Thiamine Deficiency and Alcohol Exposure Both Lead To An Impulsive Phenotype

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THIAMINE DEFICIENCY AND ALCOHOL EXPOSURE BOTH LEAD TO AN IMPULSIVE PHENOTYPE

BY

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Abstract

Chronic alcohol consumption is a prevalent phenomenon linked to many behavioral constructs, including impulsivity and cognitive inflexibility, as well as neuropathology in brain regions central to these tasks such as the orbitofrontal and limbic cortices. The detrimental effects of alcohol consumption are due to a confluence of factors that often occur in addition to alcoholism, importantly thiamine deficiency. This nutritional deficiency can manifest in the amnestic disorder Wernicke-Korsakoff Syndrome, most commonly seen in cases of alcoholism. In this study, we examined the effects of chronic alcohol exposure and thiamine deficiency in isolation, as well as in concert, to further our understanding of the independent effects of each treatment and how they interact. Furthermore, we included a group receiving chronic alcohol with thiamine supplementation to deduce whether the effects of long term alcohol exposure can be ameliorated with prophylactic treatment. Our results demonstrated that both chronic alcohol exposure and moderate thiamine deficiency induce an impulsive phenotype. Importantly, the increased impulsivity induced by thiamine deficiency was protracted as compared to that exhibited under conditions of chronic alcohol alone. Additionally, there may be decreased cellular activity in the posterior orbitofrontal cortex in chronically alcohol treated animals, as well as increased limbic cortical volume. However, differential pathology was observed in the chronic ethanol treated group presenting as increased limbic volume. This study has furthered our understanding of
the role of multiple factors in alcohol induced decision making deficits while illustrating
the need to look at the interaction of chronic alcohol exposure induced mild thiamine
deficiency. It also provides justification to investigate potential prophylactic treatments for
alcoholics or those in treatment to prevent further neurological insult.
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Introduction

Alcohol consumption is ubiquitous across cultures. The lifetime prevalence of alcohol use disorders in males is 8.4%, and 4.2% of females according to the Substance Abuse and Mental Health Services Administration; additionally, 70.1% of the population reported using alcohol within the past year (SAMHSA, 2015). It has been established that chronic alcohol consumption can lead to brain damage. The detrimental effects of chronic alcohol exposure are due to a confluence of factors; not only does alcohol exposure alone induce cellular death and damage to neural tissue (Goodlett and Horn, 2001), but there are also common comorbid factors that cloud our ability to ascertain the effects of alcohol consumption in isolation. Chronic compulsive alcohol consumption without a comorbid condition must be studied to increase our understanding of alcohol's damaging effects because alcoholism is often 'complicated' by the existence of other conditions such as liver disease, polydrug use, history of head trauma, or nutritional deficiency (Pitel, Zahr, Jackson, Sassoon, Rosenbloom, Pfefferbaum, and Sullivan, 2011; Sullivan and Pfefferbaum, 2005). It is important to understand the effects of chronic alcohol exposure in addition to each of these factors in isolation as well as the consequences of these common comorbidities in and of themselves.

Chronic alcohol consumption alone has also been demonstrated in human studies and animal models to elicit neuropathology due to a confluence of molecular factors. Importantly, the most vulnerable regions include the orbitofrontal and cingulate cortices, with volume loss again being an expression of pathology (for review see Sullivan and Pfefferbaum, 2005; Volkow and Fowler, 2000; Ridderinkhof, Vlut, Bramlage, Spaan, Elton, Snel, and Band, 2002). Alcohol exposure can lead to apoptosis or necrosis (cell death), interfere with cellular function and alter cellular division rates,
depending on the type of cell and its developmental stage (for review see Goodlett and Horn, 2001; Bredensen, 1996 a, b). As will be discussed below, thiamine deficiency may also play a role in these processes.

As these studies have demonstrated, chronic alcohol consumption can be highly detrimental to the integrity of the brain. Importantly, comorbid conditions can exacerbate these effects, particularly nutritional deficiency. In the following section the effects of thiamine deficiency, which can be induced by high levels of alcohol consumption, will be explored.

**Thiamine Deficiency in Alcoholism**

The confluence of nutritional deficiency in alcoholism is of particular interest, specifically in the case of thiamine deficiency, as this can result in Wernicke-Korsakoff Syndrome (WKS). WKS is a retrograde and anterograde diencephalic amnesic disorder most often seen in alcoholics, but also found in those with bulimia, gastrointestinal distress, starvation, long term IV usage, dialysis and AIDS (for review Mair, 1994; Kopelman, Thomson, Guerrini, and Marshall, 2009). Chronic alcoholics are prone to nutritional deficiency because they derive up to 41% of their calories from alcohol (Gruchow, Sobocinski, Barboriak, and Scheller, 1985). To further exacerbate this situation, chronic alcohol consumption also decreases absorption or utilization of nutrients (Galvin, Brathens, Ivashynka, Hillbom, Tanasescu, Leone, 2010) which may result in, or compound, the effects of nutritional deficiency.

It is estimated that up to 80% of alcoholics are thiamine (vitamin B1) deficient due to poor nutrition (Galvin et al., 2010), however, approximately 75% of cases of thiamine deficiency are undiagnosed in alcoholics (for review see Zahr, Kaufman, and Harper, 2011) and around 13% of alcoholics have thiamine deficiency induced neuropathology.
(for review see Harper, Giles, and Finlay-Jones, 1986). There is also graded damage between these groups; milder brain and behavioral dysfunction is seen in uncomplicated alcoholics relative to complicated alcoholics with thiamine deficiency. (Pitel, et al., 2011; Sullivan, and Pfefferbaum, 2005). Therefore, it is clear why WKS is seen in cases of alcoholism.

Thiamine deficiency can induce excitotoxic lesions to the thalamus (anterior and midline nuclei) and mammillary bodies which are a hallmark feature of the neuropathology seen in WKS (de Wardener and Lennox, 1947). Thalamic lesions are necessary to induce the amnestic disorder, however hippocampal damage is sometimes observed as well (for review see Kopelman, Thomson, Guerrini, and Marshall, 2009; Sullivan and Pfefferbaum, 2005). Additionally, frontal lobe atrophy, and white matter pathology are often detected. Particularly relevant to this study is the fact that the most vulnerable frontal regions include the orbitofrontal and cingulate cortices, which often present with decreased overall volume, reduced neuronal density, and decreased metabolism and blood flow (for review see Beaunieux, Eustache and Pitel, 2014; Sullivan, and Pfefferbaum, 2005). In addition to amnesia, WKS patients also present with impaired problem solving skills, poor content and contextual memory, deficits in spatial learning and memory, ataxia, oculomotor disturbances, and global confusion (for review see Savage, 2014; Savage, Hall, and Resende, 2012; Vetreno, Klintsova, and Savage, 2011; Roland, Mark, Vetreno, and Savage, 2008; Vetreno, Hall and Savage, 2011; van Oort, and Kessels, 2009; Pitel, et al., 2011).

A contributing factor in chronic alcohol induced cell death may be due to alterations in gastric motility, and nutritional absorption caused by alcohol consumption (for review see Martin, Singleton, and Hiller-Sturmhofel, 2003; for review see Hoyumpa, 1980). Specifically, the B vitamin, thiamine has been implicated in cellular death when
depleted as will be discussed in the following paragraphs (for review see Butterworth, 2003).

As reviewed by Martin et al. (2003), adult Homo sapiens require >0.33 mg of dietary thiamine per 1,000 calories consumed (0.66 mg per day given a 2,000 calorie diet) (Hoympa, 1980; Martin et al. 2003). However, in alcoholics, oral absorption of thiamine is minute to non-existent (Agabio, 2005; Thomson, 2000). Deficits in this vitamin disrupt a variety of metabolic pathways and the synthesis of numerous neurotransmitters and proteins.

Three enzyme systems are dependent on thiamine: transketolase, pyruvate dehydrogenase, and alpha-ketoglutarate. Transketolase is involved in the pentose phosphate pathway, which plays a role in myelination maintenance. Pyruvate dehydrogenase plays a role in energy production via the breakdown of pyruvate to the coenzyme Acetyl CoA, an enzyme also involved in the production of the neurotransmitter acetylcholine. Finally, alpha-ketoglutarate, a glutaric acid is involved in the formation of the neurotransmitters acetylcholine, GABA, and Glutamate (for review see Martin et al, 2003; Cook, Hallwood, and Thomson, 1998; Thomson and Pratt, 1992; Thomson, Jeyasingham, Pratt, and Shaw, 1986) as well as aspartate, another excitatory neurotransmitter (Martin et al, 2003). Thiamine is, therefore, central to the preservation of the myelin sheath, cellular energy reserves, and neurotransmitter synthesis. Based on these findings one can see how thiamine deficiency can lead to neuronal dysregulation on several fronts.

Adding fuel to the fire is the fact that thiamine deficiency also induces a selective downregulation of the glutamate transporters located at the astrocyte/synapse junction on the astrocytic cell in some brain regions (for review see Butterworth, 2003; Hazell, Rao, Danbolt, Pow, and Butterworth, 2001; Sheldon, and Robinson, 2007) exacerbating
the potential for both alcohol and thiamine deficiency induced cellular death. As discussed by Sheldon and Robinson (2007), extracellular metabolism of glutamate does not seem to occur once it is released. As such, it must be removed from the synaptic space by transporters, in this case by glutamate transporters (GLUTS) including GLT-1 and GLAST. These transporters are found on astrocytic processes proximal to the synaptic terminal where they are in a prime position for glutamate uptake and storage (for review see Sheldon and Robinson, 2007; Hazell et al., 2001). This down regulation of GLUTs leads to an increase in extracellular glutamate (for review see Butterworth, 2003; Hazell et al., 2001). Due to the excitatory nature of glutamate, extracellular levels need to be regulated to prevent excitotoxicity induced cell death. These observations further implicate thiamine deficiency as a factor in the cell death induced by chronic alcohol exposure.

Alcohol Addiction, Thiamine Deficiency, and Impulsivity

The propensity to consume excessive amounts of alcohol in addicted individuals, which may contribute to thiamine deficiency, also has a genetic component that should be considered. There is evidence that differential genetic backgrounds can alter susceptibility to the thiamine deficiency inducing effects of chronic alcohol consumption. Based on work by multiple researchers (for review see Martin et al., 2003; Tallaksen, Bohmer, and Bell, 1992; Hoyumpa, 1980; and Morgan, 1982) it appears that as high as 80% of alcoholics may experience thiamine deficiency. However, as discussed by Harper et al., (1988), only ~13% of this population develop WKS. This indicates that there may be a subset of the population with a higher sensitivity to thiamine deficiency that may be more vulnerable to alcohol induced thiamine deficiency related brain damage (Harper, Gold, Rodriguez, and Perdices, 1989). Work investigating this point
has returned mixed results. For example, Mukherjee and colleagues (1987) observed that both alcoholic men and their alcohol naive sons had lower levels of thiamine diphosphate (metabolically active thiamine) than controls (Mukherjee, Svoronos, Ghazanfari et al., 1987). However, another study found no differences in thiamine diphosphate, indicating that there was not a difference in the transketolase enzyme necessary to add the diphosphate bonds to thiamine and render it active (Nixon, Kaczmarek, Tate, Kerr and Price, 1984). Because some work has demonstrated that moderate thiamine deficiency increased white matter pathology in a genetic model with alcohol exposure (He, Sullivan, Stankovic, Harper, and Pefferbaum, 2007), this opens an avenue for further investigation applying a thiamine deficient protocol to genetic models of addiction. Furthermore, the addiction phenotype includes pervasive executive function deficits, which may be exacerbated by thiamine deficiency and alcohol exposure, including impulse control deficits and perseverative behavior.

Persistent, compulsive drug use without regard to consequences is a hallmark feature of addiction (for review see Hyman, Malenka and Nestler, 2006; for review see Crews and Boettiger, 2009; Hser, Hoffman, Grella, and Anglin, 2001; McLellan, Lewis, O’Brien, and Kleber, 2000). Although some regular users are able to abstain on their own, for many addiction is a chronic problem (Hyman, Malenka and Nestler, 2006). This compulsive behavior is related to deficits in reversal learning (Jentsch, Olausson, De La Garza and Taylor, 2002), perseverative behavior (related to reversal learning) (Hyman, 2005), and impulsivity (for review see Jentsch and Taylor, 1999; Oberlin and Grahame, 2009; Crews and Boettiger, 2009) and has long been associated with addiction as demonstrated by these animal models of drug exposure and genetic susceptibility as well as human studies examining decision making in similar tasks.
Executive Function Deficits: Impulsivity and Reversal Learning

Impulsivity is a maladaptive inability to delay receipt of reward resulting in a net loss of reward (e.g. food, money) for the organism (Ainslie, 1975). Delay discounting paradigms are a highly translational behavioral assessment of impulsive choice behavior (referred to as impulsivity or impulsive choice here) contingent on the fact that the preference for a large immediate reward over a small one is conserved between species (Mazur, 1987; Oberlin and Grahame, 2009; Odum, 2011), and that as the wait to receive the reward increases over time the perceived value of said reward subjectively diminishes (Bechara, Dolan, and Hindes, 2002; Odum, 2011; Oberlin and Grahame, 2009; Mars and Robbins, 2007; Mazur, 1987), hence “Delay Discounting”.

Delay discounting can manifest as a maladaptive behavior leading to an inability of the organism to optimize the results of goal directed behavior due to an inordinate preference for small immediate gain regardless of the potential for overall long term gain and greater overall rewards accrued (Ainslie, 1975). Because immediate rewards are excessively valued during decision making, there is a tendency in some individuals to quickly change their preferences and decision making styles to favor an immediate short term reward over a deferred, but greater, reward (Mazur, 1987).

In populations with a genetic predisposition towards addiction, drug naive impulsivity can be predictive of future drug consumption (Oberlin and Grahame, 2009; Beckwith and Czachowski, 2014). However, drug exposure has also been demonstrated to correlate with impulsivity. Beckwith and Czachowski (2014) demonstrated that high ‘alcohol seeking’, but not ‘consumption’, positively correlates with increased delay discounting. This study used high alcohol drinking (HAD2) and alcohol-preferring (P) rats, both genetic models of alcoholism. Interestingly, only the ‘high seeking’ P rats demonstrated greater delay discounting than control Long-Evans rats, indicating that
there is a link between drug seeking and impulsivity that is independent of consumption (Beckwith and Czachowski, 2014). Furthermore, P rats have also been shown to have increased choice impulsivity (Perkel, Bentzley, Andrzejewski, and Martinetti, 2015 (alcohol naïve); Linsenbardt, Smoker, Janetsian-Fritz, and Lapish, 2016 (vs parent strain)). Other rodent models selected for alcohol drinking have also exhibited differences in impulsivity subtypes. For example, the high drinking STDRH12 mouse has increased motor, but not choice, impulsivity (Wilhelm, Reeves, Philips, and Mitchell, 2007). These studies indicate that, although impulsivity plays a role in addiction, it is not the only factor involved in the presentation of an addictive phenotype and that certain types of impulsivity and drug ‘seeking’ or ‘consumption’ may have separable roles.

In human males with alcohol use disorders increased choice impulsivity is also observed. Additionally, these subjects exhibited hyperactivity in brain regions related to cognitive control and prospection; the dorsolateral prefrontal cortex and posterior parietal cortex, respectively (Amlung, Sweet, Acker, Brown, and MacKillop, 2014). A study examining females with alcoholism also demonstrated that there is increased choice impulsivity as well as increased activity in multiple brain regions related to cognitive control including the insula/orbitofrontal cortex (Claus, Kiehl, and Hutchison, 2011). This finding is in line with studies demonstrating the roles of the medial and lateral orbitofrontal cortex, anterior cingulate, and anterolateral prefrontal cortex in reward dependent decision making (for review see Rushworth, Noonan, Boorman, Walton, and Behrens, 2011, for OFC specific reviews see Wallis, 2007 and 2012). Another study, examining women, found increased impulsivity in heavy drinkers as well as decreased sensitivity to the aversive effects of alcohol consumption, which may increase abuse potential (Reed, Levin, and Evans, 2012). Increased impulsivity is also seen in numerous other addictive phenotypes ranging from nicotine use (Baker and Bickel,

Additionally, alcohol exposure during development and adolescence may increase impulsive choice behavior (Banuelos, Gilbert, Montgomery, Fincher, Wang, Frye, Setlow, and Bizon, 2012) as well as relate to other associated behaviors such as adolescent anxiety (Mejia-Toiber, Boutros, Markou, and Semanova, 2014; (Stein, Renda, Barker, Liston, Shahan, and Madden, 2015) and risk taking (Roman, Stewart, Bertholomey, Jensen, Colombo, Hyytia, Badia-Elder, Grahame, Li and Lumeng, 2012). Other decision making deficits also appear to contribute to this behavioral phenotype. Cognitive flexibility is also an executive function metric closely related to impulsivity and governed by many of the same brain regions, as will be discussed below. Deficits in cognitive flexibility, measured by reversal learning tasks, are also seen in models of addiction and alcohol exposure.

Reversal learning tasks are another way to assess OFC function and evaluate potential decision making deficits. Successful reversal learning requires reflection on past stimulus-reward associations and the adaptation of behavior to accommodate changing reward contingencies, which are considered an essential element of emotional and social behavior. Reversal learning tasks examine both affective (emotion based) decision making and learning, in addition to assessing the ability to alter behavioral response when presented with changes in stimulus-reward associations (Franken, van Strien, Nijs and Muris, 2008). Reversal learning is also used to assess both compulsive and impulsive behavior, and OFC function in addiction (for review see Izquierdo and Jentsch, 2012). Additionally, in the case of delay discounting tasks, reversal learning may also be an inherent part of the task as the perception of the ‘preferred lever’ is
altered with increasing delay, which may confound the discrimination between reversal learning deficits and a propensity toward impulsive choice behavior.

**Brain Regions Governing Impulsivity and Cognitive Flexibility**

Impulsivity and cognitive flexibility are both governed largely by the orbitofrontal cortex as has been demonstrated by numerous studies. For example, lesions to the orbitofrontal cortex result in the inability of animals to modify their responding following alterations in reward contingencies (Chudasama, Passetti, Rhodes, Lopian, Desai, and Robbins, 2003; for review see Clark, Cools, and Robbins, 2004; Boulougouris, Dalley, and Robbins, 2007) because this area encodes for violations of reward expectation, with reversal responding supported by the lateral OFC specifically (Ghahremani, Monterosso, Jentch, Bilder, and Poldrack, 2010). Damage to the OFC has been shown to lead to impulsivity, behavioral inflexibility, and emotional disorder (Roberts and Wallis, 2000). Loss of inhibitory control of affective responding has been implicated in impaired reversal learning. Deficits may also present themselves as a failure to avoid short term reward that leads to long term negative consequences (choice impulsivity) (Clark, Cools, and Robbins, 2004). There is substantial evidence suggesting that there is orbitofrontal dysfunction in addicts, which may contribute to compulsive behavior (Everitt, Belin, Economidou, Pelloux, Dalley, and Robbins, 2008). In cases of frontotemporal dementia with OFC pathology, compulsive behaviors have been reported including hyperphagia, gambling, and substance abuse. OFC damage in humans, as well as non-human primates and rodents, has led to addictive behavior (Crews and Boettiger, 2009). Additionally, there is evidence from human neuroimaging studies, as well as animal studies, examining cellular activity that corroborates these findings.
Neuroimaging studies performed on healthy subjects have shown that the prefrontal cortex is activated during reversal learning tasks, and that deficits in reversal learning are correlated with damage to the OFC (Franken et al, 2008). The role of these areas in reversal learning is further supported by studies recording the activity of single cells in the orbitofrontal cortex, which have confirmed that the firing of neurons in this region changes in response to alterations in reward contingencies. Importantly, neurons in the macaque OFC have been shown to reverse from firing at the presentation of previously rewarded stimulus to a currently rewarded stimulus (for review see Clark, Cools, and Robbins, 2004; Rolls, Critchley, Mason, and Wakeman, 1996; Thorpe, Rolls, and Maddison, 1983) implicating them in cognitive flexibility when reward contingencies change.

Shoenbaum and colleagues have demonstrated that the orbitofrontal cortex plays a particularly pivotal role in the assessment of expected and received outcomes (for review see Shoenbaum, Roesch, and Stalnaker, 2006), important in decision making tasks central to this study (delay discounting and reversal learning). Human neuroimaging studies have demonstrated that there are changes in blood flow in the OFC both while anticipating outcomes and when the expected outcome is altered or not attained (Gottfried, O’Doherty, and Dolan, 2003; Gottfried, O’Doherty, and Dolan 2002; O’Doherty, Deichman, Critchley, and Dolan, 2002; Nobre, Coull, Frith, and Mesulam, 1999). This change appears to be associated with reward value and is observed when this information is being processed in decision making (Arana, Parkinson, Hinton, Holland, Owen, and Roberts, 2003). Animal studies have demonstrated that the neurons in the OFC fire differentially depending on reward salience including expected size, time to attainment, and possible consequences of incorrect behavior (Schoenbaum, Stelow, Saddoris, and Gallagher, 2003; Schoenbaum, Chiba, and Gallagher, 1998; Tremblay
and Schultz, 1999; Roesch and Olson, 2004; Roesch and Olson, 2005) making them critical in delay discounting tasks which require the assessment of reward size and time to attainment. If an expected reward is not obtained, or is devalued (ex: by time), there is a decrease in this responding (Tremblay, and Schultz, 1999). Thus, outcome expectancies encoded by the OFC are critical to adaptive learning (Saddoris, Gallagher, and Schoenbaum, 2005). Additionally, there is evidence that the maladaptive decision making processes associated with drug addiction may result from drug induced changes to this region (London, Ernst, Grant, Bonson, and Weinstein, 2000; Rogers, Everitt, Baldacchino, Blackshaw, Swainson, Wynne, Baker, Hunter, Carthy, Booker, London, Deakin, Sahakian, and Robbins, 1999; Porrino, and Lyons, 2000; for review see Dom, Sabbe, Hulstiin, and Van Den Brink, 2005). This possibility indicates that an underlying deficit in this behavior could be exacerbated by drug and alcohol exposure, as well as thiamine deficiency.

The limbic cortex (infralimbic and prelimbic regions encompassing the medial prefrontal cortex) is implicated in response inhibition, which also relates to an impulsive phenotype as the organism’s inability to suppress the impulse to respond although it has been learned that no reward can be elicited (Chudasama, Passetti, Rhodes, Lopain, Desai, and Robbins, 2003). Studies have demonstrated that the limbic region is not involved in successful reversal learning, however. These experiments have shown that there is functional selectivity in this type of task. While the OFC and limbic cortices both play a role in impulsivity, the OFC alone was implicated by these lesion studies (Boulougouris, Dalley, Robbins, 2007).

Recent work in this lab, using an animal model, has demonstrated that thiamine deficiency in conjunction with chronic ethanol exposure impaired cognitive flexibility. This study used spontaneous alternation and attentional set shifting to assess this aspect of
executive function. Additionally, these results supported the potential that sub-clinical thiamine deficiency may exacerbate the cognitive deficits induced by chronic alcohol consumption (Vedder, Hall, Jabrouin, and Savage, 2015). Here, we further this line of inquiry into executive function assessments and how this behavior can be altered by a variety of treatments. We investigated the effects of chronic ethanol consumption, thiamine deficiency, thiamine supplementation, and the combination of those variables on behavioral tasks that are highly dependent on the frontal cortex. We assessed impulsivity using an operant delay discounting task and incorporated a reversal of reward contingencies to examine differences in impulsivity and cognitive flexibility induced by these treatments. Following these tasks, we examined the neuropathology induced by thiamine deficiency; thalamic cell loss using NeuN to stain neuronal cell bodies. The immediate early gene Arc was then used to examine neuronal activity in the OFC and infralimbic/prelimbic cortices. Additionally, Cavalieri volumetric analysis was used in conjunction with NeuN staining to assess possible changes in the volume of the OFC and limbic cortices. The results of this investigation allow us to further tease apart the roles of the OFC and limbic cortices in impulsivity and cognitive flexibility as well as the effects of alcohol exposure, thiamine deficiency, and the comorbid treatments on these brain regions and the associated behavioral constructs.

**Methods**

**Subjects**

This experiment used 56 adult male Sprague-Dawley rats (Envigo, Indianapolis, IN, USA) that where 3-4 months at the start of treatment. Rats were randomly assigned to one of 6 treatment groups (see Vedder et al, 2015); Pair Fed (PF) control group; Chronic Ethanol Treatment (CET); Chronic Ethanol Treatment with IP Thiamine
supplementation (CET-Th); Chronic Ethanol Treatment in conjunction with IP injections of Pyrithiamine hydrobromide (CET-PTD); PTD-EAS (Pyrithiamine induced Thiamine Deficiency-Early Acute Stage; and PTD-MAS (Pyrithiamine induced Thiamine Deficiency-Middle Acute Stage). The initial N consisted of 8-9 per group, though some subjects were lost due to failure to thrive post-treatment or advanced age resulting in a final N of 48. Additionally, tissue damaged during processing resulted in the loss of some subjects and varied group sizes in immunohistological assays. All experiments were conducted according to the Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the State University of New York at Binghamton.

Treatments

Chronic Ethanol Treatment: Ethanol treated groups (CET, CET-Th, CET-PTD) were exposed to increasing concentrations of ethanol using a ‘Fading On’ procedure during which subjects receive 6%, 9%, 12% and subsequently 20% ethanol in tap water in five day increments. Following six months of 20% ethanol in tap water, animals were titrated off using the reverse of this procedure, termed ‘Fading Off’. During ethanol exposure, tail blood samples were taken and analyzed for blood ethanol concentration (BEC) via ANALOX at month 1, 2/3, 4, and 6. Consumption was assessed twice a week (by taking bottle weights) for these animals. PF animals also had their fluid consumption (tap water) monitored and had blood samples taken at the above-mentioned times. CET-Th subjects received IP injections of thiamine hydrochloride (0.4 mg/kg IP) three times a week, in addition to chronic ethanol exposure, and had BECs taken at the same time as samples were collected from other ethanol groups (Figure 1).
Pyrithiamine-induced Thiamine Deficiency (TD): Thiamine deficient groups (CET-PTD, PTD-EAS, and PTD-MAS) received ad libitum thiamine deficient chow (Envigo Diets, IN, USA) and received daily injections of pyrithiamine hydrobromide, which prevents thiamine from becoming metabolically active, (0.25 mg/kg IP; Sigma-Aldrich, St. Louis, MO) beginning 1 month into ethanol exposure in CET treated groups. About 12-13 days after the start of PTD treatment neurological symptoms (anorexia, ataxia, loss of righting reflexes, and ultimately opisthotonus (spasm) were recorded hourly. Rats were treated to different severity levels of TD, consisting of: (i) an early acute stage (PTD-EAS and CET-PTD) in which rats were reversed with a bolus of 0.5 cc of thiamine hydrochloride (100 mg/ml, IP; Sigma-Aldrich) within 1 hour after the appearance of opisthotonus and (ii) a moderate acute stage (PTD-MAS) when rats were reversed after 4.25 hours following seizure.

Combined Treatments of CET and PTD: To assess the potential synergistic interactions between CET and TD, a subset of rats (CET-PTD) were exposed to the PTD-EAS stage while they were exposed to 20% v/v ethanol. Following reversal, all PTD rats were returned to normal rat chow and given a second thiamine reversal injection 24 hours later.

Pair fed (PF) control subjects also received thiamine deficient chow during this symptom onset dependent 14-17 day period, however, they also received daily injections of thiamine hydrochloride (0.4 mg/kg IP; Sigma-Aldrich, St. Louis, MO). To mimic the anorexic effects of thiamine deficiency, PF animals were food deprived during the period of weight loss prior to the onset of symptoms experienced by thiamine deficient groups to match weight loss experienced by these subjects. All subjects were given three weeks to recover and regain their free feeding weights prior to behavioral testing. This paradigm has been previously described in work from this lab (Vetreno, Klintsova, and Savage, 2011; Pitkin and Savage, 2000) and has been demonstrated to
produce no differences, relative to untreated controls, in progenitor cell proliferation, neurogenesis, (Vetreno, Klintsova, and Savage, 2011) or thiamine dependent enzymes (Butterworth and Heroux, 1989).

**Behavioral Assessments**

Prior to behavioral testing all treatment groups underwent 7 days of food restriction to achieve weights of 85 +/-5% free feeding weight, at which point delay discounting testing commenced. Additionally, all animals were handled for five minutes a day for 5 days prior to the initiation of testing and acclimated to BioServe (Flemington, NJ, USA) Dustless Precision reward pellets (.5 g, unflavored) in their home cages during this time.

**Operant Delay Discounting and Reversal Learning**

Operant boxes consisted of 12 large rodent testing chambers (Med Associates Inc, St. Albans, VT, USA) measuring 55.9 cm X 55.9 cm X 35.6 cm. Each operant box was contained within a sound and light attenuated chamber with a ventilation fan. Head entry detectors were centered on the 35.6 cm side connected to external pellet dispensers which deposited pellets in the head entry trough. Levers were located 3 cm away from the head entry detector on either side at approximately 11 cm high. A single house light was centered on the opposite wall 27 cm high.

**Behavioral Testing**

**Initial and Reversal Delay Discounting**

Following home cage exposure to reward pellets, subjects were trained on programs of ascending difficulty; beginning with habituation, autoshaping, Fixed Ratio-
(FR1), Fixed Ratio-2 (FR2). Habituation consisted of 90-minute session during which the rats were exposed to the operant boxes. The following day autoshaping was conducted; this consisted of a 60 min session during which levers were randomly presented individually and presses were rewarded until 60 trials were completed. Failure to lever press during this 60 min task resulted in extended autoshape training done overnight. The side that received the majority of presses during this procedure was deemed the preferred side and designated the ‘delayed reward lever’ in later trials.

After completion of this task, rats were presented with a FR1 program during which the house light would come on signaling the start of a trial. They then had to place their head into the nose poke, breaking a photoreceptor beam to start the trial. At that time the levers extended and the rat would have to press once to receive a 1 pellet reward. The levers retracted and the house light went out for a 100 sec inter-trial interval (ITI). In order to move on to the next phase, the rat had to have a side preference of less than 75%.

If a rat had a side preference greater than 75%, remedial training was done consisting of a FR1 task in which only one lever at a time was pseudo-randomly presented. Completion of this task with choice behavior below the 75% ceiling moved the animal on to FR1 where their performance was evaluated again to ascertain that they would not exhibit a substantial side preference.

The FR2 program differed only in that the rat now had to make two lever responses to receive the reward. The previously described 75% ceiling on side preference remained in place and if the rat failed to perform below this criterion it was again given remedial training consisting of an amended program that presented the animals with a pseudo-randomized combination of 30 forced trails that presented the
non-preferred lever and 30 random trials. Following this task, the rat was again presented with the FR2 program and given another opportunity to reach criterion.

These pretraining programs culminated in a 0 Delay version of the Delay Discounting program, during which reward discrimination was assessed. Once it was established that each subject could discriminate reward magnitude, determined to be a preference for a large, immediate (4 pellet) versus small, immediate (1 pellet) reward 75% of the time, they were exposed to the delay discounting program.

The delay discounting task was adapted from Mar and Robbins (2007) and presented each subject with the opportunity to receive a small or large reward by pressing one of two levers with predetermined designations. This was an ascending delay paradigm beginning with a block at a 0 second delay and progressing to 10, 20, 40, and 60 seconds. Each block of delays consisted of 10 delay trials daily. The first session began with 4 forced choice trials (2 forced trials occurred prior to subsequent delay blocks) where levers were presented individually, which ensured that the subject was exposed to both reward magnitudes.

Each delay block was initiated by the presentation of the house light, which signaled the opportunity for the rat to nose poke, breaking a photoreceptor beam and initiating the trial (failure to nose poke was coded as a ‘nose poke omission’). At this point both levers would extend simultaneously, affording the animal the ability to press either lever to obtain a reward. The rats had 10 seconds to respond by lever press, if they failed to respond this would be counted as a “lever press omission”. Following a 6-s consumption interim, the house light would go off for a 100 second inter-trial interval prior to the initiation of the next trial. Once the subject reached behavioral stability, deemed no differences in choice behavior greater than 25% for each consecutive delay
for two three-session blocks, they progressed to the next phase of behavioral testing (Reversal Learning).

Latency to nose poke and press levers were also recorded for later assessment of individual/group differences in motivation. At the point of behavioral stability, determined by no choice variation greater than 25% over the course of 6 consecutive testing days when comparing across discrete delay intervals, the reversal phase commenced the following day with delay times remaining consistent. The number of sessions required to reach behavioral stability was recorded.

Importantly, which lever was pressed at each delay point was recorded allowing for the assessment of changes in lever preference as a function of delay; determining the subjective value of the reward for each rat as the time to receive said reward increased incrementally across trials. This is the critical component of this task which allows for interpretations regarding the impulsive choice behavior of each treatment group.

Reversal Learning

Reversal learning was evaluated using the same program as the delay discounting task, however reward contingencies were changed such that the lever previously associated with receiving the large reward was now providing the smaller reward. All other contingencies being equal, this forced the subjects to re-evaluate the reward parameters of the task and alter behavioral responses accordingly. This assessment continued until the subjects again reached behavioral stability, defined as no choice variation greater than 25% over the course of 6 consecutive testing days when comparing across discrete delay intervals. After the subject met this criterion, the reversal phase was initiated. The number of sessions required to reach behavioral
stability was recorded, or a 40 day testing ceiling, resulting in a post-reversal delay discounting curve.

Nose poke and lever omissions were also recorded for later assessment of individual/group differences in motivation. At the point of behavioral stability, determined by no choice variation greater than 25% over the course of 6 consecutive testing days when comparing across discrete delay intervals, the reversal phase commenced. The number of sessions required to reach behavioral stability was recorded.

**Behavioral Data Analysis**

Behavioral data was analyzed using a two-way ANOVA followed by Dunnett’s test in order to compare each group to controls, and LSD analysis for general group comparisons.

**Immunohistochemistry**

Whole brains were extracted approximately 90 minutes following behavioral testing. Rats were first euthanized with Sleep Away (0.5 mg/kg i.p. [26.0% sodium pentobarbital in 7.8% isopropyl alcohol and 20.7% propylene glycol], Fort Dodge Animal Health, Fort Dodge, IA) and transcardially perfused (Masterflux Cole-Parmer Instrument Company, Trumpers Way, London) with a saline solution (9%) followed by a 4% paraformaldehyde solution (in 0.1 M PBS). Brains were post fixed in 4% paraformaldehyde followed by immersion into a 30% sucrose solution. Tissue was sliced on a freezing sliding microtome (Leica SM2000R, Munich, Germany) at 40 μM and stored in cryoprotectant at -20 degrees C in preparation for immunohistochemistry to assess Arc and NeuN staining, and Cavalieri volumetric analysis.
NeuN Staining

Slice selection commenced at the most anterior sample collected containing the areas of interest (OFC, IL and PL, between 5.64 and 4.20 from Bregma, according to Paxinos and Watson, 1982). Every 5th section was collected for staining. Sections were rinsed 3 times in TBS for 5 minutes each at room temperature (RT). (Note: Adapted from Cold Spring Harbor Protocols (2009); 50mM Tris-Cl and 150mM NaCl (pH 7.5 +/-0.02)). Subsequently, sections were rinsed in a 0.3 % Hydrogen Peroxide solution for 30 minutes at room temperature. Then sections were again rinsed 3 times for 5 minutes in tris-buffered saline (TBS) at RT and blocked with Normal Horse Serum (NHS) (Vector Labs (S-2000) Burlingame, CA, USA). Following this step, slices were rinsed for another 3 cycles in TBS for 5 minutes each at room temperature and incubated in NeuN primary antibody monoclonal mouse anti-NeuN (Millipore (MAB337; Billerica, MA) for 48 hours at 4 degrees C (1:500). Sections were again rinsed 3 times in TBS for 5 minutes each, then incubated in secondary antibody biotinylated horse anti-mouse IgG (Vector Labs; BA-2001). Following this step, slices were incubated in ABC solution (ABC Elite kit standard Vector Labs) at room temperature for 120 minutes then rinsed again for another 3 cycles in TBS for 5 minutes each. Slices were then immersed in 3,3’-Diaminobenzidine (DAB substrate kit, Sigma Aldrich) for visualization for approximately 3 minutes, a time period determined by the color of the chromagen (DAB). Finally, slices were washed for another three 10 minute cycles in TBS prior to mounting on slides. Slices were left to dry overnight and coverslipped using Paramount (Fisher Chemicals, Hampton, NH).

Arc Staining

This procedure is adapted from Chia & Otto. (2013). Sections were washed 3 times in phosphate buffered saline (PBS) for 10 minutes each at room temperature and subsequently blocked in 1% Bovine Serum Albumin (BSA) in PBS + (0.1% Triton-X) at
room temperature for 1 hour. Following this step, slices were incubated in Primary rabbit polyclonal Arc 1:1000 (Synaptic Systems, Goettingen, Germany). Thereafter, sections were washed 3 times in PBS for 10 minutes and incubated in secondary solution (Vector-Vectastain ABC kit (PK-6012)) anti-mouse at room temperature for 1 hour. Afterwards, sections were again washed 3 times for 10 minutes each in PBS prior to immersion in 3,3’-Diaminobenzidine (DAB substrate kit Sigma Aldrich St. Louis) solution for visualization (approximately 3 minutes). Finally, slices were washed for another three 10 minute cycles in PBS prior to mounting on slides. Slices were left to dry overnight and coverslipped using Paramount (Fisher Chemicals).

**Cavalieri Volumetric Analysis (CVA)**

Mounted 40 micron slices of NeuN stained tissue were examined using a Zeiss Axioskop 2 plus microscope (Oberkochen, Germany) and CX9000 MBF Bioscience camera (Willeston, VT), and analyzed using StereoInvestigator MBF Biosciences. Sections were determined using a systematic sampling technique in which there was analysis of equidistant sections commencing with the last section collected at the confluence of the corpus callosum (Examples of region determination for volumetric analysis of the OFC and PL/IL can be seen in Figure 2) in which the region of interest last appears in a randomly chosen pre-collected series continuing for every 5th section until 9 sections of orbitofrontal and 4 sections from the limbic cortex were analyzed for each subject. These slices ranged from 2.52 to 4.20 from Bregma (Paxinos and Watson, 1982). CVA data was analyzed using a univariate ANOVA followed by a Dunnet’s test to compare each group to controls, as well as LSD tests to compare across groups more broadly.

**Interventricular Distances (IVD) Measures**
In addition to quantitative analysis of cortical volumes, measures of interventricular distances were taken as a metric of thalamic shrinkage (Robinson & Mair, 1992; Hall & Savage, 2016). This was completed by measuring the distance from the floor of the dorsal third ventricle to the top of the third ventricle at three locations (-2.76mm, -3.24mm, -3.60mm from Bregma according to Paxinos and Watson, 1986) thereby measuring the size of the thalamus. IVDs were analyzed using a univariate ANOVA. Additionally, Pearson’s correlations were done to analyze the relationship between IVDs and delay discounting behavior.

**Cell Counting**

Arc stained slices were counted at 7.6X magnification to evaluate cellular activity in the medial and lateral orbitofrontal cortices as well as the prelimbic and infralimbic regions. ROIs were .8 x .6 mm and omitted the neuron sparse plexiform layer of the cortex (see Figure 3 for an exemplar). Slices were determined using the sampling technique described in the CVA section and analyzed using ImageJ to automatically count the regions depicted using a Nikon Optishot-2 microscope (Melville, NY) equipped with an Infinity HD Lumenera camera (Nepan, ON, Canada). Counts were analyzed based on anterior and posterior orientation using a repeated measures ANOVA and Dunnett’s test as well as by individual slice using LSD analysis to compare across all groups.

**Results**

**Treatment Data:**

Weight: There were overall differences in weights across groups (F(5,55)=5.286, p<.05). PF subjects had a greater average body weight (544.79 g) than CET-TH (455.96 g) and CET-PTD (457.70 g) animals (p<.05) (Figure 4).
Consumption: There were no significant differences in liquid consumption by body weight across ethanol treated groups (CET; CET-Th; CET-PTD) (F(2, 29)=3.064, p>.05) (Figure 5).

BEC: Blood ethanol concentration data analysis did not show any significant differences across ethanol treated groups (Figure 6 A-C). All ethanol treated groups were significantly higher than PF, the ethanol naïve control group (F(3,28)=28.321, p<.001). In Month 1 all CET groups had means above 34 g/kg, this increased to means above 45 g/kg in Month 2/3, rising again in Month 4 to means above 70 g/kg, at which point there seems to be a ceiling effect with means topping out at above 85 mg/dl. This effect of time was significant for all groups (F(3, 28)=28.321, p<.001).

**Behavioral Data**

**Phase 1: Initial Acquisition (Delay Discounting)**

There were significant group differences in the number of days required to reach stability on the Delay Discounting task (Figure 7 A) (Main effect of treatment (F(5, 43)=3.33, p<.05). Dunnett’s test revealed that CET and CET-Th groups took significantly longer than PF controls to reach criterion.

**Exploratory Analysis**

Exploratory post hoc LSD analysis showed differences between PF and CET (p<.01), CET-TH (p<.01), CET-PTD (P<.05), and PTD-EAS (P<.05) illustrating that these treatment groups took significantly more days to attain the behavioral stability criterion than PF (Figure 7 A). Although PTD-MAS subjects did not differ significantly from PF animals, they exhibited greater variability as expressed by their standard deviation.

Delay Discounting
A significant overall effect of Treatment was seen ($F(5,43)=2.21, p< 0.028$). In addition there was a general effect of Delay ($F=4,172)=74.14, p<.001$). There was a significant Treatment x Delay interaction ($F(20,172)=2.01, p<.05$) (Figure 7 B). Dunnett’s test revealed that there was a slight trend toward fewer delay lever choices in CET treated groups ($p=.080$) and a significant reduction in PTD-MAS subjects ($p<.05$) when compared to PF controls. Additionally, when examining discrete delay points there was no significant difference at the 0 second time point ($F(5, 49)=1.169, p>.05$); at the 10 second delay there was no significant difference between groups ($F(5, 49)=2.081, p>.05$). However there was a significant reduction in the preference for the delay lever in PTD-MAS subjects at this time point as compared to controls using the Dunnett’s test ($p<.05$); at the 20 second time point there were no significant differences overall ($F(5,49)=2.238, p>.05$), however again Dunnett’s post hoc analysis revealed a significant difference in PTD-MAS animals delay lever choices demonstrating a reduction as compared to controls ($p<.05$); at the 40 second delay point there was a significant effect of delay overall ($F(5,49)=3.014, p<.05$) as well as a significant reduction in delay lever preference in PTD-MAS subjects as compared to PFs using the Dunnett’s test ($p<.01$); finally, at the 60 second time point there was a significant effect of delay ($F(5,49)=3.385, p<.05$) as well as a significant reduction in delay lever choice behavior in the PTD-MAS ($p<.01$) and CET treated ($p<.05$) animals as compared to controls revealed by the Dunnett’s test. There were no significant differences among other groups ($p>.05$).

**Exploratory LSD:**

Exploratory post hoc LSD (which allows for comparisons across all groups as opposed to controls as in the Dunnett’s test) analysis shows differences between groups at several delay intervals. PF rats are significantly different from rats in the CET and CET-TH groups at 0-s delay (both $F$'s$(1, 13)=5.879, p<.05$). At the 10-s, 20-s and 40-s
delay intervals only the PTD-MAS group differed from PF controls (F(1, 15)=5.907, p’s<.05). Furthermore at the 60-delay interval both the CET and PTD-MAS groups were different from PF controls (both F’s(1,15),(1,14))=5.880, p’s<.01). The PTD-MAS subjects differ from PF controls starting at every protracted delay point, demonstrating a more pronounced increase in impulsive choice behavior, as described in order of increasing delay in the following paragraph. Neither the PTD-EAS or the CET-Th groups differed from the PF control group at any delay interval. Because of the potential, indicated by work from the lab previously discussed, for thiamine supplementation to prevent the effects of CET and the potential differences between CET and PTD treated groups, these metrics were also analyzed. Based on these analyses further testing may be warranted. There were no significant differences among other treatment groups (p>.05) (Figure 7 B).

Nose Poke Omissions

An additional metric observed, nose poke omissions, constitute the failure of an animal to initiate the trial. There were no Treatment X Delay interactions (F(20, 168)=0.92, p<.3). There was an effect of Delay (F(4,268)=13.75, p<.05). There were no significant differences across groups during the stability phase of the initial delay discounting task (F(5,42)=.618,p>.05). (Figure 7 C and D)

Lever Omissions

A final metric, lever omissions, deemed a subject’s failure to press a lever and thereby make a choice, showed no significant effects of Treatment during the last 6 days for delay discounting (F(5,42)=1.471, p>.05). There was an effect of Delay (F(4,168)=2.45, p<.05). There was no Treatment X Delay interaction (F(20, 168)=1.14, p<.3) (Figure 7 E and F).
Phase 2: Switched Contingencies (Reversal Learning)

There were no differences across treatment conditions during the Reversal Learning Phase, defined as the first six days after the change in reward contingencies (F(11.175)=.817, p>.05) (Figure 8 A and B).

Lever Omissions

There were no significant differences (5,42)=.269, p>.05) in lever omissions across groups (Figure 8 C and D).

Nose Poke Omissions

There were no significant differences (F5,43)=1.45, p>.05) in nose poke omissions across groups (Figure 8 E and F).

Task Reacquisition

Time to behavioral stability during the Reversal Delay Discounting was not different across the treatment conditions (F(5,40)=1.609, p>.05) (Figure 9 A). Additionally, Dunnett’s test revealed no significant differences across groups as compared to PF controls (all p’s >.05).

Stable Performance:

Reversal Delay Discounting

The reversed delay discounting curves were attenuated by previous exposure to the task resulting in an effect of delay (F(1.89, 77.57)=120.131, p<.001) (adjusted for sphericity violation using Greenhouse-Geisser) and no effect of treatment
Dunnett’s test did not reveal any significant differences in treatment groups as compared to PF controls (p>.05) (Figure 9 B)

**Exploratory Analysis:**

LSD analysis showed that there was a difference between PF and PTD-MAS treatments at the 20 second time point, PTD-MAS rats were more impulsive than PF subjects (F(1, 17 )=4.612, p<.05) (as noted in Figure 9 B).

**Nose Poke Omissions**

There were no significant Treatment differences in nose poke omissions across groups during the stability phase of the reversal phase delay discounting task (F(5,42)=1.45,p>.05). There were no Treatment X Delay interactions (F(10.17, 87.07)=1.08, P>.05). There was an effect of Delay (F(2.02, 87.007)=23.39, p<.0001) (Figure 9 C and D).

**Lever Omissions**

Finally, there were no significant effects of treatment on lever omissions during the reversal phase of discounting (F(5,42)=0.741,p>.05) There was also no effect of Delay (F(3.03, 127.23)=1.41, p>.15) (Figure 9 E and F).

**Histological Data**

**Interventricular Distances**

There was no main effect of IVDs over all treatment groups (F(5,30)=2.07,p>.05). (Figure 10). However, Dunnett’s test revealed that the IVD of CE T-PTD subjects were significantly reduced as compared to PFs (p<.05).

**Exploratory IVD and Behavioral Correlations**
Phase 1: Delay Discounting

Pearson’s r revealed no correlation between IVD and delay discounting behavior at any time point. At 0 delay ($r=-.234, n=25, p=.130$); at 10 seconds ($r=-.038, n=25, p=.429$); at 20 seconds ($r=-.047, n=25, p=.412$); at 40 seconds ($r=-.056, n=25, p=.395$) and at 60 seconds ($r=-.054, n=25, p=.398$). (Figure 11)

Phase 2: Reversal Learning

Pearson’s r revealed no correlation between IVD and delay discounting behavior at any time point. At 0 delay ($r=.319, n=25, p=.060$); at 10 seconds ($r=.012, n=25, p=.478$); at 20 seconds ($r=-.066, n=25, p=.377$); at 40 seconds ($r=-.188, n=25, p=.184$) and at 60 seconds ($r=-.090, n=25, p=.334$). (Figure 12)

Cavalieri Volumetric Analysis

Orbitofrontal Cortex

There were no significant differences in cortical volume in the OFC across groups ($F(5,38)=.287, p=.919$) (Figure 13).

Limbic Cortex

There were no significant differences in limbic volumes across groups in the limbic cortex ($F(5,38)=1.366, p=.259$) (Figure 14). Dunnett’s test revealed no significant differences in any groups as compared to PF controls (all p’s greater than .05).

Exploratory Analysis

Although there were no significant differences in orbitofrontal cortical volumes across groups (all p’s>.05), LSD comparative analysis of limbic cortical volume revealed
a significant increase in volume in CET subjects as compared to PF animals (p=.015). Additionally, there was a trend (p=.06) toward larger limbic volumes in CET animals as compared to those with thiamine supplementation (CET-TH) (Figure 14).

**Cellular Activity**

**Medial OFC Activity**

Cellular activity in the medial OFC (Figure 15 and 16) was not significantly different overall across treatment groups (F(5, 33)=.332, p=.890). However, there was a significant effect of anterior versus posterior regions which revealed higher Arc immunoreactivity in the posterior region of the mOFC in all groups (F=1,33)=31.130, p<.01). Dunnett’s test revealed that there were no significant differences in any treatment groups as compared to PF controls (all p’s >.05).

**Exploratory Analysis: Medial OFC**

Slices were also analyzed individually to inform further analysis, those with significant differences are reported here. For Slice 8: there were no overall differences (F(5,1)=1.237, p=.166), again the PF subjects exhibited more cellular activity than CET subjects (p=.042) and PTD-EAS animals (p=.042) (Figure 17). Additionally, for Slice 9 (Figure 18): there were no overall differences (F(5,1)=1.077, p=.394), yet there was still a significant decrease in cellular activity in CET animals as compared to PFs (p=.037) (Figures 17 and 18).

**Lateral OFC Activity**

Arc immunoreactive cell counting revealed that there were no overall significant differences in the lateral OFC across groups (F(5, 31)=.535, p=.748). However, there were still differences across the anterior (Figure 19) versus posterior (Figure 20) axis
with significantly greater overall Arc immunoreactivity in the posterior region \((F=(5, 31)=28.688, p<.01)\). Dunnett's test found that there were no significant differences in treatment groups as compared to controls (all p’s >.05).

**Exploratory Analysis: Lateral OFC**

LSD analysis revealed that there were differences in cellular activity in posterior slices, which will be discussed specifically. For Slice 7 (Figure 21): PF groups had more active cells than CET (\(p=.018\)), PTD-EAS (\(p=.010\)), and PTD-MAS (\(p=.010\)), additionally there was a trend toward more activity in PFs compared to both the CET-PTD subjects (\(p=.082\)) and CET-TH animals (\(p=.055\)). For Slice 8 (Figure 22): there were no overall significant differences \([F(5,1)=2.011, p=.106]\) however PF subjects had significantly more cellular activity than all treatment groups; CET (\(p=.020\)); CET-PTD (\(p=.034\)); CET-TH (\(p=.019\)); PTD-EAS (\(p=.010\)); PTD-MAS (\(p=.014\)). Finally, in Slice 9 (Figure 23): there were no overall significant differences \((F(5,1), p=.085)\) though PF animals again had higher cellular activity than other groups; CET-PTD (\(p=.007\)); CET-TH (\(p=.042\)); PTD-EAS (\(p=.010\)); PTD-MAS (\(p=.014\)). In this case, there was also a trend toward PFs having higher activity than CET subjects (\(p=.076\)).

**Infralimbic Cortex**

There were no overall differences in Arc immunoreactivity in the infralimbic cortex \((F(5,31)=.853, p=.524)\), however there were significant differences across the anterior-posterior axis; the anterior portions again had greater Arc immunoreactivity \((F(1,30)=14.869, p<.01)\). Dunnett’s test revealed that there were no significant differences in treatment groups as compared to PFs (all p’s>.05) (Figures 24 and 25).

**Exploratory Analysis: Infralimbic Activity**
In the case of the infralimbic cortex (Figure 26) in Slice 3: although there were no overall differences ($F(5,1)=.264, p=.264$) there was significantly reduced activity in the PTD-EAS subjects as compared to PFs ($p=.038$). In Slice 4 (Figure 27): there were no significant overall differences across groups ($F(5,1)=1.037, p=.414$), however due to a reduction of activity in PTD-EAS animals, and an increase in PTD-MAS subjects as compared to PFs, there was a strong trend ($p=.051$) according to LSD analysis.

Prelimbic Cortex

There were no overall differences in Arc staining in the prelimbic cortex across treatment groups ($F(5,31)=504, p=.771$). However, again there were significantly more Arc positive cells in the anterior regions ($F(1,30)=411,420, p=.019$). Dunnett’s test revealed no significant differences across groups when compared to PFs (all $p's>.05$) (Figures 28 and 29).

**Exploratory Analysis: Prelimbic Activity**

In the prelimbic cortex (Figure 30) there was again differential cellular activity in the anterior region. In Slice 1: there were no overall differences ($F(5,1)=1.086, p=.390$), and PF animals had significantly more cellular activity than PTD-MAS subjects ($p=.046$).

**Discussion**

The differential delay discounting curves demonstrated by CET and PTD-MAS animals indicate that both of these treatments result in increased impulsive choice behavior. Additionally, post reversal delay discounting curves demonstrated that this behavior can be ‘trained out’ (reduced) with task exposure in CET subjects. That is to say that, with practice, these impulsive choice tendencies can be ameliorated. An important difference is that in PTD-MAS subjects there was an increase in impulsive
behavior in the initial delay discounting task and this impulsivity may not be alleviated by training during the post reversal phase as indicated by exploratory LSD analysis.

Another key point is that the animals exposed to chronic ethanol that received thiamine supplementation (CET-Th) did not develop an impulsive phenotype. Additionally, there is a non-significant, but visible, downward shift in the discounting curve of subjects with milder thiamine deficiency (PTD-EAS) indicating a possibility that mild thiamine deficiency could lead to a slightly more impulsive tendency, which combined with CET, but not seen in CET-PTD, could result in significant differences in impulsive choice behavior.

However, as illustrated in figures 11 and 12, in this study the CET-PTD subjects demonstrated a unique phenotype with potential subpopulations with dissimilar approaches to the task. This may be due to differences in neuropathology induced by these comorbid treatments, an avenue for further investigation. Although there were not statistically significant differences in CET-PTD subjects’ delay discounting curves from those of PF subjects, this seems to have changed when examined with regard to IVD or thalamic pathology. Subjects with reduced interventricular distance had a pattern of slightly higher, though non-significant, levels of impulsivity than members of their group whose IVDs were not two standard deviations below the mean of the PF group in the initial delay discounting phase (Figure 11). This difference persisted during the reversal phase as well (Figure 12), though there were no observable differences by the 60 second time point in this case.

Additionally, PTD-EAS subjects also exhibited this behavioral dichotomy when compared based on IVD, illustrating a distinct difference in these two ‘subpopulations’ during the initial delay discounting phase (Figure 11) with those with thalamic pathology being more impulsive. These observations suggest that thalamic damage is associated
with the generation of the pervasive impulsive phenotype. Importantly, thalamic lesions (a hallmark of WKS) which induce reductions in IVD are a direct consequence of thiamine deficiency (for review see Savage, 2014), further implicating this nutritional deficiency in the production of the impulsive phenotype.

Volumetric data indicated that there is a significant increase in the volume of the limbic cortex, but not OFC, in CET animals compared to PF, based on LSD analysis. This could be due to a compensatory or inflammatory response to chronic ethanol treatment. Additionally, there was a trend toward larger limbic volumes in the CET group as compared to CET-Th animals which should be noted. This may indicate that this increase can be prevented with thiamine supplementation. Although there is established evidence that chronic ethanol exposure can result in cortical shrinkage, there is also differential vulnerability to ethanol exposure across brain regions (Archibald, Fennema-Notestine, Gamst, Riley, Mattson, and Jernigan, 2001; for review see Butterworth, 2003; for review see Goodlett, and Horn, 2001; for review see Sullivan and Pfefferbaum, 2005; Volkow and Fowler, 2000; Ridderinkhof, Vlut, Bramlage, Spaan, Elton, Snel, and Band, 2002). Because volumetric analysis in this study encapsulated each region from anterior to posterior as well as medial to lateral as a single data point, regional differences across the tissue may not have been evident in the overall analysis. Therefore, further studies could address the possibility that shrinkage in these regions is selective across these dimensions, and if some regions increase in volume due to a compensatory mechanism as may be suggested in our exploratory analysis in the case of CET subjects.

This point is furthered by the IHC data observing cellular activity using the immediate early gene Arc, according to ANOVA (anterior-posterior differences) and exploratory (LSD) analysis. Although these were low powered analyses, and are therefore highly speculative, we observed a significant decrease in cellular activity in the posterior
orbitofrontal cortex of all treatment groups compared to controls. In the limbic cortices, however, differences were seen in the more anterior regions in the PTD-MAS animals as compared to all other groups. This could be an artifact of region specific vulnerability that could be explored in future work.

Importantly, however, this differential cellular activity is also consistent with past literature, which informed the decision to conduct post hoc analyses in this study, that indicates functional specificity within the anterior-posterior and medial-lateral axis of the OFC. Studies have demonstrated that the medial OFC is implicated in the processing of reward value, while the lateral portion is involved in punishment evaluation (for meta-analysis and review see Kringlebach and Rolls, 2004; O’Doherty, Kringlebach, Rolls, Hornak, and Andrews, 2001). Differences in medial OFC activity demonstrated in the most posterior, 8th and 9th, slices of the CET subjects, significantly less than PF, may indicate a deficit or alteration in the processing of reward value. Also consistent with the literature is the posterior location of these differences, as this region is implicated in the processing of simple rewards (e.g., food) as opposed to the anterior region which processes complex or learned rewards (e.g., cue, money) (for meta-analysis and review see Kringlebach and Rolls, 2004; O’Doherty et al., 2001; De Araujo, Kringlebach, Rolls, and McGlone, 2003b; De Araujo, Kringlebach, Rolls, McGlone, and Phillips, 2003c).

It is important to note that the Arc immunoreactivity indicating cellular activity monitored in this study is specific to cellular activity at the 60 second time point, because subjects were sacrificed at the culmination of their last day of stability (or at ceiling) 90 minutes after completion. This is the critical window for optimal Arc immunoreactivity (Lonergan, Gafford, Jarome, and Helmsetter, 2010); however, it only demonstrates the most recent activity. Because of the 60 second delay most recently presented to the animals, there could have been reduced cellular activity inherent to that phase of the
task, as 41% of neurons in the OFC fire in response to rewards received after a short delay, whereas only 17% respond in anticipation of delayed rewards. This means that there is inherently more activity for short delay rewards (Roesch, Taylor, and Schoenbaum, 2006). However, at the 60 second time point there were no statistically significant differences between groups indicating that this differential activity may be due to reward processing differences rather than exposure to delay, because all subjects were choosing the small delay for the immediate reward.

Because in this study we observed, according to limited exploratory analysis, that there was significantly more cellular activity in the posterior three sections of the lateral OFC, with the PF subjects displaying significantly more activity, there may be an alteration in the processing of the aversive effects of delay (if considered punishing) across all treatment groups.

Based on both behavioral and histological data, there is a possibility that thiamine supplementation could slow or ameliorate the effects of chronic alcohol exposure on choice impulsivity and potentially rescue some of the neuropathological changes that occur in instances of chronic alcohol consumption. For example, there is a visually evident, though non-significant, increase in impulsivity in the CET-Th group as evidenced in the apparent increase in slope during delay discounting. This may indicate that the progression of developing an impulsive phenotype is partially due to thiamine deficiency because of the malabsorption of nutrients induced by chronic alcohol consumption which seems to be slowed by thiamine supplementation. Bolstering this argument is the apparent change in the behavior of the PTD-EAS subjects toward a more impulsive phenotype, though not significantly different from PF animals. Importantly, excessive thiamine deficiency at the level seen in the PTD-MAS animals not only results in an impulsive phenotype, these subjects become more impulsive than
those with chronic alcohol exposure alone and the ‘practice effects’ due to protracted
task exposure seen in CET subjects are absent from this group. This indicates that there
is a more persistent change in impulsive choice behavior that is dependent on thiamine
deficiency.

An additional point that warrants further investigation is the role of the limbic
cortex in the pervasive impulsive phenotype exhibited by the PTD-MAS group. This was
the only group that retained impulsive decision making behavior after excessive training.
They were also unique in their differential activity in the anterior portion of this region,
specifically the anterior prelimbic cortex. Because of this, the limbic cortical insults may
be critical in prolonged impulsive choice behavior. The differences in PTD-EAS cellular
activity in this region may have been driven by subjects in the low IVD ‘subpopulation’,
another point of further investigation.

The results observed in the CET-Th group demonstrated attenuated levels of
impulsivity and no significant differences from PF controls on neurohistological
assessments. This indicates a protective effect of thiamine supplementation in chronic
ethanol exposure. As discussed by Martin et al. (2003), chronic alcohol exposure can
culminate in an inadequate uptake of thiamine due to decreased absorption in the
gastrointestinal tract (Martin, Singleton and Hiller-Sturmhofel, 2003; for review see
Hoyumpa, 1980). Although in this study nutritional uptake was not measured (a potential
avenue for further investigation), there are two mechanisms involved. These were
discussed by Hoyumpa (1980); first a reduction in gastric absorption, and therefore a
potential for reduced thiamine intake into cells, and secondly impaired cellular utilization
of nutrients, in this case thiamine, may have been a factor in our CET groups. This may
have been prevented by thiamine supplementation in the CET-Th group, attenuating the
negative side effects of alcohol induced thiamine deficiency. Additionally, in animal
studies it has been demonstrated that when thiamine is in low concentrations active transport is necessary for thiamine to traverse the cellular membrane. In higher concentrations, there is a chemical gradient which allows for passive transport of the molecule into the cell (for review see Martin et al., 2003, Hoyumpa, 1980). This differential mechanism may play a role in allowing for thiamine absorption in the CET-Th subjects. However, this was not explored in this study and leaves another venue open for investigation. Another factor may have been differences in food consumption across both this group, as well as CET-PTD, as compared to PF subjects, who weighed significantly more on average (Figure 4).

This research bolsters previous studies which have indicated that thiamine supplementation may be beneficial in cases of chronic alcohol consumption. For example, studies exploring the effect of thiamine supplementation administered to chronically ethanol treated lab animals and alcoholic human patients have demonstrated that this treatment ameliorated both metabolic and behavioral consequences of alcohol induced thiamine deficiency (Lee, Tarter, Holburn, Price, Winestein and Martin, 1995; Victor, Addams, and Collins, 1989). Additionally, experiments examining the effects of thiamine supplementation on human subjects in recovery have demonstrated that the most benefit is garnered from high dose thiamine treatment when considering working memory performance (Ambrose, Bowden, and Whelan, 2001).

In line with these investigations, the use of thiamine supplementation as a prophylactic treatment has been recommended in the past. It has been suggested that preventative thiamine supplementation of 250 mg IM should be administered to at risk patients (Cook and Thomson, 1997; Thomson and Cook, 1997; Cook, 2000; Thomson, 2000; Hope, Cook and Thomson, 1999). Prophylactic thiamine treatment has also been suggested by Bligh and Madden (1983). Our current study bolsters this argument by
demonstrating that thiamine supplementation concomitant with alcohol exposure ameliorates the chronic alcohol induced impulsive phenotype as well as the neuropathological changes seen in this treatment group.

This study also provides justification to investigate potential prophylactic treatments for alcoholics or those in treatment to prevent further neurological insult. Additionally, we have demonstrated separable, but linked, roles for the OFC and limbic cortices that provide a foundation for further inquiry.
Figure 1. Treatment and behavioral testing timeline depicting chronic ethanol and PTD treatment, blood sample collection, recovery, and behavioral testing.
Figure 2. Example regions for Cavalieri Volumetric Analysis of the orbitofrontal cortex (OFC), prelimbic (PL), and infralimbic (IL) cortices on representative NeuN stained frontal tissue.
Figure 3: Example regions for Arc immunoreactive cellular counting analysis of medial and lateral OFC (mOFC and IOFC) as well as prelimbic and infralimbic cortex (PL and IL). All regions were .8 x .6 mm.
Figure 4. Average weights for PF and ethanol treated groups across the period of ethanol treatment encompassing fade on and fade off demonstrate no differences. (n per group: PF=8; CET=8; CET-TH=8; CET-PTD=9)
Figure 5. A) Ethanol consumed in g/kg body weight for PF subjects and all CET groups. B) Weeks 1-3 encompass the ‘Fade On’ portion of CET titrating up from 0 (6%, 9%, 12% EtOH). C) Weeks 29-31 are the ‘Fade Off’ of treatment prior to recovery. (n per group: PF=8; CET=8; CET-TH=8; CET-PTD=9)
Figure 6. BEC levels measured at months 1, 2/3, 4, and 6 were not different as a function of group within CET treated animals. However, the BECs of rats exposed to EtOH increased across time. The BECs from these groups were significantly above PF rats given water. (n per group: PF=8; CET=8; CET-TH=8; CET-PTD=9)
A. Days to Reach Criterion  

B. Delayed Discounting Curve (Last 6 Days)

C. Nose Poke Omissions by Delay  

D. Nose Poke Omission Averages

E. Lever Omissions by Delay  

F. Lever Omission Averages

Figure 7. A). Days to reach behavioral stability during initial delay discounting phase. B) Delay lever choice behavior (%) across increasing delays (s) C) Nose poke omissions across increasing time points. D) Average nose poke omissions during the last 6 days of delay discounting. E) Lever omissions by delay. F) Average lever omissions. (n per group: PF=8; CET=8; CET-TH=8; CET-PTD=7; PTD-EAS=9; PTD-MAS=9)
A. Reversal Average Delay Lever Choice  B. Reversal-First 6 Days

C. Lever Omissions by Delay  D. Lever Omission Averages

E. Nose Poke Omissions by Delay  F. Nose Poke Omission Averages

Figure 8. A). Days to reach behavioral stability. B) Delay lever choice behavior (%) across increasing delays (s) (*) indicate significant differences from PF at a particular time point). C.) Lever omissions by delay D.) Average lever omissions E.) Nose poke omissions across increasing time points illustrating that PTD-MAS and CET-Th rats are less likely to fail to initiate trials F). Average nose poke omissions during reversal learning phase. (n per group: PF=8; CET=8; CET-TH=8; CET-PTD=7; PTD-EAS=8; PTD-MAS=10)
Figure 9. A) Days to reach reversal phase delay discounting stability criterion (i.e., behavioral stability). B) Delay lever choice behavior (%) across increasing delays (s) (* indicate significant differences from PF at a specific time point). C) Nose poke omissions across increasing time points. D) Average nose poke omissions during the last 6 days of delay discounting. E) Lever omissions as delay to reward increases. F) Average lever omissions by treatment. (n per group: PF=8; CET=7; CET-TH=7; CET-PTD=7; PTD-EAS=9; PTD-MAS=9)
Figure 10. Average interventricular distance demonstrating reductions (n.s.) in PTD-EAS and PTD-MAS treatment groups and significant reductions in CET-PTD rats. (n per group: PF=6; CET=7; CET-TH=3; CET-PTD=6; PTD-EAS=7; PTD-MAS=7)
Figure 11. Initial phase delay discounting curves with PTD subjects parsed based on interventricular distance (IVD). Groups designated ‘sm’ have IVDs 2 standard deviations below the mean of PF treated animals. (n per group: PF=8; CET=7; CET-TH=7; CET-PTD=3; PTD-EAS=6; PTD-MAS=7; C-P Sm=3; EAS Sm=2; MAS Sm=2)
Figure 12. Reversal phase delay discounting curves with PTD subjects parsed based on interventricular distance. Groups designated ‘sm’ have IVDs 2 standard deviations below the mean of PF treated animals. (n per group: PF=8; CET=7; CET-TH=7; CET-PTD=3; PTD-EAS=6; PTD-MAS=7; C-P (CET-PTD) Sm=3; EAS Sm=2; MAS Sm=2)
Figure 13. Cavalieri volumetric analysis of orbitofrontal cortex volumes demonstrating no differences across groups. (n per group: PF=7; CET=7; CET-TH=6; CET-PTD=7; PTD-EAS=8; PTD-MAS=9)
Figure 14. Cavalieri volumetric analysis of limbic cortex volume demonstrating increased volume in the CET group as compared to PF animals. (n per group: PF=7; CET=6; CET-TH=7; CET-PTD=7; PTD-EAS=8; PTD-MAS=9)
Figure 15. Average anterior Arc immunoreactive cells in the antero-medial orbitofrontal cortex. (n per group: PF=5; CET=5; CET-TH=4; CET-PTD=4; PTD-EAS=5; PTD-MAS=4)
Figure 16. Average Arc immunoreactivity in postero-medial orbitofrontal cortex neurons. 
(n per group: PF=5; CET=5; CET-TH=4; CET-PTD=4; PTD-EAS=5; PTD-MAS=4)
Figure 17. Arc immunoreactive cell counts indicating higher cellular activity in PFs as compared to CET and PTD-EAS subjects. (n per group: PF=5; CET=6; CET-TH=7; CET-PTD=5; PTD-EAS=7; PTD-MAS=7)
Figure 18. Active cell counts demonstrating decreased cellular activity in CET subjects as compared to PFs. (n per group: PF=5; CET=5; CET-TH=5; CET-PTD=6; PTD-EAS=8; PTD-MAS=6)
Figure 19. Average antero-lateral orbitofrontal cortex Arc immunoreactivity. (n per group: PF=4; CET=3; CET-TH=3; CET-PTD=4; PTD-EAS=7; PTD-MAS=4)
Figure 20. Average postero-lateral orbitofrontal cortical Arc immunoreactivity. (n per group: PF=4; CET=3; CET-TH=3; CET-PTD=4; PTD-EAS=7; PTD-MAS=4)
Figure 21. Cellular activity counts demonstrating higher activity in PF animals as compared to other groups. (n per group: PF=5; CET=5; CET-TH=5; CET-PTD=6; PTD-EAS=8; PTD-MAS=7)
Figure 22. Counts of active cells demonstrating that PFs had higher cellular activity as compared to all other groups. (n per group: PF=5; CET=6; CET-TH=5; CET-PTD=6; PTD-EAS=7; PTD-MAS=7)
Figure 23. Counts of active cells demonstrating that PFs had higher cellular activity as compared to all other groups. (n per group: PF=5; CET=3; CET-TH=5; CET-PTD=6; PTD-EAS=8; PTD-MAS=6)
Figure 24. Average anterior infralimbic cortical Arc immunoreactivity. (n per group: PF=2; CET=2; CET-TH=2; CET-PTD=3; PTD-EAS=3; PTD-MAS=5)
Figure 25. Average posterior infralimbic Arc immunoreactivity. (n per group: PF=2; CET=2; CET-TH=2; CET-PTD=3; PTD-EAS=3; PTD-MAS=5)
Figure 26. Counts of active cells demonstrating that PFs had significantly more cellular activity than PTD-EAS subjects. (n per group: PF=5; CET=6; CET-TH=5; CET-PTD=5; PTD-EAS=7; PTD-MAS=6)
Figure 27. Active (Arc positive) cell counts demonstrating that PTD-EAS animals differed from PTD-MAS animals as they decreased and increased as compared to controls, respectively. (n per group: PF=5; CET=6; CET-TH=5; CET-PTD=6; PTD-EAS=8; PTD-MAS=7)
Figure 28. Average anterior prelimbic Arc immunoreactivity. (n per group: PF=4; CET=5; CET-TH=4; CET-PTD=5; PTD-EAS=6; PTD-MAS=5)
Figure 29. Average posterior prelimbic Arc immunoreactivity. (n per group: PF=4; CET=5; CET-TH=4; CET-PTD=5; PTD-EAS=6; PTD-MAS=5)
Figure 30. Active cell counts demonstrating that PF subjects had significantly more activity than PTD-MAS animals. (n per group: PF=5; CET=5; CET-TH=5; CET-PTD=5; PTD-EAS=8; PTD-MAS=6)
References:


