Neuronal and Molecular Adaptations of GABA Neurons in the Ventral Tegmental Area to Chronic Alcohol

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NEURONAL AND MOLECULAR ADAPTATIONS OF GABA NEURONS
IN THE VENTRAL TEGMENTAL AREA TO CHRONIC ALCOHOL

by

Kimberly Hales

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

Master of Neuroscience

Department of Physiology and Developmental Biology
Brigham Young University
December 2007
GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

NEURONAL AND MOLECULAR ADAPTATIONS OF GABA NEURONS
IN THE VENTRAL TEGMENTAL AREA TO CHRONIC ALCOHOL

Kimberly Hales
Department of Physiology and Developmental Biology
Master of Neuroscience

The purpose of this thesis project was to examine the effects of chronic alcohol on the excitability and molecular adaptation of GABA neurons of the ventral tegmental area (VTA). GABA neurons are of interest with regards to ethanol intoxication, reinforcement, and dependence due to their widespread distribution and connectivity to mesocorticolimbic dopamine (DA) neurons implicated in alcohol reward and addiction. Since we have previously shown adaptation of VTA GABA neuron firing rate to chronic ethanol (Gallegos, Criado et al. 1999) and suppression of gap-junction (GJ) mediated coupling between these neurons by acute ethanol (Stobbs, Ohran et al. 2004), we wanted to further characterize the effects of chronic ethanol on VTA GABA neuron excitability, electrical coupling and molecular adaptation. In particular, we analyzed the GJ mediated coupling and protein regulation of VTA GABA neurons following a three week period of continuous ethanol exposure via liquid diet. Although some animals showed tolerance,
there was no significant tolerance to ethanol inhibition of GJ-mediated electrical coupling. In addition, we were able to characterize differences in mRNA expression levels for the DA synthesizing enzyme tyrosine hydroxylase (TH), the DA D2 receptor and the NMDAR2B receptor subunit in DA versus GABA neurons, all three of which were expressed at higher levels in DA neurons. We also determined the effects of chronic ethanol on mRNA levels of these same proteins as well as µ-opioid receptors (µORs) and connexin-36 (Cx36) GJs. Most significantly, we found a down-regulation of the DA D2 receptor, confirming that molecular modification occurs in these VTA GABA neurons with chronic alcohol. While we reject our hypothesis that acute ethanol inhibition of VTA GABA neuron electrical coupling would undergo tolerance to chronic ethanol in these non-dependent rats, which was the focus of this thesis, it remains to be determined if tolerance to chronic ethanol might be obtained in ethanol-dependent rats.
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INTRODUCTION

The field of alcohol research is devoted to a vast conglomeration of labs across the country and world. One main reason for this is the wide-spread use and abuse of this psychoactive drug that results in billions of dollars of damage and millions of deaths each year. Alcohol addiction is an area of focus worth studying due to its global impact, biological nature, abuse potential and long-term relapsing effects. The tolerance and dependence that comes from repeated chronic use is of particular interest to me and its biological implications are the focus of my project. The rationale for this study is predicated on the belief that advancement in the understanding of the neuronal substrates underlying the acute and chronic effects of alcohol will pave the way for more effective treatment strategies for alcoholism that would save lives and resources throughout the world. There were two main goals in this study: 1) Evaluate whether gap junctions (GJs) and electrical coupling in the ventral tegmental area (VTA) of the midbrain, an area implicated in natural rewarding behaviors and drug addiction, play a role in the adaptation process that occurs during the transition from the acute effects of alcohol, to relapse, and then to dependence, and 2) Assess the regulation of key proteins in the VTA following chronic alcohol use. My principal hypothesis was that electrical coupling in VTA GABA neurons is involved in the acute effects of ethanol and that changes in GJ
function might play a role in the progressive increase in the motivation to seek alcohol. In addition, I predicted that protein expression in this population of VTA neurons would be modified with chronic alcohol. In this thesis I will: 1) Briefly review the mesocorticolimbic reward system and discuss the current state of knowledge regarding alcoholism; 2) Review the neural circuits implicated in the acute and chronic effects of alcohol; 3) Describe the neurobiology of GJs and evidence for DA modulation of GJs; 4) Summarize the research regarding VTA GABA neurons and their role in the mesocorticolimbic DA system; 5) Present our experimental results, and 6) Discuss the implications of these results while providing an overview of critiques to help in future studies.

BACKGROUND

The mesocorticolimbic system is an area in the brain known to be involved with natural and drug reward as well as addiction. Within this system, many different parts of the brain are linked by pathways, including the hippocampus, amygdala, medial prefrontal cortex, nucleus accumbens and ventral tegmental area (Pierce and Kumaresan 2006). The ventral tegmental area (VTA) and nucleus accumbens (NAcc) are considered to be reward centers that are affected by alcohol. Within the VTA, there are two main types of neurons: Dopamine (DA) and GABA. Pathways from both of these types of neurons are sent to other parts of the limbic system and in turn, the VTA receives GABAergic input from the NAcc. Dopamine and GABA neurons have distinctive firing patterns in anesthetized adult male rats. Dopamine neurons fire slowly and sometimes in
bursts while GABA neurons fire at a much higher frequency and do not fire in bursts (Steffensen, Svingos et al. 1998). Using this distinctive firing pattern of these neurons, we are able to distinguish between the two relatively easily in the brain in vivo.

The prevailing dogma behind reward and addiction in the brain is that a euphoric state is achieved through increased DA levels in the mesocorticolimbic system, particularly the NAcc and the VTA (Wise 2004). Thus, much of the drug and alcohol research has been dedicated to studying DA neurons in this system of the brain. Interestingly, rats will self-administer ethanol directly into the VTA (Gatto, McBride et al. 1994) and an ethanol-induced increase of DA release in the NAcc, detected by microdialysis, has been reported extensively (Imperato and DiChiara 1986; Wozniak, Pert et al. 1991; Yoshimoto, McBride et al. 1992) (see also (Di Chiara and Imperato 1985; Di Chiara and Imperato 1988; Weiss 1991; Weiss, Hurd et al. 1991)). While activation of the mesocorticolimbic system is implicated in drug reward, the DA component of the system is critical only for psychostimulant reward (Koob 1996), as rats continue to self-administer ethanol as well as opioids with severe neurotoxin-lesions of the mesocorticolimbic DA system (Koob 1992). Thus the focus of our lab is on the GABA neurons in the VTA.

We believe that GABA neurons synapse on DA neurons in the VTA, thereby providing an inhibitory input to regulate DA release. This is in agreement with the previous finding of GABAergic control over DA neurons in the substantia nigra (Tepper, Paladini et al. 1998), a structure involved more with motor activity than psychomotor activity, but analogous to the VTA. Inhibition of these inhibitory GABA neurons would result in hyperexcitability of DA neurons and an increased amount of DA release. This is
in line with the DA theory mentioned above. Previously, it has been shown that alcohol decreases the firing rate of these GABA neurons with acute administration for up to 2 weeks (Gallegos, Criado et al. 1999). In line with our theory and the DA theory, the decreased firing rate of the GABA neurons would result in more DA to be released and a euphoric state would result. After 2 weeks of daily alcohol administration however, the firing rate no longer decreases (Gallegos, Criado et al. 1999). This indicates that tolerance is occurring with these neurons, resulting in hyperactivity of the GABA neurons and thus a decrease in DA release that leads to a withdrawal state once alcohol is out of the system.

Another interesting phenomenon we have previously shown is that high-frequency stimulation of the internal capsule (IC) causes multiple spike discharge (ICPSDs) of GABA neurons in the VTA (Steffensen, Svingos et al. 1998). These discharges are blocked by gap junction (GJ) antagonists, suggesting that ICPSDs are a physiological manifestation of GJ-mediated electrical coupling between VTA GABA neurons (Steffensen, Stobbs et al. 2003). Ethanol suppresses VTA GABA neuron ICPSDs, with an IC$_{50}$ at a dose of 1.1 g/kg of ethanol (Stobbs, Ohran et al. 2004), a moderately intoxicating dose. Thus, in addition to ethanol affecting the firing rate of VTA GABA neurons, it appears that ethanol is also affecting the ability of these neurons to couple electrically. Given the previous work done in ours and other’s labs, the main focus of my project was to further characterize these GABA neurons electrophysiologically and molecularly with chronic alcohol administration.

There are six proteins that were analyzed using RT-PCR: Connexin-36 (Cx36), Scn4b, DA D2 receptor, µ-opioid receptor (µOR), NMDAR2B (NR2B), and tyrosine
hydroxylase (TH). I will briefly go over the rationalization for studying each of these proteins by showing their correlation with alcohol. The first is Cx36, a GJ protein that we have previously found to be expressed by GABA neurons in the VTA (Allison, Ohran et al. 2006). Interestingly, just as ethanol blocks GABA neuron ICPSDs, quinidine, a non-selective GJ antagonist, and mefloquine, a selective Cx36 junction antagonist, also block ICPSDs. We don’t necessarily believe ethanol is acting through the same mechanism, by blocking the GJs, but knowing whether this protein is being up or down regulated is of significant importance, suggesting changes in the GABA neuron electrical network.

Scn4b is a recently discovered β-subunit of the voltage-gated sodium channel that appears to play a role in the functional aspect of channel activation (Yu, Westenbroek et al. 2003). The gene for this protein has also been found to be correlated with strong alcohol preference in mice (Mulligan, Ponomarev et al. 2006). While this role seems to deal more with alcoholism rather than chronic alcohol abuse, we wanted to see if this β-subunit is expressed in VTA GABA and/or DA neurons and if it changes over time with ethanol exposure.

Dopamine, as we have seen thus far, plays an important role in the mesocorticolimbic system. Acute, local administration of DA activates GABA neurons, increasing their firing rate 100-200% (Stobbs, Ohran et al. 2004). This activation has been shown in our lab recently to be occurring through the D2 receptor, given that antagonists block this activation. As acute ethanol decreases the firing rate of VTA GABA neurons, but chronic ethanol increases their firing rate, it would seemed logical to look at expression levels of this protein to see if it is also affected with chronic ethanol.
Opioids have long been associated with reward in the brain. Thus, it is not surprising that receptors for µ-opioid receptors (µORs) are located within the mesocorticolimbic system. The subpopulation of VTA GABA neurons that we study expresses µORs (Steffensen, Stobbs et al. 2006). Work has been done showing that an acute dose of ethanol in vivo causes a reduction of µOR binding in the nucleus accumbens and VTA 1 hour after administration with an increase in binding in the frontal/prefrontal cortices 2 hours after administration (Mendez, Leriche et al. 2001). Many current investigations in alcohol research focus on determining associations between endogenous opioid systems and alcohol. In fact, one of the newest therapies for alcohol craving is naltrexone, an opioid antagonist. Thus the goal was to see what changes might occur in µOR expression with chronic ethanol.

Tyrosine hydroxylase is an enzyme involved in the formation of dihydroxyphenylalanine, a precursor to DA. Twelve week exposure to liquid diet with alcohol increased tyrosine hydroxylase in the VTA (Ortiz, Fitzgerald et al. 1995). Our reasoning for looking at this protein was to compare expression levels after a shorter exposure to ethanol compared to Ortiz et al using western blot analysis and to have a key molecular marker for distinguishing neuronal type in the aspirated cells when performing RT-PCR.

The final protein analyzed by RT-PCR was NMDAR2B. The NMDA receptor is made up of NR1 and NR2 subunits. There are four types of NR2 subunits (A-D) and it is the NR2B subunit that has been implicated the most with alcohol. While one study showed that after 10-12 days of ethanol exposure, no NR1 and NR2B mRNA or protein level changes were seen in the amygdala (Lack, Floyd et al. 2005), it has also been shown
that NR1, NR2A and NR2B showed elevated levels in hippocampal explants after chronic exposure to 75 mM ethanol (Hendricson, Maldve et al. 2007). It would be expected to see an up-regulation of NMDA receptors in general given that ethanol inhibits NMDA receptors (Harris 1999). In addition it has been shown that developmental decreases in ethanol inhibition of NMDA receptor function in rat cortical neurons parallel developmental decreases in inhibition by ifenprodil, in which the NR2B subunit is essential for its action (Lovinger 1995). Since we also believe NMDA receptors are involved with ethanol suppression of GABA neuron ICPSDs (Stobbs, Ohran et al. 2004), additional characterization was needed to further determine their role in acute and chronic ethanol models.

In addition to analyzing these six proteins through RT-PCR, we analyzed some of these proteins through western blotting. We were able to obtain reliable antibodies for TH, NMDAR2B, Cx36 and GAD-6. GAD, glutamic acid decarboxylase, is an enzyme that catalyzes the decarboxylation of glutamate to form GABA, the main neurotransmitter released in GABA. Though we were not able to analyze GAD via RT-PCR, we did have an antibody for this protein and opted to analyze the level of expression in the VTA, NAcc and whole rat brain.

HYPOTHESES

Since we had previously demonstrated that tolerance accrues to ethanol inhibition of VTA GABA neuron firing rat (Gallegos et al., 1999), and that acute ethanol inhibits coupling between VTA GABA neurons (Stobbs et al., 2004), I hypothesized that
tolerance would accrue to ethanol inhibition of electrical coupling between VTA GABA neurons. Since others had shown that critical gene products adapt during chronic ethanol (e.g., TH), I hypothesized that some of the gene products that we had chosen (other than TH) would adapt as well. Specifically, given the well-known rapid adaptability of µORs demonstrated in other systems (Bailey and Connor 2005), the fact that VTA GABA neurons contain µORs and that the firing rate of VTA GABA neurons is markedly inhibited by µOR agonists (Steffensen, Stobbs et al. 2006), I predicted that µOR expression would be up-regulated in chronic ethanol treated rats. Since Cx36 expression has been shown to up-regulate in association with cocaine treatment (Bennett, Arnold et al. 1999), and VTA GABA neurons express Cx36, I hypothesized that Cx36 expression would be up-regulated as well to chronic ethanol. D2 receptors appear to be mostly involved in auto-receptor inhibition of DA neurons. Thus, since D2 receptor activation by DA clearly excites VTA GABA neurons I hypothesized that it might be a critical substrate for adaptation to chronic ethanol.

METHODS

Animal Procedures

Male Sprague-Dawley rats were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Once weighing 140-160 g (34-38 days old), they were housed separately and given ad libitum access to solid food, water and a commercially available liquid diet (Dyets, Bethlehem
PA), known as the Lieber-DeCarli diet (Lieber and DeCarli 1989). The liquid diet was made up fresh every few days in a blender and stored at 4°C. After one day, the solid diet and water was removed and rats were divided into two groups, control and chronic ethanol. The following day, the control group received the standard liquid diet and the chronic ethanol group received an ethanol-containing diet. Due to the natural aversion of rats to ethanol, the rats in the latter group were introduced to the ethanol by increasing the percentage of ethanol in three increments over five days (3-5%). To accommodate for reduced food intake, the chronic ethanol rats were pair-fed with the control group to maintain equal caloric intakes in both groups, with the control group only receiving the amount their pair-fed chronic ethanol rat consumed the previous day. In addition, maltose dextrin was added to the control diet to make the diet iso-caloric with the ethanol diet. Once the chronic ethanol group reached an ethanol level of 5% in their liquid diet, they were maintained at this concentration for 2-3 weeks. Both control and ethanol liquid diets were changed daily at 5:00pm. Prior to in vivo electrophysiology experiments, chronic ethanol rats were withdrawn from ethanol for a period of 3-24 hours, depending on the group.

**Surgical Procedure and Electrophysiology**

For acute electrophysiological recordings of VTA GABA neurons, the rats were anesthetized using isoflourane and placed in a stereotaxic apparatus. Body temperature was maintained at 37.4 ± 0.4°C by a feedback regulated heating pad. With the skull exposed, holes were drilled for placement of stimulating and recording electrodes.
Extracellular potentials were recorded by 3.0 M KCl-filled micropipettes (2-4 MΩ; 1 µm inside diameter). Potentials were amplified with an Axon Instruments Multiclamp 700A amplifier (Union City, CA). Microelectrodes were oriented, via stereotaxic coordinates, into the VTA (from bregma: 5.6 - 6.5 posterior (P), 0.5 - 1.0 lateral (L), 6.5 - 7.5 ventral (V)) with a piezoelectric microdrive (EXFO Burleigh 8200 controller and Inchworm, Victor, NY). Single-unit activity was filtered at 0.3-10 kHz (-3dB) for "filtered" recordings and 0.1 Hz - 10 kHz for "unfiltered" recordings with the Multiclamp 700A amplifier and displayed on Tektronix (Beaverton, OR) digital oscilloscopes. Potentials were sampled at 20 kHz (12 bit resolution) with National Instruments data acquisition boards in Macintosh computers (Apple Computer, Cupertino, CA). Extracellularly-recorded action potentials were discriminated with a World Precision Instruments WP-121 Spike Discrimator (Sarasota, Fl) and converted to computer-level pulses. Single-unit potentials, discriminated spikes and stimulation events were captured by National Instruments NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) in Macintosh computers.

VTA GABA neurons were identified by previously-established stereotaxic coordinates and by electrophysiological criteria (Steffensen, Svingos et al. 1998). They included: relatively fast firing rate (>10 Hz); ON-OFF phasic non-bursting activity; spike duration less than 250 µsec; and multiple post-stimulus spike discharges (PSDs) produced by stimulation of the internal capsule (-1.0-1.3 P, 2.3-3.0 L, 5.0-6.0 V). We evaluated only those spikes that had greater than 4:1 signal-to-noise ratio. Corticotegmental activation of VTA GABA neurons was accomplished by stimulation of the internal capsule (IC) with insulated, bipolar stainless steel electrodes. This activation
of VTA GABA neuron spikes is referred to as IC stimulus-induced PSDs (ICPSDs). Square-wave constant current stimulus pulses (50-2000 µA; 0.15 ms duration; average frequency, 0.1 Hz) were generated by an AMPI IsoFlex isolation unit controlled by an AMPI MASTER-8 Pulse Generator (Jerusalem, Israel) or by computer. Stimulation was performed at an intensity that produced 50% maximum VTA GABA neuron ICPSDs with 10 pulses at 200 Hz (Steffensen, Svingos et al. 1998; Steffensen, Stobbs et al. 2003). Systemic administration of 1.0 g/kg ethanol (16% ethanol in saline) was accomplished by intraperitoneal injections.

**Analysis of Responses**

Discriminated spikes and stimulation events were processed with National Instruments LabVIEW and IGOR Pro software (Wavemetrics, Lake Oswego, OR). Peri-stimulus spike histograms (PSHs) were constructed for determinations of the number of VTA GABA neuron ICPSDs. The histograms were normalized to number of IC stimulations before and after drug treatment (12 stimulation trains at 10 sec intervals, 1 sec epoch, 2 ms bin width). The number of driven spikes following IC stimulation was determined by rectangular integration using IGOR Pro software. As the number of discharges varies across neurons within each animal and across animals, we integrated spikes on PSHs falling in bins immediately after the stimulation epoch and extending to a point on the PSH where the discharges appeared to be just above the floor of spontaneous activity (range between 250-600 ms beyond the stimulus artifact). To further reduce variability across treatment groups, we standardized ICPSDs to percent control. The
results for control and chronic ethanol groups were derived from calculations performed on PSHs and expressed as means ± S.E.M. The results were then compared between ethanol diet and pair-fed control groups using the paired two-sample for means t-test.

**Tissue Sectioning**

After the *in vivo* electrophysiology was performed, the rats were anesthetized and decapitated. The brain was quickly removed and placed in ice-cold slushy artificial cerebral spinal fluid (ACSF) (124 mM NaCl, 2 mM KCl, 1.25 mM NaH2PO4-H2O, 26 mM NaHCO3, 11.5 mM glucose, 1 mM MgSO4-7H2O, 2 mM CaCl2-H2O). Horizontal sections were cut in ice-cold ACSF using a sapphire blade on a vibratome (Series 1000, Ted Pella Inc, Redding CA, USA), then immediately placed into 36 °C ACSF and bubbled with 95% O2 / 5% CO2 for at least 30 minutes to allow for cell recovery. Brain slices were then placed in a recording tissue chamber (Warner RC27) and held in place by a slice anchor composed of a stainless steel bridge with Lycra™ cross fibers. The recording chamber was mounted on a heated chamber platform (Warner PH-6) with constant flow (1.5 ml/min) of ACSF perfusing the slices throughout the recording period. ACSF was maintained at 33-35 °C by an in-line solution heater (Warner SH-27B) with both the platform and in-line solution heaters being regulated by a dual temperature controller (Warner TC-344B). The location of the VTA in the slice was visually identified using infrared differential interference contrast (IR-DIC) microscopy at low power (4X objective) by triangulation from anatomical landmarks including the mammillothalamic tract, oculomoter nerve, and the substantia nigra pars compacta.
Recording/aspiration electrodes were made from filaments of glass (Sutter Instrument, Novato CA) using a P-97 micropipette puller (Sutter Instruments) to a tip diameter of approximately 3 microns. Intra-pipette solution consisted of (in mM): 5 NaCl, 147 KCl, 20 HEPES, 1.2 MgCl₂, 11 glucose and 5 Mg-ATP. Electrodes were positioned near the cells via triple-axes piezoelectric microdrives (SD Instruments MX7630L&R). Neurons were then characterized with whole-cell recordings in current-clamp mode via hyperpolarizing and depolarizing currents, using an Axon Instruments Multiclamp 700A amplifier and pClamp software suite.

In addition to the sections used for patch-clamp and aspiration of cells, a 0.7 mm horizontal section of tissue was taken and frozen immediately on dry ice in a Petri dish. Tissue samples of both the nucleus accumbens and VTA were taken using a 1.0 mm tissue punch (EMS, Hatfield PA); whole parts of the brain were kept as well for control purposes. All tissue was stored at -80°C until further use.

**Blood Alcohol Level Analysis**

Immediately following decapitation of the rats, blood samples were taken and frozen in microcentrifuge tubes at -80°C until further analyses. Samples were then thawed and centrifuged at 4°C for 40 minutes at a speed of 2000 g. The serum was removed and analyzed immediately for alcohol content. Blood alcohol levels were determined using an ethanol assay (Diagnostic Chemicals, Oxford CT). Absorbance was read with a spectrophotometer at a wavelength of 340. Alcohol concentration was
determined based on a standard curve analyzed at the same time as the samples. Concentrations were then arranged on a scatter plot to see the overall range of BALs.

**Single-cell RT-PCR**

Neurons from control and ethanol treated rats were aspirated under visual observation by application of suction using a 10 cc syringe attached to the recording pipette, and were then added to a reverse transcription (RT) reaction mixture. The iScript cDNA synthesis kit (Biorad) was used for a total volume of 10 µl per reaction. Reactions were run at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min in a PTC-200 thermal cycler (MJ Research Inc., Watertown MA). Reactions were then stored at -20°C until running the PCR. A preamplification round of multiplex PCR was performed by adding iTAQ Supermix with ROX (Biorad) and a cocktail of primers to the completed RT reaction, for a final volume of 50 µL. The reactions were held at 94°C for 30 seconds then cycled 20 times. Each cycle consisted of: 92°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. One µl samples of the initial multiplex PCR were then used as substrate for each reaction in the subsequent real-time quantitative PCR. Real-Time quantitative PCR using gene specific primers with FAM-TAMRA TaqMan® probes (Applied Biosystems) for Connexin-36, Scn4b, DA D2 receptor, NMDAR2B, µ-opioid receptor, and tyrosine hydroxylase cDNA and 18s rRNA were performed using the iTaq Supermix with ROX (Bio-Rad) with an iCycler IQ (Bio-Rad) real-time PCR System. Samples were amplified in triplicate, together with a negative control for each subunit (an ACSF-only aspiration taken from the brain slice recording chamber when the cells were
aspirated). The amplification protocol was 50°C for 2 minutes, 95°C for 5 minutes, then 50 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Cycle threshold (Ct) values were calculated automatically by the iCycler IQ software, with threshold values set between 5 and 20. Relative fold expression was calculated using the 2^ΔΔCT method as described in (Livak and Schmittgen 2001). The results were then compared between groups using the unpaired two-sample for means t-test.

**SDS-PAGE and Western Blot**

Brain tissue from punches previously taken was homogenized using a disposable BioMasher® in a lysis buffer containing 150mM sodium chloride, 1.0% TX-100, 50 mM Tris, pH 8.0 and protease inhibitor cocktail (BD Biosciences, San Jose CA). Total protein concentration was measured using the DC Protein Assay Kit (Biorad, Hercules CA). Brain homogenate was diluted to equal concentrations, added to a reducing sample buffer and loaded onto a 4-20% Tris-Glycine gel (Pierce, Rockford IL). Gels were run for 45 minutes at 130 V in a running buffer (100 mM Tris, 100 mM HEPES and 3 mM SDS) and then transferred for 90 minutes onto polyvinylidene difluoride membranes in a transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Following transfer, the membranes were blocked in a blocking solution (5% non-fat dairy milk, 25 mM Tris HCl, 150 mM NaCl and 0.05% Tween-20) and probed with primary antibodies for two hours at room temperature. The primary antibody dilutions consisted of: GAD-6, mAb tissue culture supernatant, neat, (DSHB, University of Iowa; a gift from Dr. Matthew Rasband), anti-NMDAR2B 1:333 (Zymed, San Francisco CA), anti-tyrosine hydroxylase
1:500 (Zymed, San Francisco CA), anti- µOR 1:2000 (Chemicon, Temecula CA) and anti-DA D2 receptor (Chemicon, Temecula CA and Millipore, Billerica MA). Immunoblots were then washed 3 times for 5 minutes each with a tris buffered saline (25 mM Tris HCl and 150 mM NaCl, pH 7.2) and probed with goat anti-mouse/rabbit secondary antibody for one hour (Pierce, Rockford IL). Following this incubation, membranes were again washed and soaked in SuperSignal West Pico/Femto substrate (Pierce, Rockford IL). Visualization took place using FluorChem 8900 (Alpha Innotech) and was subsequently analyzed for band intensity with FluorChem software. Values for each band were based on pixel intensity and designated as an “integrated density value” (IDV). Background was automatically subtracted. The IDVs for the ethanol diet rats were then calculated as a percentage of the pair-fed control rats IDVs.

**Immunohistochemistry**

Adult male rats were sacrificed with an overdose of isoflourane and perfused with 4% formaldehyde. Brains were immediately dissected out and placed at 4°C in 4% formaldehyde for 1-2 hours, then transferred to 20% sucrose and kept at 4°C until equilibrated. A cryostat was used to make longitudinal sections 40 µm in thickness. Sections were immediately placed on gel-coated cover slips and stored at 4°C. To permeabilize the tissue, a solution of 0.1 M PB, pH 7.4, containing 0.3% Triton X-100 and 10% goat serum (PBTGS) was placed on the sections for 1 hour at room temperature. Commercially obtained antibodies were diluted in PBTGS and kept on tissue sections 2 hours-overnight at room temperature. Antibody dilutions were as follows: Gad-6, neat;
anti-tyrosine hydroxylase 1:1000 (Affinity BioReagents, Golden CO); and anti-DA D2 Receptor 1:100 (Abcam Inc., Cambridge MA). Sections were washed 3 times for 5 minutes each with PBTGS and then covered with the secondary antibody for 1 hour. Secondary antibody dilutions were as follows: Alexa Flour 546 goat anti-mouse IgG (H+L) 1:500 and Alexa Flour 488 goat anti-rabbit IgG (H+L) 1:500 (Molecular Probes, Carlsbad CA). Sections were again washed with PBTGS, 0.1 M PB and 0.05 M PB for 5 minutes each, dried, and mounted onto slides using Fluoromount-G (Southern Biotech, Birmingham AL). Slides were visualized using an Olympus FluoView FV 300 confocal laser scanning microscope and pictures were taken at a magnification of 200x.

RESULTS

*Chronic ethanol consumption: Effects on weight*

The liquid ethanol diet procedure is a new approach in the Steffensen lab. As we did not have any experience with this form of chronic ethanol treatment it was important to replicate previous studies using the diet and assure that it was a feasible treatment strategy that did not adversely affect the overall health of the rats. We found that ethanol-treated rats had body weights that did not differ significantly from their pair-fed controls ($P=0.85$, $F_{(1,19)}=0.036$), suggesting that the chronic ethanol treatment did not adversely affect the dietary intake of calories (Fig. 1). This is consistent with previous studies using the Lieber-DeCarli diet and the average daily growth rate for both groups
was approximately 5 grams per day. The average consumption of ethanol during the 3 week period for the ethanol diet rats was 10.1 g/kg/day.

Figure 1. Chronic ethanol consumption rates: Effects on weight. Ethanol-treated rats were fed a 5% v/v solution of ethanol in a liquid diet. They consumed approximately 10.1 g/kg of ethanol/day (n=12). Pair-fed control and alcohol-fed rats maintained similar weight gains throughout the 3 week feeding period. Both groups had normal growth rates of approximately 5 grams/day while on the liquid diet. There was no significant difference in weight gain between chronic ethanol-treated rats and their pair-fed controls.
Effects of chronic ethanol treatment on ethanol inhibition of VTA GABA neuron ICPSDs

In our first experimental run with the chronic ethanol diet we recorded VTA GABA neurons in vivo from 4 ethanol-treated rats and their pair-fed controls. Since this was our first run through the procedure we decided to record two rats 2-3 hours into ethanol withdrawal and 2 rats 6-8 hours into ethanol withdrawal. In the second group of rats, all 4 ethanol-treated rats were recorded at 6-8 hours into ethanol withdrawal. To make sure that all of the alcohol was out of their system in the third group, we recorded the ethanol-treated rats at 24 hours into ethanol withdrawal.

Brief, high-frequency stimulation of the internal capsule (IC) evoked VTA GABA neurons post-stimulus spike discharges (ICPSDs) that persisted for 300-600 msec after termination of the stimulus train (Figure 2A,B). The mean number of VTA GABA neuron ICPSDs at 50% maximum stimulus level (200 Hz and 10 pulses) in ethanol-treated rats was 150.8 ± 24.6, which was not significantly different from pair-fed controls (P=0.26, t(1,8)= 1.20; mean pair-fed control = 103.3 ± 28.5; n=9). We then administered 1.0 g/kg ethanol, an acute intoxicating dose of ethanol which typically reduces ICPSDs approximately 50% (Stobbs, Ohran et al. 2004), and recorded VTA GABA neuron ICPSDs at 5, 10, 15, and 30 min intervals (Figure 2B,C). Although in some rats there appeared to be tolerance to an acute dose of ethanol (Figure 2B,D), as previously reported for firing rate (Gallegos, Criado et al. 1999), there was no significant difference between ethanol-treated rats and pair-fed controls for acute ethanol inhibition of VTA GABA neuron ICPSDs (P=0.44, t(1,8)=0.81; mean pair-fed % baseline= 59 ± 5 % and
mean ethanol diet \% baseline = 66 \pm 8\% at the 15 min time point; n=9). Please note that ICPSD data from ethanol diet rats withdrawn for only 3 hours and their pair-fed controls was not included in this statistical analysis.
Figure 2. No tolerance to ethanol inhibition of VTA GABA neuron ICPSDs. (A,B) The top inset shows a representative recording of a VTA GABA neuron spike in a pair-fed control rat and an ethanol diet rat respectively during high frequency stimulation of the IC (200 Hz, 10 pulses). Note that spiking extends beyond the stimulus train for hundreds of msec. The peri-stimulus interval spike histogram (PSH) shows the cumulated spiking associated with 12 high frequency IC stimulation epochs demonstrating IC-evoked post-stimulus spike discharges (ICPSDs). (C,D) The top inset shows the same neurons following IP administration of 1.0 g/kg ethanol, a moderately intoxicating dose. (C) This PSH shows the effects of ethanol on cumulated ICPSDs in the pair-fed control rat. Ethanol reduced ICPSDs about 50% at this level of ethanol as previously reported (Stobbs, Ohran et al. 2004) (D) An acute challenge of ethanol did not appear to affect ICPSDs as shown in the top inset and PSH of this ethanol-diet rat. (E) This graph summarizes the effects of acute ethanol (1.0 g/kg) on VTA GABA neuron ICPSDs in chronic ethanol treated rats vs pair-fed controls. Although some chronic ethanol treated rats exhibited tolerance to ethanol (B,D) there was no significant difference in acute ethanol inhibition of ICPSDs between ethanol treated and pair-fed controls (n=9).

To ensure both the pair-fed and ethanol diet rats received adequate IP injections of ethanol during the *in vivo* recording, we measured the blood alcohol levels between 30 to 70 minutes post-injection and found that the mean blood alcohol level was 48.61 ± 1.31 mg% (Figure 3). All rats showed a blood alcohol level within range of this mean, suggesting that the IP injection was suitable.
Figure 3. Blood Alcohol Level Distribution. Blood samples were taken from rats after in vivo experiments were performed. Blood alcohol levels were calculated for each rat to verify IP injection reliability. As seen here, all rats received a substantial amount of ethanol, ensuring that injections were credible. Not all blood samples were taken at the same time post-injection, likely accounting for the range of BALs seen here.

Electrophysiological characterization of neurons in the VTA

VTA GABA neurons were distinguished from DA neurons by specific electrophysiological criteria, in agreement with previous studies from other labs (Grace 1988; Grace and Onn 1989; Mercuri, Calabresi et al. 1990; Johnson and North 1992). While this analysis was not performed on all aspirated cells, it did give another input in addition to PCR analysis to characterize these neurons (Figure 4).
Figure 4. Electrophysiological characterization of VTA GABA neurons in the midbrain slice preparation. (A,B) Under current-clamp recording conditions, VTA GABA neurons had higher input resistance measured with hyperpolarizing currents, a more negative resting membrane potential shorter spikes (measured at half-maximum peak amplitude) and a relative lack of spike accommodation (C,D) compared to DA neurons. (E,F) Under voltage-clamp recording conditions, VTA GABA neurons were characterized by a lack of hyperpolarization-driven mixed cationic inward current ($I_h$) compared to DA neurons.
Expression of select genes in dopamine versus GABA neurons: Quantitative single-cell RT-PCR

In order to determine if differences in molecular signature exist between GABA and DA neurons in the VTA, we evaluated the expression of select gene products in GABA neurons and compared them to DA neurons. We evaluated the gene expression of 50 VTA GABA neurons, aspirated from the pair-fed control rats, and 25 VTA DA neurons, aspirated from rats on a regular solid diet. Although the rats were on different diets (solid versus liquid), it is unlikely that this variation would evoke the extremely significant differences we saw in certain genes. Figure 5 summarizes the differences between VTA DA and GABA neurons for the expression of TH, D2, Cx36, NMDAR2B, and µOR mRNA transcripts. Tyrosine hydroxylase expression in DA neurons was significantly greater than GABA neurons (P=0.0002, t(1,23)=4.5; mean DA neuron TH expression = 6.12 ± 0.77 and mean GABA neuron TH expression = 0.07 ± 0.01; n=33). The cycle threshold for 18s, the housekeeping gene that was used for the quantification of the relative expression of each of the gene products, was not significantly different between DA and GABA neurons (P=0.35, t(1,31)=0.95; mean DA cell 18s CT = 12.53 ± 0.18 and mean GABA cell 18s CT = 12.87 ± 0.34). The expression of D2 was significantly greater in DA neurons than GABA neurons (P=0.019; mean DA cell D2 expression = 3.39 ± 0.59 and mean GABA cell D2 expression = 0.26 ± 0.03; n=29). The cycle threshold for 18s was not significantly different between groups (P=0.82, t(1,27)=0.23; mean DA cell 18s CT = 12.53 ± 0.18 and mean GABA cell 18s CT = 12.63 ± 0.35). Connexin-36 expression in DA neurons was not significantly different than
GABA neurons (P=0.24, t_{(1,15)}=1.2; mean DA cell Cx36 expression = 1.68 ± 0.22 and mean GABA cell Cx36 expression = 1.02 ± 0.19; n=17). The cycle threshold for 18s was not significantly different between groups (P=0.88, t_{(1,15)}=0.16; mean DA cell 18s CT = 12.71 ± 0.25 and mean GABA cell 18s CT = 12.64 ± 0.39). The expression of NMDAR2B was significantly greater in DA than GABA neurons (P=0.050, t_{(1,10)}=2.2; mean DA cell NMDAR2B expression = 5.19 ± 1.13 and mean GABA cell NMDAR2B expression = 0.65 ± 0.22; n=17). The cycle threshold for 18s was not significantly different between groups (P=0.77, t_{(1,15)}=0.29; mean DA cell 18s CT = 12.81 ± 0.28 and mean GABA cell 18s CT = 12.68 ± 0.37). Expression of μOR in DA neurons was not significantly different than GABA neurons (P=0.59, t_{(1,4)}=0.59; mean DA cell μOR expression = 1.18 ± 0.33 and mean GABA cell μOR expression = 1.71 ± 0.38; n=6). The cycle threshold for 18s was not significantly different (P=0.14, t_{(1,4)}=1.85; mean DA cell 18s CT = 11.92 ± 0.31 and mean GABA cell CT = 13.65 ± 0.88).
**Figure 5. Expression of select genes in dopamine versus GABA neurons.**

Single-cell quantitative RT-PCR was performed on neurons in the VTA of adult male rats. Both dopamine and GABA neurons expressed TH, D2, Cx36, NR2B and µOR transcripts. However, the expression of TH, D2 and NR2B was much higher in DA neurons than GABA neurons. This differential expression of some gene products may provide a way to distinguish DA and GABA neurons.

**Immunohistological characterization of the VTA**

In order to better understand the expression levels of the gene products analyzed by PCR, immunoflorescent labeling of midbrain sections was performed using antibodies against Gad-6, an enzyme precursor to GABA, TH, an enzyme precursor to DA, and D2, a receptor located on VTA GABA neurons. As seen in Figure 6, TH clearly labels what we assume would be DA neurons. Gad-6, while its labeling is quite diffuse labeling both cell bodies and terminals, appears to have a complete lack of staining for many of these cells. There are some cells though, indicated by white arrows, where both Gad6 and TH are seen co-localized. This co-localization provides an explanation for the presence of TH mRNA in GABA neurons, as revealed through RT-PCR. Co-labeling is also seen with D2 and Gad-6 as indicated by white arrows.
Figure 6. Immunohistological characterization of the VTA. Tissue sections in the area of the VTA taken from adult male rat brains were stained for the following antibodies: Gad-6, TH and D2. (A) Gad-6 shows diffuse terminal labeling as well as cellular labeling. TH clearly labels cell bodies in the VTA (B) and can be seen co-labeled with Gad-6 in select neurons in (C), indicated by arrows. Gad-6 also shows co-localization with Dopamine D2 receptors (D, E, F) as indicated by arrows.

Effects of chronic ethanol treatment on expression of select genes in VTA GABA neurons: Quantitative single-cell RT-PCR

In order to determine if molecular adaptation occurs with chronic ethanol, we evaluated the expression of select gene products in VTA GABA neurons in chronic ethanol-treated rats and compared them to pair-fed controls. We evaluated the gene expression in 109 GABA neurons: 59 from the 12 ethanol diet rats and 50 from the 12
pair-fed controls. Figure 7 summarizes the differences between VTA GABA neurons from ethanol-treated rats and pair-fed controls for the expression of TH, D2, Cx36, NMDAR2B, and μOR. TH expression in ethanol treated rats was not significantly different than pair-fed controls (P=0.90; mean pair-fed TH expression = 1.44 ± 0.23 and mean ethanol diet TH expression = 2.39 ± 0.35; n=37). The cycle threshold for 18s, the housekeeping gene that was used for the quantification of the relative expression of each of the gene products, was not significantly different between pair-fed and ethanol diet rats (P=0.41, t_(1,35)=0.83; mean pair-fed 18s CT = 12.87 ± 0.34 and mean ethanol diet 18s CT = 12.56 ± 0.18). The expression of D2 in ethanol treated rats was significantly less than pair-fed controls (P=0.0068, t_(1,16)=3.1; mean pair-fed D2 expression = 1.13 ± 0.14 and mean ethanol diet D2 expression = 0.52 ± 0.06, n=18). The cycle threshold for 18s was not significantly different between groups (P=0.08, t_(1,16)=1.85; mean pair-fed 18s CT = 12.63 ± 0.35 and mean ethanol diet 18s CT = 12.02 ± 0.16). Connexin-36 expression in ethanol treated rats was not significantly different than pair-fed controls (P=0.83, t_(1,18)=0.22; mean pair-fed Cx36 expression = 1.45 ± 0.28 and mean ethanol diet Cx36 expression = 1.62 ± 0.29; n=20). The cycle threshold for 18s was not significantly different between groups (P=0.59, t_(1,18)=0.55; mean pair-fed 18s CT = 12.64 ± 0.39 and mean ethanol diet 18s CT = 12.90 ± 0.28). The expression of NMDAR2B in ethanol treated rats was not significantly different than pair-fed controls (P=0.90, t_(1,13)=0.12; mean pair-fed NMDAR2B expression = 2.17 ± 0.73 and mean ethanol diet NMDAR2B expression = 2.45 ± 0.89; n=15). The cycle threshold for 18s was not significantly different between groups (P=0.51, t_(1,13)=0.67; mean pair-fed 18s CT = 12.68 ± 0.37 and mean ethanol diet 18s CT = 13.13 ± 0.48). Expression of the μOR in ethanol treated rats
was not significantly different than pair-fed controls (P=0.88, \( t_{(1,8)} = 0.15 \); mean pair-fed µOR expression = 1.36 ± 0.30 and mean ethanol diet µOR expression = 1.53 ± 0.38; n=10). The cycle threshold for 18s was not significantly different between groups (P=0.19, \( t_{(1,8)} = 1.42 \); mean pair-fed 18s CT = 13.65 ± 0.88 and mean ethanol diet CT = 12.42 ± 0.43). Scn4b was expressed in only 5 neurons aspirated from the alcohol rats and did not show up in any neurons from pair-fed control rats.

![Figure 7](image)

**Figure 7.** Effects of chronic ethanol treatment on expression of select genes in VTA GABA neurons. Single-cell quantitative RT-PCR was performed on GABA neurons aspirated from the VTA of chronic ethanol diet and pair-fed control rats. While TH expression in GABA neurons was increased, it did not reach significance. However, there was a significant decrease in D2 receptor expression in VTA GABA neurons of chronic ethanol-treated rats compared to pair-fed controls. All other gene expressions were comparable for ethanol diet and pair-fed control rats.
Protein Expression in the VTA

Since mRNA expression levels do not always correlate with protein expression levels, we took tissue punches of the VTA, NAcc and whole rat brain, pooled samples for each group and compared levels of certain proteins using western blot analysis. Proteins that were analyzed include TH, NMDAR2B, Gad-6, µOR and D2. While several different D2 antibodies were tested, none produced a strong enough signal for analysis (data not shown). Pixel intensity for each band was determined using Flourchem software and designated as an integrated density value (IDV). Results in Figure 8 were calculated by making the IDV values from the ethanol diet tissue samples a percentage of the pair-fed control IDV value. In the VTA, the ethanol diet tissue expressed as a percent of control: 72.34% for TH, 112.7% for NMDAR2B, 78.94 % for µOR, and 107.8% for Gad-6. In the NAcc, the ethanol diet tissue expressed as a percent of control: 91.03% for TH, 109.6% for NMDAR2B, 111.11 % for µOR, and 96.34% for Gad-6. In the whole brain control, the ethanol diet tissue expressed as a percent of control: 101.51% for TH, 75.53% for NMDAR2B, 82.01 % for µOR, and 89.44% for Gad-6. Tissue samples analyzed were those taken from the contralateral side of the IC stimulation in vivo experiment.
Figure 8. Protein expression in ethanol treated rats as a percent of pair-fed controls. Western blot analysis was performed on tissue taken from ethanol treated rats and pair-fed controls. Specifically, tissue samples were taken from the VTA, NAcc and whole rat brain, combined into corresponding groups and homogenized for analysis. Equal amounts of protein were loaded into each well of the gel, and following SDS-PAGE separation and transfer, PVDF membranes
were probed for TH, NMDAR2B, µOR and GAD-6. Note there are no error bars because the tissue samples were pooled for each brain region.

DISCUSSION

The ethanol liquid diet we chose to use, the Lieber-DeCarli diet, was critical to the success of this project. Since it was the first time for our lab to use this procedure, we were careful to measure consumption and weights of each rat on a regular basis. By doing this, we were able to show that each group of rats consumed a consistent amount that kept them on a normal growth schedule. In addition, the ethanol-diet rats consumed approximately 10.1 g/kg/day of ethanol, an amount shown by other labs to be enough to induce molecular changes in the brain (Lack, Floyd et al. 2005). However, because this amount of ethanol is consumed slowly over a 24 hour period, and likely titrated by the animal to not produce intoxication, it is questionable whether the dose was strong enough to induce dependence and withdrawal that has been seen with twice-daily intraperitoneal injections of ethanol (Gallegos, Criado et al. 1999). This could be due to the fact that the rats are never forced into withdrawal periods since they have continuous access to the ethanol. The signs of withdrawal checked for were ventromedial flexion, tail stiffness, and abnormal gait. While there may have been tail stiffness, none of these signs stood out as a clear indicator of withdrawal. At one point during the last group of rats, we tried to adjust the feeding schedule for a few days to force the ethanol-diet rats to drink more in an eight hour period before the diet was removed for 16 hours, but this only resulted in a low amount of liquid diet consumption. In the future, the lab will have to consider how to increase this amount of ethanol intake to a point that will induce clear signs of withdrawal, indicating alcohol dependence, if it wants to completely match past
experiments. This could include forced ethanol injections, gavage or inhalation. In fact, this study is ongoing at this time. For this project, the Lieber-DeCarli diet was sufficient for the ethanol-treated rats to be considered as chronic, though likely non-dependent.

The first physiological experiment that was performed on rats was to evaluate the effects of acute ethanol on electrical coupling (i.e., ICPSDs) between VTA GABA neurons in chronic ethanol-treated rats. Since we have previously demonstrated that VTA GABA neuron firing rate adapts to an acute challenge of ethanol (Gallegos, Criado et al. 1999), we predicted a similar result with the ICPSDs, which are normally inhibited 50% by a 1.1 g/kg dose of ethanol. While there were a few ethanol rats that seemed to show a smaller degree of inhibition, overall there was no difference between groups in the inhibition of ICPSDs caused by an acute ethanol injection. To make sure that each rat received a viable injection, we took blood samples upon completion of the experiment and measured the blood alcohol level (BAL). Ethanol elimination is first order kinetics and is eliminated at 25 mg%/hr. Because not all the ethanol diet rats were withdrawn for the same amount of time, it is likely that two of the twelve ethanol-diet rats recorded at 3 hours after withdrawal still had ethanol in their system while the other ten rats, recorded 8 and 24 hours after withdrawal should have been cleared of all ethanol. This variability in withdrawal times was a pitfall for this in vivo experiment. It is well known that ethanol withdrawal peaks around 8 hours after withdrawal, when all the alcohol is out of the system. For this reason, we decided not to include the 3 hour withdrawn rats in the ICPSD statistics. In future experiments, we will likely record 24 hours after withdrawal in order to maximize effects and assure the systems are clear of alcohol in every rat in spite of their drinking pattern. Regardless of this pitfall, we still saw variability in the
response to acute ethanol in rats that were clearly withdrawn from ethanol. This could mean that some rats tolerated more quickly to the ethanol, perhaps due to some genetic influence differing between the rats. Much more work would need to be done to look into this possibility; however, we take it as a sign that tolerance to acute ethanol inhibition of electrical coupling might be forthcoming in rats that are clearly dependent on ethanol. Although not an aim of my thesis, these studies are currently ongoing.

The molecular side of this project came with some discrepancies and further characterization of DA and GABA neurons was needed, due to much controversy in the field. Originally it was assumed that DA and GABA neurons would be distinguished by the presence or absence of TH. However, many of the GABA neurons characterized electrophysiologically expressed TH at a low level compared to electrophysiologically characterized DA neurons. In consequence of this finding, we analyzed a large number of DA neurons and compared them to the GABA neurons and found that they differed significantly in the expression of TH, D2 and NMDAR2B. All three of these were expressed in much lower levels in GABA neurons compared to DA neurons, with TH being extremely different. While some would suggest that the finding of TH in GABA neurons could simply be a result of contamination from surrounding cells, despite proper controls taken, there is evidence from other labs that TH is present in GABA neurons (Klink, Exaerde et al. 2001; Olson and Nestler 2007). To further support this evidence, immunofluorescent experiments were performed and included staining of the VTA in adult male rats with both TH and GAD. Evidence of co-labeling was seen (see Figure 6). Thus, it is apparent that both DA neurons and GABA neurons are expressing TH. The GAD antibody we used labels both GAD65 (terminal/fiber) and GAD67
(cellular), explaining why there is such a large amount of positive staining. It could be that the GAD co-labeled with TH is the staining of GAD terminals on a DA neuron, but the GAD labeling on the neuron to the right in A of Figure 6 appears to be more than just terminal staining.

After setting a base standard to distinguish DA from GABA neurons, we were able to more accurately analyze and compare mRNA expression levels between ethanol diet and pair-fed control GABA neurons. Though it has been previously shown by other labs that TH expression increases with chronic ethanol (Ortiz, Fitzgerald et al. 1995), we did not see a significant difference between the chronic and control group. It did appear to be higher in the chronic group but statistically, did not reach an acceptable P-value. This lack of statistical significance could be due to various neurons that did not quite fit in with the rest and could be remedied by running more cells (despite the large number we already ran). We did however find that DA D2 receptor mRNA expression was significantly lower in ethanol diet rats compared to their pair-fed controls. Although it is well-known that DA neurons are inhibited by D2 auto-receptor activation by DA, we have recently found that VTA GABA neurons are excited by DA via D2 receptors (paper in submission). We have postulated that DA neurons regulate GABA neuron excitability via DA release and D2 activation, although there is no anatomical evidence for this synaptic arrangement in the VTA. Regardless, D2 receptor activation is an important regulator of VTA GABA neuron excitability, as tonic firing rate of VTA GABA neurons is nearly abolished by D2 antagonists. Thus, modulation of D2 receptors on VTA GABA neurons by chronic ethanol is an important finding. This finding corresponds well with findings from other labs showing up-regulation in TH, the precursor enzyme to DA.
While a change in Cx36 expression was hypothesized, it remained at similar levels in both groups, as did NMDAR2B and μOR. Expression of μOR was more rare than any of these other genes and thus our sample size was small. However, this low mRNA expression rate did correlate with the low protein expression in the VTA as seen in Figure 8. One other gene that was analyzed in some of the cells was Scn4b. Interestingly, it was only expressed in cells and bath controls from ethanol-diet rats. While only a third of the total number of cells was analyzed for Scn4b, it is worth noting that not a single cell or bath control from the pair-fed rats expressed Scn4b. Indeed this may be worth further investigation in the future, but for our present purpose we chose to dedicate the remainder of the cells for analyzing μOR expression in placement of Scn4b.

In order to compare and correlate mRNA expression with actual protein expression in the VTA, we analyzed tissue samples taken from the VTA, NAcc and whole brain. Since the tissue samples were so small, we combined individual samples for all ethanol-diet and pair-fed control rats into a pools for analysis. Protein levels were determined and loaded equally onto a gel for both groups. Though we found differences between the various parts of the brain, we did not see any significant differences between ethanol diet and pair-fed control samples in any part of the brain. Had we been able to analyze each tissue punch separately and obtain a large sample size, we may have been able to more accurately distinguish differences in band intensity. However, the band intensity fluctuates too much in the controls for there to be any conclusions drawn from the data. In the future, a different approach would need to be taken in order to retrieve and maintain an adequate amount of tissue sample from a single punch so that multiple western blots could be run for each group. We hoped to be able to correlate the D2
down-regulation seen using RT-PCR with down-regulation in actual protein seen by western blot, but were not able to find a sufficient antibody to detect D2 in our tissue samples.

While there are many unanswered questions left behind by this project, there are several findings that are significant. The first was the configuring of the liquid diet. We confirmed that the rats maintain healthy consistent eating habits even on a 5% ethanol liquid diet and also determined that the rats need to be withdrawn from the ethanol for 24 hours before doing in vivo electrophysiology experiments. In addition, because there were signs of tolerance to ethanol inhibition of ICPSDs in some rats, future studies similar to this one would be valuable to further evaluate electrical coupling in ethanol-dependent rats. These studies are now being run in the Steffensen lab. Another important part of this project was determining molecular differences between VTA GABA and DA neurons. We only looked at a few of a long list of gene products and in the future, it would be valuable to continue this study to resolve controversy in distinguishing GABA and DA neurons in the VTA. Most significantly, we found that chronic ethanol for the 3 week time period induced a down-regulation of DA D2 receptor mRNA. This down-regulation could be part of a chain of events that leads to the cravings that come during withdrawal and future studies should be aimed at analyzing in depth the role of D2 receptors on VTA GABA neurons. Finally, we learned from the protein analysis that there was no substantial change in each area of the brain for each protein examined and that more samples need to be analyzed to obtain statistical relevancy. In conclusion, I have found that D2 receptor expression in VTA GABA neurons down-regulates in association with chronic ethanol diet. However, there were no
statistically-relevant changes in VTA GABA neuron physiology in non-dependent rats. It may be that dependence to ethanol is necessary for physiological manifestations of tolerance in VTA GABA neurons, as well as full adaptation of gene products other than D2. These findings will serve as a foundation for future studies in ethanol-dependent rats, which is currently an ongoing project in the Steffensen lab.
REFERENCES


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**REFERENCES**
*Available upon request*