(4, 45) = 17.978$, $p < .0001$], as well as a non-significant treatment x concentration interaction [$F(4, 45) = .794$, $p = .535$]. One-way ANOVA showed that muscimol inhibited the firing rate of VTA GABA neurons in saline injected animals only at the 100 nM concentration of muscimol [$F(1, 10) = 4.359$, $p = .063$], but there were no differences with other muscimol concentrations.

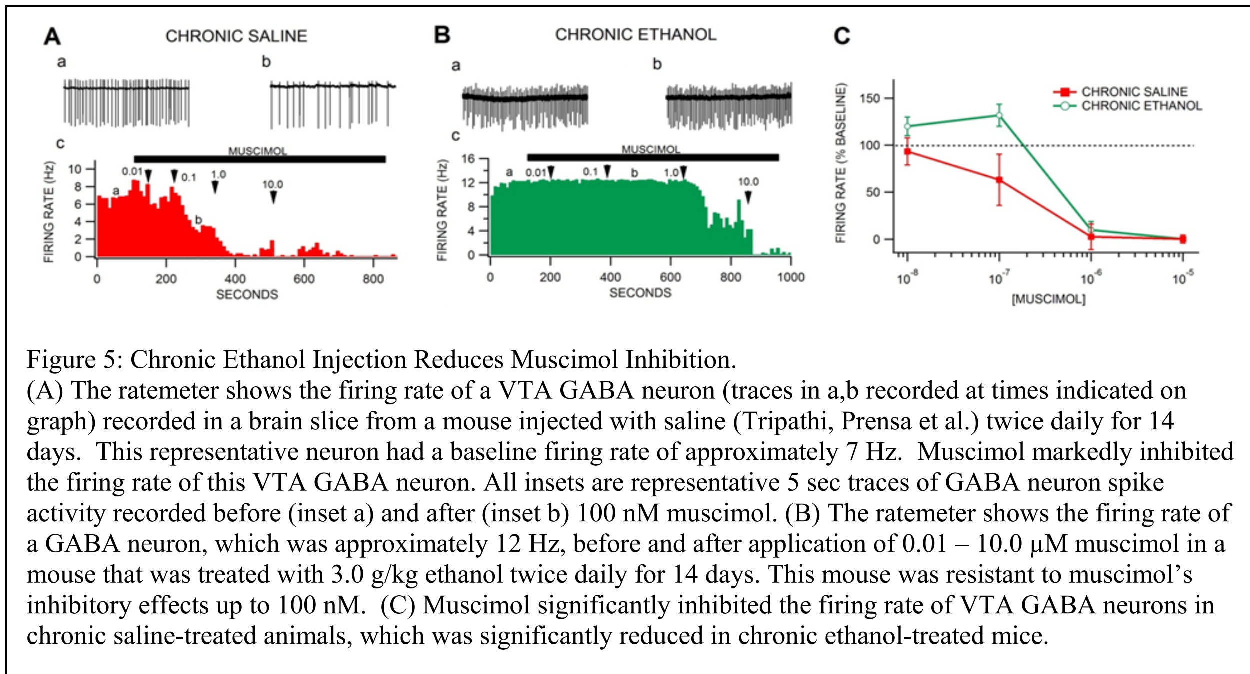

Figure 5: Chronic Ethanol Injection Reduces Muscimol Inhibition.
(A) The ratemeter shows the firing rate of a VTA GABA neuron (traces in a,b recorded at times indicated on graph) recorded in a brain slice from a mouse injected with saline (Tripathi, Prensa et al.) twice daily for 14 days. This representative neuron had a baseline firing rate of approximately 7 Hz. Muscimol markedly inhibited the firing rate of this VTA GABA neuron. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 100 nM muscimol. (B) The ratemeter shows the firing rate of a GABA neuron, which was approximately 12 Hz, before and after application of 0.01 – 10.0 µM muscimol in a mouse that was treated with 3.0 g/kg ethanol twice daily for 14 days. This mouse was resistant to muscimol's inhibitory effects up to 100 nM. (C) Muscimol significantly inhibited the firing rate of VTA GABA neurons in chronic saline-treated animals, which was significantly reduced in chronic ethanol-treated mice.

We further continued these firing rate studies in animals treated with alcohol in the vapor chambers. Animals were exposed to chronic intermittent ethanol in the alcohol vapor chambers where blood alcohol levels (BALs) were monitored and adjusted. They were exposed to 200 mg% BAL for 8 h (1000-1800 hrs) during their dark cycle (i.e., reverse cycle light). After a minimum of 2 wk of chronic intermittent exposure to alcohol, we evaluated muscimol effects on VTA GABA neuron firing rate in GAD GFP mice in horizontal brain slices and compared them to their air-exposed controls. Figure 6 illustrates that there was also less sensitivity to muscimol in animals treated with ethanol vapor versus air-exposed controls. A two-way ANOVA (treatment vs. muscimol concentration) revealed non-significant main effects of treatment (air control vs. ethanol vapor) [$F(1, 113) = 2.689$, $p = 0.104$], significant muscimol concentration [$F(4, 113) = 21.166$, $p < 0.0001$], as well as a non-significant group x concentration interaction [$F(4, 113) = 1.395$, $p = .240$]. One-way ANOVA showed that muscimol significantly inhibited the firing rate of VTA GABA neurons in air-exposed animals only at the 1 µM concentration of muscimol [$F(1, 28) = 5.493$ $p = .027$], but there were no differences with other muscimol concentrations.

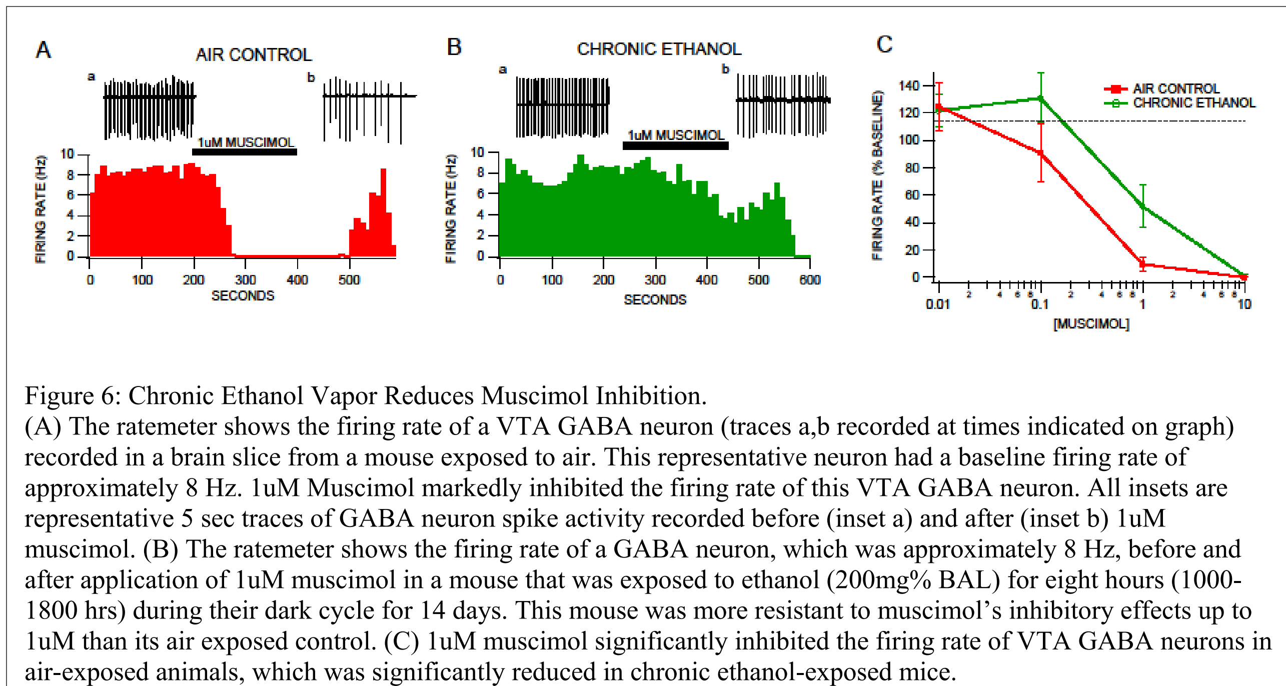

Figure 6: Chronic Ethanol Vapor Reduces Muscimol Inhibition.
(A) The ratemeter shows the firing rate of a VTA GABA neuron (traces a,b recorded at times indicated on graph) recorded in a brain slice from a mouse exposed to air. This representative neuron had a baseline firing rate of approximately 8 Hz. 1uM Muscimol markedly inhibited the firing rate of this VTA GABA neuron. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 1uM muscimol. (B) The ratemeter shows the firing rate of a GABA neuron, which was approximately 8 Hz, before and after application of 1uM muscimol in a mouse that was exposed to ethanol (200mg% BAL) for eight hours (1000-1800 hrs) during their dark cycle for 14 days. This mouse was more resistant to muscimol's inhibitory effects up to 1uM than its air exposed control. (C) 1uM muscimol significantly inhibited the firing rate of VTA GABA neurons in air-exposed animals, which was significantly reduced in chronic ethanol-exposed mice.

Effects of Withdrawal on GABA(A)R-Mediated Synaptic Responses

In order to test ethanol's withdrawal effects on GABA(A)R-mediated synaptic responses, we compared eIPSCs and eIPSC paired-pulse responses in ethanol exposed versus air-exposed mice as well as the effects of a challenge of ethanol (5 mM) on eIPSCs. Alcohol dependency was achieved as detailed in the prior experiment. For synaptic studies involving GABA(A)R responses, we included APV and CNQX to block excitatory synaptic transmission. Notably, a recent study has shown interactions with ethanol and CNQX (Brickley, Farrant et al. 2001, Maccaferri and Dingledine 2002). Thus, in some studies we used kynurenic acid (3 mM) to block glutamate (GLU) transmission in place of APV and CNQX. In these slice studies we utilized GAD GFP mice to unequivocally identify VTA GABA neurons. We also employed a variation of the conventional criteria to classify GABA neurons. Mainly, GABA neurons were subjected to a GABA spike command waveform [spikes at 200 Hz for 500 msec; (Steffensen, Taylor et al. 2008)]. It has been found that GABA neurons will follow the command waveform while DA neurons will not. GABA neuron spike activity is enhanced or not affected by DA

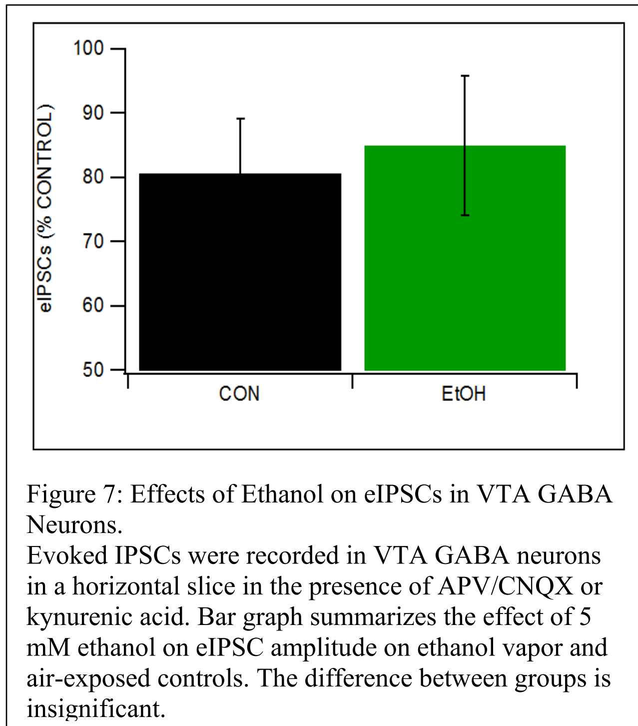

Figure 7: Effects of Ethanol on eIPSCs in VTA GABA Neurons.
Evoked IPSCs were recorded in VTA GABA neurons in a horizontal slice in the presence of APV/CNQX or kynurenic acid. Bar graph summarizes the effect of 5 mM ethanol on eIPSC amplitude on ethanol vapor and air-exposed controls. The difference between groups is insignificant.

(Stobbs, Ohran et al. 2004, Lassen, Brown et al. 2007) while DA neurons are well-known to be inhibited by DA or D2 agonists. Figure 7 illustrates that there was no significant difference in eIPSC amplitude in animals treated with ethanol vapor versus air-exposed controls when given a challenge dose of 5 mM ethanol [$F(1, 10) = .078$, $p = .786$].

Effects of Withdrawal on VTA DA Neuron Firing Rate

The dogma is that local circuit GABA neurons inhibit DA neurons in the VTA. In order to determine how ethanol may be influencing DA activity and release via the changes we found in VTA GABA neurons, we studied VTA DA neuron firing rate. For firing rate studies, we performed cell-attached, voltage-clamped studies on VTA DA in slice preparation for GAD-GFP mice. We used the same technique as described above in our VTA GABA firing rate studies, except here on VTA DA neurons. VTA DA neurons were identified as non-glowing cells and exhibited their characteristically slow and regular firing rates. In addition, they did not follow the command waveform like GABA neurons. We found that there was no significant difference in firing rate activity in animals treated with ethanol vapor versus air-exposed controls when given a challenge dose of ethanol (5 mM, [$F(1, 15) = .592$, $p = .454$]; and 50 mM, [$F(1, 9) = .457$, $p = .518$]), as illustrated in Figure 8.

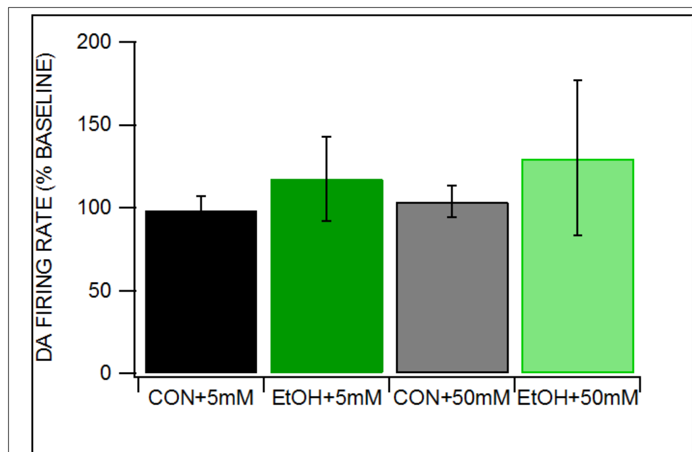

Figure 8: Effects on Ethanol on VTA DA Firing Rate. Firing rate was recorded in VTA DA neurons in a horizontal slice. Bar graph summarizes the effect of 5 and 50mM ethanol on DA firing rate on ethanol vapor and air-exposed controls. The difference between groups is insignificant at both ethanol doses.

GABA(A)Rs on VTA GABA Neurons Switch their Function via a Change in Chloride Gradient

The Steffensen lab has shown previously that the firing rate of VTA GABA neurons is inhibited by acute ethanol, that tolerance develops to chronic ethanol and that the excitability of VTA GABA neurons is markedly excited during withdrawal from chronic ethanol when animals are alcohol dependent (Gallegos, Criado et al. 1999). The mechanism underlying the adaptation of VTA GABA neurons to chronic ethanol remains to be elucidated. Although not yet published, the Steffensen lab has recently found that a single exposure to ethanol (either incubated for 1 h in 30 mM ethanol or injected in vivo 24 h previous at 2.5 g/kg) results in GLU plasticity in VTA GABA neurons during withdrawal, characterized by markedly enhanced sEPSCs and AMPA/NMDA ratio. In addition, a single exposure to ethanol results in lowered sIPSCs in VTA GABA neurons. Thus, excitatory GLU synaptic transmission is enhanced and inhibitory GABA synaptic transmission is reduced which may explain why GABA neuron firing rate is enhanced during withdrawal. Although not the focus of this study, we felt that this short-term plasticity is an harbinger for more persistent long-term changes involving GABA(A)Rs. Indeed, we hypothesized that alcohol dependence results in a switch in GABA(A) function, as per the van der Kooy model involving the chloride transporter, which we have demonstrated with opiate dependence.

Effects of Chronic Ethanol on Chloride Reversal Potentials

Using gramicidin antibiotics we studied GABA currents and chloride reversal potential without altering the intracellular ionic milieu. We attempted to compare chloride reversal potentials in VTA GABA neurons in ethanol-exposed mice to air-exposed controls using perforated patch recordings. Unfortunately, we were unsuccessful in obtaining a perforated patch, and if a perforated patch was believed to be obtained, an inhibitory current could not be evoked. Because of this, we were unable to compare chloride reversal potentials in VTA GABA neurons in ethanol-exposed mice to air-exposed controls. After consulting with Dr. Jie Wu from the Barrow Institute, our collaborator who has extensive knowledge about the perforated patch protocol, we learned of possible explanations for our ill success. Dr. Wu typically performs perforated patch recordings on dissociated neurons from the VTA of 2 – 3 wk old mice (Taylor 2013). According to him, this method is preferred because there are fewer synaptic inputs and the neurons are more likely to remain healthy. This is not the case with brain slices, which is the method that we employ. Another difficulty with perforated patch in the brain slice is the required positive pressure applied to the pipette in order to get a tight seal. This positive pressure will force the gramicidin out of the pipette and compromise the cell membrane before a tight seal is created. Additionally, because we must expose our animals to ethanol vapors for two weeks after weaning, our mice are rather aged in comparison (4 – 6 wk old).

DISCUSSION

As shown in our results, muscimol significantly inhibited the firing rate of VTA GABA neurons at low concentrations (100 nM and 1 μM) in mice given IP injections of ethanol and ethanol vapor mice, respectively. This is consistent with our previous studies demonstrating a switch in GABA neuron response to muscimol in BDNF treated rats (Vargas-Perez, Kee et al. 2009) and chronic morphine treated rats (Ting-A-Kee, Vargas-Perez et al. 2013). It is interesting to note the difference in concentration at which muscimol significantly inhibited firing rate in mice given IP injections and ethanol vapor mice. Perhaps this is due to the difference in ethanol administration between mice, or by chance due to a higher variance in smaller sample sizes. It is also interesting that while our muscimol studies support a switch in the GABA(A)R function, our synaptic responses with GABA eIPSCs and DA firing rate do not reflect this switch. Because ethanol is known to have multiple target receptors and effects in the brain, perhaps ethanol is compensating for the switch in GABA(A)R functionality through alternative mechanisms.

A significant ongoing concern is that there are multiple populations of VTA GABA neurons, both of which exhibit fluorescence in the slice preparation. There is no approach currently to unequivocably differentiate them. There is nothing electrophysiologically (i.e., whole cell currents, firing rate, spike waveform, input resistance, or depolarization spiking), at least in mice, that might serve as a marker that might differentiate VTA GABA neurons.

There is also a dearth of direct evidence for inhibition of DA neurons by local circuit GABA neurons. Thus, it should be kept in mind that most VTA GABA neurons may not directly inhibit DA neurons at cell bodies. I continue to work under the theoretical framework that some VTA GABA neurons inhibit DA neurons. However, it is becoming increasingly evident that VTA GABA neurons may exert their effects more at DA terminals in the NAc rather than at cell bodies in the VTA.

Another concern is that the animals in the alcohol vapor chambers were given access to running wheels. There has been recent evidence suggesting that voluntary exercise alters GABA(A)R subunit and GAD67 gene expression in rat forebrains in a region-specific manner (Hill, Droste et al. 2010). While our experiments were performed in mice, because our study focuses on a potential change in GABA(A)Rs, it would be best to remove exercise as a possible confounding variable to our results. Thus, in future experiments animals will not have access to running wheels.

Additionally, there are slight concerns with the alcohol vapor chambers. There have been several occasions where animals that have been exposed to ethanol vapors for over two weeks failed to show typical withdrawal symptoms. This concern may address why there was no significance between ethanol vapor and air-exposed groups in the eIPSC and DA firing rate studies. Additional troubleshooting should be done in order to ensure alcohol dependency and withdrawal in the ethanol vapor exposed animals. While not explicitly looked at in this study, there appears to be a discrepancy in results between air-exposed mice and naïve mice, as done in previous studies. A separate study comparing naïve mice and air-exposed mice should be done in order to determine if such a difference does indeed exist. If a difference is to exist, it is possible that the air-exposed mice may be receiving some, albeit slight, ethanol exposure. Ethanol may be leaking from either the tubing or the chambers which would then disperse throughout the vapor chamber room. This is problematic because the air-exposed mice are pumped with air from the vapor chamber room. A possible solution is to place the pumps that provide the animals with air into a separate room, that way there is no chance of accidental ethanol exposure.

Another possibility explaining the discrepancy in results the two mice populations is that there may be a difference of alcohol sensitivity in GAD GFP+ and GAD GFP- mice. Future

experiments will use GAD GFP+, GAD GFP- , and wild type mice. If there appears to be a significant difference in results, GAD GFP- or wild type mice will be utilized instead.

Although not the focus of this study, we believe that there may persistent long-term changes involving GABA(A)Rs, due to an observed short-term plasticity given a single exposure to ethanol in previous studies. While for this thesis project, we hypothesized that alcohol dependence results in a switch in GABA(A) function, there are other equally plausible mechanisms that could underlie the plasticity. For example, GABA(A)R subunits may be differentially expressed during dependence. Also, recent studies have shown that GABA(A)Rs may undergo internalization (Luscher, Fuchs et al. 2011). In future studies, the quantitative expression of GABA(A)R subunits α1, α2, β1, γ2, ρ, δ; as well as TH, Cx36,and TrkB receptors in VTA GABA neurons from GAD GFP mice inhaling ethanol vapors versus their paired controls should be compared. Clathrin, a protein that mediates endocytosis, has been shown to be involved in the internalization of GABA(A)Rs (Luscher, Fuchs et al. 2011). Experiments to evaluate GABA currents before and after ethanol treatment in VTA GABA neurons from naïve animals treated with pitstop, a clathrin inhibitor, should be done to see if internalization of GABA(A)Rs occur. These two experiments would help further outline the longer-term changes involving GABA(A)Rs.

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