Glutamatergic mechanisms in behavioural sensitization to ethanol

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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University of Toronto

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Abstract

Repeated exposure to ethanol (EtOH) in mice produces behavioural sensitization. However, not all mice sensitize to EtOH and in any cohort high-sensitizers (HS) can readily be distinguished from low-sensitizers (LS). The neural mechanisms mediating vulnerability and resistance to EtOH sensitization remain unclear. Given the prominent role of glutamate in neuroplasticity, we examined whether this system might explain the variable response to EtOH.

In the first set of experiments, we measured nucleus accumbens (NAc) glutamate levels in LS and HS mice following an EtOH challenge two weeks after sensitization development. EtOH-induced glutamate release in the NAc was present in HS mice and use of a glutamate release blocker prevented the expression of sensitization. To ascertain whether NAc glutamate was also elevated in HS mice during the development of sensitization, we measured levels after the 1st and 5th EtOH injection and found them unchanged in HS mice. These results suggest that NAc glutamate may not be necessary for the development of EtOH sensitization, but it appears essential for the expression of a sensitized response.
We next studied the role of NMDARs in EtOH sensitization. LS mice demonstrated elevated NMDAR subunit gene expression across the brain compared to HS and saline-treated mice. However, NMDAR antagonism with MK-801 and CGS 19755 did not interfere with EtOH sensitization, indicating perhaps that NMDARs may not be crucial to sensitization.

A final goal was to examine whether LS and HS mice differ in the expression of the plasticity markers pCREB, Bdnf, trkB, and Arc, and if they show structural changes in NAc neurons. HS mice showed increased pCREB expression in the NAc. LS mice showed reduced trkB and Bdnf expression across the brain, however inhibition of BDNF-TrkB signaling had no effect on EtOH sensitization. LS mice also presented greater stubby spine density in the NAc and increased Arc mRNA in several brain areas when compared to saline controls and HS mice.

Taken together, these results demonstrate variation in the glutamate system between LS and HS mice. This thesis discusses these differences and how they may contribute to individual differences in sensitization response.
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>Arc</td>
<td>Activity-regulated cytoskeleton-associated protein</td>
</tr>
<tr>
<td>Bdnf</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BEC</td>
<td>Blood ethanol concentration</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response-element binding protein</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>GABA</td>
<td>Gamma-amino butyric acid</td>
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<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>HS</td>
<td>High sensitized</td>
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<tr>
<td>IEG</td>
<td>Immediate early gene</td>
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<td>LMA</td>
<td>Locomotor activity</td>
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<td>LS</td>
<td>Low sensitized</td>
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<td>MSN</td>
<td>Medium spiny neuron</td>
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<td>NAc</td>
<td>Nucleus accumbens</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
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<tr>
<td>NR1 KD</td>
<td>NR1 Knockdown</td>
</tr>
<tr>
<td>SAL</td>
<td>Saline</td>
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Introduction

1. Behavioural sensitization

1.1 Definition

Early laboratory drug studies reported an interesting phenomenon whereby repeated, intermittent exposure to psychostimulants produced progressive increases in response to that drug, a phenomenon now referred to as behavioral sensitization (Post and Rose, 1976; Rebec and Segal, 1979; Segal and Mandell, 1974; Segal et al., 1980). Since its emergence, many laboratories across the world have successfully produced sensitization following repeated drug treatment of abused drugs, highlighting the robustness of this phenomenon (Vanderschuren and Kalivas, 2000b).

Behavioural sensitization is thought to reflect various neurobiological changes that have been implicated in addiction processes (Robinson and Berridge, 2000a). Sensitization has been shown to persist for weeks and months after the last drug treatment and this has led to its classification as a form of pathological experience-dependent plasticity (Hyman and Malenka, 2001). Indeed, sensitizing regimens of psychostimulants have been shown to induce long-term changes in brain regions involved in reward and motivation (Luscher, 2013; Robinson and Kolb, 2004).

1.2 Features of behavioural sensitization

Many drug effects become sensitized following repeated, intermittent exposure. Behavioural responses that become enhanced include: stereotypic behaviours; repetitive movements such as head bobbing, circling, grooming, sniffing, and nail biting; and locomotor activity (Leith and Kuczenski, 1982; Robinson and Berridge, 2000b). Subjective states such as a drug’s euphoric and reinforcing effects also become sensitized with repeated treatment.
Locomotor activity is the most common behaviour used to assess sensitization because it can be easily be measured with activity monitors and reliably increases following chronic drug treatment, thereby indicating that a change in behaviour has taken place (Eilam and Szechtman, 1989; Masur and Boerngen, 1980a).

Repeated drug treatment not only sensitizes behavioral responses, but also enhances neurochemical responses, and it is believed that behavioral sensitization is a manifestation of these drug-induced neuronal adaptations in the brain (Kalivas, 1995; Pascoli et al., 2012; Pierce and Kalivas, 1997).

1.3 Development and expression of behavioural sensitization

The neural mechanisms underlying behavioural sensitization have been conceptualized into two distinct anatomical and temporal phases, namely a development and expression phase (Kalivas and Stewart, 1991). The development phase refers to the progressive increases in behaviour and the accompanying molecular and/or cellular effects of the drug, while the expression phase refers to the long-term consequences of these effects as manifested during a drug challenge after a period of no exposure (Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000b).

Studies suggest that the development of sensitization is mediated largely by the ventral tegmental area (VTA), whereas the expression of sensitization involves largely the nucleus accumbens (NAc) (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000b). Several studies show that repeated microinjections of psychostimulants in the VTA results in behavioural sensitization to a systemic psychostimulant challenge, an effect not seen with repeated infusions in the NAc (Dougherty and Ellinwood, 1981; Hitzemann et al., 1980; Hooks et al., 1992a; Kalivas and Weber, 1988; Vezina and Stewart, 1990). Also,
sensitization induced by repeated VTA administration of amphetamine is associated with increased extracellular dopamine (DA) in NAc following a drug challenge and an increased behavioural response to amphetamine microinjected in the NAc (Perugini and Vezina, 1994; Vezina, 1993). Intriguingly, repeated drug infusion into the NAc elicits locomotor activation that does not produce an enhanced response following repeated intra-NAc infusions, highlighting the anatomical separation of the development and expression phases constituting sensitization (Cador et al., 1995). In the VTA, it is largely enhanced somatodendritic dopamine release that is believed to play a critical role in the development of sensitization, whereas changes in the mechanisms regulating presynaptic release in the NAc account for the expression of sensitization (Kalivas and Stewart, 1991).

Although dopamine neurotransmission in the VTA and NAc have received considerable attention for their role in behavioral sensitization, other neurotransmitters and neuroanatomical regions interacting with these structures are also involved, as highlighted in Figure 1 (Pierce and Kalivas, 1997).
Figure 1. Behavioural sensitization circuitry. This diagram depicts the major neurotransmitter pathways and anatomical regions involved in behavioural sensitization, adapted from Pierce and Kalivas, 1997. VTA ventral tegmental area, VP ventral pallidum, NAc, nucleus accumbens, PFC prefrontal cortex, DA dopamine, Glu glutamate, GABA gamma-aminobutyric acid.
1.4 Relation to addiction

The notion that the behavioural stimulant effects of abused drugs might be related to their positive reinforcing properties was first proposed by Wise and Bozarth in their psychomotor theory of addiction in 1987. According to this theory, drugs of abuse have psychomotor stimulant properties, as well as positive reinforcing effects, and that both of these characteristics arise from activation of the same mesolimbic dopamine (DA) pathway (Wise and Bozarth, 1987b). In 1993, Robinson and Berridge extended this theory to include behavioural sensitization—if psychomotor activation shared a common neural basis with drug reward, then psychomotor responses that become sensitized by repeated drug exposure might be indicative of a sensitized DA system and therefore increased positive reinforcing effects of the drug (Robinson and Berridge, 1993). This theory, the incentive sensitization theory of addiction, has been one of the most prominent theories of drug addiction, arguing that repeated exposure to addictive drugs results in persistent neuroadaptations in brain regions regulating reward and motivation. Consequently, these brain systems become sensitized to the drug and drug-associated cues, increasing the incentive to consume a drug or to engage in drug-seeking behaviour. As a result, the drug and its associated stimuli have increased incentive salience; they become highly desired/wanted, without necessarily being highly liked, leading to increased drug intake and drug seeking behaviour.

The incentive sensitization theory can explain several components of addiction, including its persistent nature, the presence of drug craving, the associative nature of addiction, the compulsive nature of drug use (i.e., highly wanted), individual vulnerability in the risk to develop addiction, and relapse (Robinson and Berridge, 2001). According to this theory, persistent drug-induced neuroadaptations make neural circuits regulating reward and
motivation highly sensitive to the drug and drug-associated stimuli, such that there is a recurring desire to take the drug (i.e., craving), even after years of abstinence. Indeed, the high rates of relapse in abstinent drug-users, and the desire to use the drug when surrounded by environmental cues that have become associated with the drug (by virtue of repeated drug-environment pairings) have been attributed to this persistent change in neural circuitry. Furthermore, not everyone who takes an addictive substance will develop an addictive disorder; there is variability in the risk to develop addiction. The incentive sensitization theory posits that factors conferring susceptibility to sensitization will also contribute to the variation in susceptibility to addiction, which could contribute to the transition from controlled, casual drug use to compulsive drug use and addiction (Robinson and Berridge, 2000b; Robinson and Berridge, 2001).

Pre-clinical research demonstrates that behavioural sensitization shares many characteristics with addiction. Indeed, individual variability in the sensitization response exists, sensitization is long-lasting and has been shown to persist for months or years after cessation of the inducing drug, and it is context-dependent, being most robustly expressed in the same environment where repeated drug exposure took place (Robinson and Berridge, 2001). Furthermore, it has been associated with drug self-administration and relapse to drug-seeking behaviour, although there are inconsistencies in the literature in this respect (De Vries et al., 2002; Marinelli et al., 1998; Piazza et al., 1990; Schenk and Partridge, 1997; Steketee and Kalivas, 2011).
1.5 Sensitization in humans

Several reports have demonstrated evidence of behavioural and neurochemical sensitization in humans. Some measures showing progressive increases after repeated psychostimulant exposure include psychomotor responses (vigor and eye-blink rates), subjective ratings of euphoria, vigor, drug-liking, elation, arousal and positive mood, as well as changes in physiological measures such as heart rate and blood pressure have been reported (Boileau et al., 2006; Johanson and Uhlenhuth, 1981; Kollins and Rush, 2002; Strakowski and Sax, 1998). In addition, neurochemical sensitization has also been documented. The ability of amphetamine and cocaine to elevate DA levels in the striatum was reported to increase with previous history, and the magnitude of cocaine-induced DA release in the NAc was found to be positively correlated with lifetime stimulant (cocaine and amphetamine) use (Boileau et al., 2006; Cox et al., 2009). However, despite these findings that demonstrate behavioural and neurochemical sensitization following repeated drug administration in humans, there have been reports that failed to demonstrate enhanced responses following repeated psychostimulant exposure (Gorelick and Rothman, 1997; Kegeles et al., 1999; Kelly et al., 1991; Nagoshi et al., 1992; Rothman et al., 1994; Strakowski et al., 1997; Wachtel and de Wit, 1999).

Inconsistencies in the evidence for behavioural sensitization in humans is likely to arise from differences in dose (5-30 mg, p.o.), dosing regimen (2-5 doses, with 1 day-12 months between doses), subject population (healthy controls vs. substance dependent), and the dependent measures (Leyton, 2007). In addition, it seems that there are optimal conditions for inducing behavioural sensitization that are specific to the drug of interest. In the case of amphetamine, sensitization can be demonstrated after 2 to 3 exposures, administered intermittently in the 20-30 mg range, and can last up to one year (Boileau et al., 2006; Leyton, 2007). However, such optimal conditions for identifying cocaine sensitization have not yet
been established, perhaps because studies were conducted on cocaine dependent subjects who may already be maximally sensitized, or perhaps more than two drug administrations are needed to observe sensitization (Gorelick and Rothman, 1997; Nagoshi et al., 1992; Rothman et al., 1994). These findings suggest that there is evidence to support the existence of behavioural sensitization to psychostimulants in humans and that further studies may be required to determine the optimal parameters/ideal protocol for inducing sensitization to a particular drug.

2. Ethanol sensitization

2.1 Characteristics

Nearly every drug of abuse leads to increased DA release in the NAc, which is believed to be critical to the development of addictive behaviours (Di Chiara and Imperato, 1988; Koob, 1992; Volkow and Morales, 2015). Although this elevation of DA is common among drugs of different classes, the mechanism by which this comes about differs. For example, cocaine inhibits and amphetamine reverses the DA transporter on presynaptic terminals in the NAc, thereby increasing DA levels in this area directly (Schmitt and Reith, 2010). Other drugs of abuse elevate NAc DA levels indirectly by activating VTA (nicotine) or disinhibiting VTA DA neurons (EtOH and morphine), ultimately leading to increased VTA DA neuron activity and subsequently increased DA release in the NAc (Chen et al., 2015; Liu et al., 2012; Nestler, 2005). Both types of drugs, those directly and those indirectly elevating accumbal DA levels, produce behavioral sensitization. Although behavioral sensitization has been demonstrated following repeated intermittent administration of a variety of drugs of abuse, it has been most extensively studied using the psychostimulants cocaine and amphetamine (Robinson and Berridge, 2001).
Although there is a paucity of data examining the neural mechanisms underlying behavioural sensitization to EtOH in comparison to the psychostimulants, several reports demonstrate that robust EtOH sensitization does indeed occur and is replicable across laboratories (Abrahao et al., 2009; Meyer et al., 2005; Nona et al., 2015b; Stevenson et al., 2008; Umathe et al., 2009).

Several interesting characteristics unique to EtOH sensitization have been reported. The stimulant effects of EtOH have a narrow time frame of onset and testing duration must be within this time. In particular, the stimulant effects of EtOH in mice are evident within the first 0-15 minutes of administration, peaking within 5 minutes after administration, after which the sedative effects predominate (Crabbe et al., 1982; Lessov and Phillips, 1998; Masur and Boerngen, 1980b; Phillips et al., 1991). As such, measuring the locomotor response to EtOH within the first 15 mins of administration isolates the stimulant effects of EtOH.

Another interesting characteristic of EtOH sensitization is that it is elicited within a narrow dose range. Generally, doses in the range of 1.8-2.5 g/kg have consistently been shown to produce a progressive increase in locomotor activity following repeated treatment (Bahi and Dreyer, 2012; Crabbe et al., 1982; de Araujo et al., 2009; Masur and Boerngen, 1980b; Palmer et al., 2003). This enhanced, sensitized response did not diminish, persisting even after 5 months of repeated treatment (Masur and Boerngen, 1980b). Chronic treatment of lower doses of EtOH, ranging from 1.0-1.5 g/kg, do not alter locomotor activity, whereas higher doses, ranging between 3.5-4.0 g/kg, acutely produce depressant effects which show tolerance after repeated treatment and are then replaced by a slight increase in activity (Masur and Boerngen, 1980a). These findings demonstrate the long-lasting nature of sensitization and suggest the possibility that tolerance to the depressant effects of EtOH may unmask its stimulant effects.
Interestingly, sensitization to EtOH has been most reliably demonstrated in mice only. Low doses of EtOH which elicit stimulation in mice fail to do so in rats, who seem to be insensitive to the stimulant effects of EtOH (Duncan and Baez, 1981; Frye and Breese, 1981; Masur et al., 1986). Although rats demonstrate tolerance to the sedative effects of EtOH, an unmasking of its stimulant effects does not occur (Masur et al., 1986). This is interesting given the robustness of this phenomenon in mice, which has been demonstrated in several laboratories, using different injection protocols, time courses, and routes of administration (Abrahao et al., 2011; Meyer et al., 2005; Nona et al., 2015b; Tabakoff and Kiianmaa, 1982; Zapata et al., 2006). Masur and colleagues (1986) have suggested that the difference in EtOH (locomotor) sensitization response between rats and mice is largely under genetic control. This is highly plausible given that there are even differences in sensitization response among mouse strains. For example, the DBA/2 (DBA) strain is more susceptible to the development of EtOH sensitization than C57Bl/6 (C57) mice (Hitzemann and Hitzemann, 1997; Phillips et al., 1994; Phillips et al., 1995b). It is important to note, however, that there has been one report suggesting the possibility of EtOH sensitization in rats, therefore it may be possible to induce sensitization in this species once an optimal protocol has been identified (Hoshaw and Lewis, 2001).

2.2 Variability in the EtOH sensitization response

Not only do differences in the sensitization response to EtOH exist between species and across mouse strains, there is also individual variability in the response within strains that do show robust sensitization. Not all mice treated with a sensitizing regimen of EtOH will demonstrate a robust locomotor response (Abrahao et al., 2014; Masur and Lodder Martins dos Santos, 1988; Nona et al., 2013b). Those mice that do sensitize are referred to as high-
sensitizers, HS, or sensitized, whereas those who show only modest sensitization or fail to sensitize at all are known as low-sensitizers, LS, or non-sensitized. In this thesis, HS and LS will be used to identify the sensitization response. The LS/HS classification is based on ranking the locomotor activity scores obtained after the final injection such that mice in the lowest 33% of the distribution are classified as LS, whereas those in the upper 33% are classified as HS (Nona et al., 2013b; Quadros et al., 2002b; Souza-Formigoni et al., 1999). This variability is not due to altered peripheral metabolism of EtOH, suggesting that central mechanisms mediate the divergent response (Quadros et al., 2005). Comparisons between LS and HS allows for the possibility of distinguishing between general pharmacological adaptations induced by chronic EtOH exposure and a subset of those adaptations that might be specific to the process of sensitization.

Behavioural variability in the sensitization response to other drugs has been reported in both mice and rats (Abrahao et al., 2009; Boudreau et al., 2007; Camp and Robinson, 1988; Churchill et al., 1999; Pierce et al., 1996; Scholl et al., 2009). In general, it appears that high locomotor response to novelty is often a reliable predictor of the locomotor stimulant and reinforcing effects of drugs of abuse (Bevins and Peterson, 2004; Hooks et al., 1992a; Hooks et al., 1992b; Hooks et al., 1991; Hoshaw and Lewis, 2001; Kalinichev et al., 2004; Piazza et al., 1989; Wooters et al., 2006). However, there currently appears to be no reliable predictors of the EtOH sensitization response. There have been some reports that mice classified as LS or HS following the final injection also differed in the locomotor response to the first EtOH injection (i.e., acute response), suggesting that the acute response might be a predictor of sensitization (Abrahao et al., 2013; Nona et al., 2013b). However, several reports have failed to find a relationship between the first injection and the response to sensitization (Abrahao et al., 2011; Nona et al., 2015a; Nona et al., 2014; Phillips et al., 1995b). This inconsistency in acute
response predicting sensitization has also been demonstrated with psychostimulants (Badiani et al., 1995; Crombag et al., 2000; Yamamoto et al., 2013).

Very few studies have examined predictors of EtOH sensitization in mice. One study looked at the association between performance in a contextual conditioning task and the development of sensitization (Quadros et al., 2003). It was found that mice demonstrating a greater freezing time on the contextual test were mice that later developed sensitization to EtOH, suggesting that contextual learning may be positively associated with the development of EtOH sensitization. A further study assessed whether basal anxiety levels in mice could predict the sensitization response to EtOH (Botia et al., 2015). The authors found that mice demonstrating low basal anxiety levels developed more robust EtOH sensitization than mice that had higher basal anxiety levels. These reports demonstrate the possible existence of predictors of EtOH sensitization and highlight the need for future research in this area.

In contrast to the few reports examining predictors of sensitization, there have been several studies probing the neural mechanisms associated with individual variability to EtOH sensitization. Differences in several neurotransmitter systems between LS and HS mice have been reported and further studies are needed to examine in detail how these systems contribute to the individual variability present in EtOH sensitization (Camarini and Pautassi, 2016).

2.3 EtOH sensitization in humans

The stimulant effects of EtOH typically begin at relatively low blood alcohol levels (BALs), during the ascending portion of the BAL curve, whereas the sedative effects predominate during the descending limb of the curve (King et al., 2002; Martin et al., 1993). Human studies have shown that increased sensitivity to the stimulant effects and decreased sensitivity to the sedative effects of EtOH are associated with a history of EtOH consumption.
and an increased risk for developing alcoholism. For example, moderate to heavy drinkers appear more sensitive to the stimulant effects and less sensitive to the sedative effects of EtOH in comparison to light drinkers (Fromme et al., 2004; Holdstock et al., 2000; King et al., 2002). Furthermore, non-alcoholic sons of alcoholics (SOA), who are at a high risk for alcoholism, are more sensitive to the stimulant effects of EtOH and less sensitive to its sedative effects compared to sons of nonalcoholics (SONA) (Newlin and Thomson, 1991; Newlin and Thomson, 1999). These studies suggest a potential link between sensitivity to the stimulant effects of EtOH and predisposition to alcohol abuse disorders. Interestingly, human reports of EtOH-induced elation and euphoria have been demonstrated 10-30min after EtOH consumption, a timepoint where the stimulant effects of EtOH peak and when EtOH-induced DA release into the NAc is evident (Boileau et al., 2003). These findings demonstrate that the stimulant effects of EtOH appear to express themselves concurrently with EtOH-induced euphoria and reinforcement, lending support for the claim that EtOH-induced stimulation in rodents may serve as a model of the euphoric effects of EtOH in humans (Phillips and Shen, 1996).

Studies assessing risk factors for alcoholism in humans have typically involved comparisons between SOA and SONA. In the first, and to our knowledge only, EtOH sensitization study in humans, Newlin and Thomson (1991) gave SOA and SONA an oral dose of 0.5g/kg of EtOH once every two days for a total of 3 sessions and assessed a series of peripheral physiological indicators of autonomic arousal (Albus et al., 1982). They reported that SOA, but not SONA, showed an increase in finger pulse amplitude, motor activity, and a trend for increased heart rate across sessions, indicative of sensitization. These results demonstrate that compared to SONA, SOA show increased autonomic reactivity to EtOH.
Interestingly, SOA and SONA do not only differ in degree of autonomic arousal. SOA appear to have less intense depressant responses to EtOH and experience a more heightened reward response to the drug, traits which predict future alcohol problems (Finn and Justus, 1997). Not surprisingly, SOA are 3-5x more likely to develop alcoholism than SONA (Cotton, 1979; Goodwin, 1985). Taken together, such results suggest a close relationship between sensitization to EtOH’s stimulant effects and risk for developing alcohol abuse disorders. Therefore, it is not surprising that differences in the tendency to develop EtOH sensitization may serve as a predictor of future alcoholism (Brodie, 2002; Schmidt et al., 2000).

### 2.4 The Importance of studying EtOH sensitization

Sensitization to EtOH has been implicated in contributing to enhanced alcohol consumption in mice, although there exist inconsistencies in the literature regarding this correlation (Camarini and Hodge, 2004; Grahame et al., 2000; Lessov et al., 2001; Ribeiro et al., 2008). Similarly, in humans, it has been suggested that sensitizing to the stimulant effects of EtOH may serve as a predictor for future alcoholism, suggesting EtOH sensitization may have relevance to alcohol consumption (Hunt and Lands, 1992; Newlin and Thomson, 1991; Newlin and Thomson, 1999; Schmidt et al., 2000). Given that EtOH-induced stimulation of locomotor activity co-occurs with the euphoriant and positive reinforcing effects of EtOH (as discussed above), elucidating the neural mechanisms mediating EtOH sensitization may clarify the role played by neuroadaptations in sustaining a complex behavior like alcoholism.

### 3. Glutamate

#### 3.1 The glutamate synapse

The excitatory neurotransmitter glutamate is a widely distributed, abundant neurotransmitter mediating as much as 70% of synaptic transmission in the CNS (Gass and
The typical glutamate synapse is shown in Figure 2. Glutamate in the presynaptic terminal is packaged into synaptic vesicles by vesicular glutamate transporters (vGluTs). Following the arrival of an action potential, Glutamate is released via exocytosis and binds to the three ionotropic receptors (iGluRs): N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and kainate (KA) receptors. These ligand-gated ion channels mediate fast excitatory neurotransmission. Glutamate also binds to metabotropic receptors, mGluR 1-8, which are G-protein coupled receptors that mediate slower, modulatory glutamatergic neurotransmission. Simultaneous activation of iGluRs and mGluRs stimulates many second messenger systems, leading to the events underlying plasticity, such as changes in transcription factor activity and gene expression, dendritic mRNA translation, and cytoskeletal remodeling (Gass and Olive, 2008).

In addition to being released by exocytosis, glutamate is released into the extracellular space by transporters on glial cells, such as the cystine-glutamate exchanger (x_c), and goes on to bind to its receptors. Excitatory amino acid transporters (EAATs) located on presynaptic terminals, glial cells, and on the postsynaptic neuron remove glutamate from the extracellular space. Glutamate taken into glial cells is converted into glutamine, released, and taken up by the neighbouring presynaptic neuron to begin the cycle again (Gass and Olive, 2008).

The iGluRs bring Na⁺ and Ca^{2+} into the neuron, while expelling K⁺. The NMDA receptors (NMDARs) are very permeable to Ca^{2+}, while AMPA/KA receptors more permeable to Na⁺. NMDARs have been extensively implicated in mediating neuronal plasticity and learning and memory processes, while AMPA receptors are required for the induction of synaptic plasticity (Castellano et al., 2001; Derkach et al., 2007; Paoletti et al., 2013; Perez-Otano and Ehlers, 2005; Santos et al., 2009). Less is known about the role of KA receptors in synaptic plasticity, although they appear to influence cell excitability and modulate
neurotransmitter release when located presynaptically (Contractor et al., 2011; Huettner, 2003; Sihra et al., 2014). The influx of Ca\textsuperscript{2+} brought about by the activation of iGluRs stimulates several intracellular signaling molecules and is sufficient for the propagation of the action potential by the postsynaptic neuron (Ron and Jurd, 2005).
Figure 2. The glutamate synapse. In the presynaptic terminal glutamine gets converted to glutamate by the enzyme glutaminase. Glutamate is packaged into synaptic vesicles by vesicular glutamate transporters (vGluTs). Upon arrival of an action potential at the terminal, glutamate is released into the synaptic cleft via exocytosis and binds to ionotropic and metabotropic receptors, leading to the activation of numerous signaling cascades and transcription factors, ultimately creating changes in the neuron that mediate neuroplasticity. Glutamate is removed from the synapse by excitatory amino acid transporters (EAATs) located postynaptically and on glial cells. In glial cells, the uptaken glutamate is converted to glutamine, expelled, and taken up into the presynaptic terminal where it gets converted to glutamate and the cycle continues. See text for further details. Taken from Gass and Olive 2008.
Metabotropic receptors, on the other hand, are G-protein coupled receptors (GPCRs) which are divided into three groups based on their signal transduction pathways. Group I mGluRs includes mGluR1 and 5 and stimulate Gαq. Gαq activates several phospholipases as well as mobilizing the release of calcium from intracellular stores, resulting in the activation of intracellular messengers and important calcium-dependent kinases. In contrast, Group II (mGluR 2 and 3) and Group III (mGluR4, 6, 7, and 8) mGluRs activate Gαi which is negatively coupled to adenylate cyclase, inhibiting its activity and in doing so reducing the levels for cyclic adenosine monophosphate (cAMP), a crucial second messenger (Pomierny-Chamiolo et al., 2014).

3.2 NMDA receptors and EtOH

NMDARs are heavily implicated in several neuroplasticity-related phenomena (Baldwin et al., 2002; Bouvier et al., 2015; Landa et al., 2014; Nakazawa et al., 2004; Xie et al., 1992). NMDARs are heteromeric protein complexes with three families of subunits that aggregate in different combinations to form a functional receptor: the obligatory NR1 subunit, needed to form functional channels, consists of 8 splice variants; the NR2 subunits have four distinct isoforms (A-D); and the NR3 subunit exists in two isoforms (A and B; Paoletti, 2011). The structural organization of a single subunit is shown in Figure 3. The conventional NMDAR exists as a heteromer of two NR1 and two NR2 subunits, while complexes containing the NR3 subunit form either diheteromer (NR1/NR3) or triheteromer (NR1/NR2/NR3) receptors (Paoletti, 2011). The NR1 and NR3 subunits contain the glycine binding site, while the NR2 subunits contain the glutamate binding site. The subunit composition of NMDARs has been known to vary during development and across different brain areas, thus providing functional diversity to the receptor complex.
Figure 3. The NMDA receptor. The conventional NMDAR is composed of 2 obligatory NR1 subunits that bind the co-agonists glycine or D-serine, and 2 NR2 subunits that bind glutamate. Membrane depolarization removes the Mg$^{2+}$ in the channel (not shown) and binding of the co-agonists leads to receptor activation and the influx of Ca$^{2+}$ and Na$^+$ as well as the expulsion of K$^+$. 
The NMDAR is a ligand-gated, voltage-dependent ion channel that requires activation by both glutamate and glycine or D-serine (Figure 3). At resting membrane potentials, the receptor is inactivated due to ion channel blockade by extracellular Mg$^{2+}$. Upon depolarization of the neuronal membrane, Mg$^{2+}$ is expelled, and the binding of glutamate and glycine triggers opening of the ion channel, allowing for the influx of Na$^+$ and Ca$^{2+}$ and the expulsion of K$^+$. The critical factor that makes NMDARs major players in plasticity is the large Ca$^{2+}$ influx into the neuron following their stimulation. This leads to activation of several intracellular signaling pathways that result in phosphorylation of ion channels, kinases, and transcription factors leading to changes in transcription factor function, gene transcription, protein synthesis and translation, structural remodeling of the cytoskeleton, and strengthening of synapses (Gass and Olive, 2008). Hence an important role for NMDARs in CNS excitability, cognition, motor coordination and in synaptic plasticity processes underlying learning and memory (Paoletti et al., 2013). Given their role in synaptic plasticity, it is not surprising that NMDARs have been heavily implicated in addiction, which many investigators consider to be an aberrant form of learning and memory (Kauer, 2004; Mameli and Luscher, 2011).

EtOH dose-dependently and noncompetitively antagonizes NMDAR function (Lovinger et al., 1989). The onset of inhibition is rapid and includes reduction in the mean open time and open frequency of the channel but not in single channel conductance. This has led to the hypothesis that EtOH directly interacts with NMDAR subunits to regulate channel gating in mediating its antagonist effects, likely by interacting with domains regulating channel gating (Moykkynen and Korpi, 2012). Furthermore, external factors influence the receptor’s sensitivity to EtOH inhibition, such as presence of cofactors, intracellular signalling and scaffolding proteins, and posttranslational receptor modifications (Ron, 2004). The precise
mechanisms by which alcohol inhibits channel activity as well as the subunits and specific amino acids involved are only beginning to be understood.

Acutely, EtOH inhibits NMDAR function, whereas chronic treatment upregulates NMDAR expression and activity as a result of repeated blockade (Holmes et al., 2013; Hu and Ticku, 1995). It has been hypothesized that this enhanced receptor function might serve as a mechanism for EtOH tolerance by decreasing receptor sensitivity to EtOH’s effects (Krystal et al., 2003). This suggests that EtOH’s actions at the NMDAR may contribute to the acquisition and maintenance of alcohol use disorders.

A plethora of evidence indicates that EtOH’s behavioural and subjective effects are due to its actions at the NMDAR. For example, NMDA antagonists mimic the subjective and intoxicating effects of EtOH in rats, mice, pigeons, monkeys, and humans (Grant and Colombo, 1993; Grant et al., 1991; Krystal et al., 1998; Sanger, 1993; Vivian et al., 2002). That this is evident in various species solidifies the hypothesis that EtOH’s behavioural effects are attributed at least in part to NMDAR antagonism. Furthermore, alcohol dependent patients and individuals at risk for alcohol use disorders have altered NMDAR responses to antagonists of this receptor (Krystal et al., 2003; Petrakis et al., 2004; Phelps et al., 2009). Such studies support an important role for NMDAR antagonism in EtOH’s effects and suggest that altered NMDAR responses may arise prior to the development of alcoholism and/or be caused by long term alcohol use.

3.3 Glutamate in behavioural sensitization

As shown in Figure 4, the glutamate system strongly interacts with the mesolimbic DA system, the latter of which consists largely of dopaminergic neurons in the VTA that project to the NAc, amygdala, and frontal cortex (Gass and Olive, 2008).
Figure 4. Glutamate and dopamine interactions within the mesolimbic DA system.

The VTA sends dopaminergic projections to the nucleus accumbens (NAcc), frontal cortex (FC) and amygdala (Amyg). These same dopamine neurons receive glutamatergic innervations from the Amyg, FC, laterodorsal tegmentum (LDT) and pedunculopontine tegmentum (PPT).

Take from Gass and Olive 2008.
Glutamatergic afferents from the frontal cortex (FC), amygdala, pendunculopontine tegmentum (PPT) and laterodorsal tegmentum (LDT) innervate DA neurons in the VTA, thus playing an important role in controlling their activity (Geisler et al., 2007; Omelchenko and Sesack, 2007). The NAc also receives glutamatergic innervations, specifically from the amygdala, hippocampus, thalamus, and frontal cortex, which regulate the activity of accumbal medium-spiny neurons (Carnes et al., 1990; Groenewegen et al., 1999).

Given the extensive interaction between the glutamate and DA system, and the interplay between these neurotransmitters in brain regions comprising the circuitry underlying behavioural sensitization, it is not surprising that glutamate neurotransmission appears to play a prominent role in sensitization, particularly involving NMDARs. For example, exposure to psychostimulants and morphine increases the phosphorylation levels of the NR1, NR2A, and NR2B subunits, modifies subunit expression, and promotes NMDAR activation and associated signal transduction pathways (Brunk et al., 2010; Kalivas and Alesdatter, 1993; Lee et al., 2008; Licata and Pierce, 2003; McGinty et al., 2008; Pierce and Kalivas, 1997; Schumann and Yaka, 2009; Sepehrizadeh et al., 2008). These biochemical changes appear to have functional implications for behaviour, as NMDAR blockade has generally been shown to disrupt the development and/or expression of behavioural sensitization as well as the cellular manifestations of sensitization (Kalivas and Alesdatter, 1993; Trujillo and Akil, 1995; Wolf, 1998b). It is highly likely that modulation of NMDAR function and expression by drugs of abuse is a key step in the development of behavioral sensitization and the acquisition of drug dependence (Luscher, 2013; Vanderschuren and Kalivas, 2000b).

In addition to changes at the receptor level, changes in extracellular NAc glutamate levels following behavioural sensitization have been reported. It has been most consistently
demonstrated that a challenge injection of cocaine elevates accumbal glutamate levels only after an extended withdrawal from a sensitizing regimen (Pierce et al., 1996; Pulvirenti et al., 1994; Reid and Berger, 1996; Robinson et al., 1997). In the case of amphetamine, a drug challenge following a 2 day withdrawal period had no effect on NAc glutamate levels (Xue et al., 1996).

Lesion studies have helped to elucidate whether or not NAc glutamate is required for the sensitized response and have also aided in determining which accumbal glutamatergic afferents are crucial to behavioural sensitization. Lesions of the prefrontal cortex (PFC) prevent the development of cocaine and opioid sensitization, whereas results have been mixed with respect to effects of PFC lesions on amphetamine sensitization. Lesions of the PFC have no effect on the expression of amphetamine sensitization, but they block the expression of cocaine sensitization and prevent the sensitized glutamate release into the NAc following a challenge injection (Cador et al., 1999; Pierce et al., 1998; Tzschentke and Schmidt, 2000; Vanderschuren and Kalivas, 2000b; Wolf, 1998b; Wolf et al., 1995). Amygdala lesions have been reported to block or have no effect on the development of amphetamine sensitization, and results with cocaine sensitization have also been unclear. The expression of cocaine sensitization was not affected by an amygdala lesion (Cador et al., 1999; Chambers et al., 2007; Wolf, 1998b; Wolf et al., 1995). Lesions of the fimbria-fornix, which destroy hippocampal input into the NAc, have been reported to block the development of amphetamine sensitization, however there has also been conflicting results in this respect (Wolf, 1998b). Contradictions in the literature likely arise from differences in the methods and extent of lesions. Additionally, given the role of the amygdala and hippocampus in context-dependent processes, discrepancies in the literature may also arise from differences in the degree to which sensitization protocols consist of context-dependent and –independent components of
sensitization (Belujon and Grace, 2011; Fuchs et al., 2009). Collectively, these lesion studies highlight the complexity of glutamatergic involvement in sensitization and demonstrate that accumbal glutamatergic afferents have different degrees of importance in the two phases of sensitization, depending on the drug and brain region in question. Despite some inconsistencies, the general consensus is that glutamate neurotransmission is important in behavioural sensitization to psychostimulants (Tzschentke and Schmidt, 2003).

Although EtOH is known to alter the functioning of the glutamate system, the role of this system in EtOH sensitization has been underexplored. Emerging evidence suggests that EtOH sensitization is associated with adaptations in glutamate transmission, particularly involving NMDARs, which warrant further investigation (Abrahao et al., 2013; Abrahao and Souza-Formigoni, 2012; Broadbent and Weitemier, 1999; Camarini et al., 2000b; Meyer and Phillips, 2003; Quadros et al., 2002b). Furthermore, glutamate neurotransmission in the NAc may be important in the expression of EtOH sensitization, however there have been inconsistencies in this respect (Carrara-Nascimento et al., 2011b; Szumlinski et al., 2005). To this end, the objective of this project was to examine the glutamatergic mechanisms underlying behavioural sensitization to EtOH and interindividual variability, looking at glutamate transmission in the NAc, the involvement of NMDARs, and post-NMDAR processes.
The glutamatergic mechanisms underlying EtOH sensitization and the variability in this response are poorly understood. A greater understanding of these mechanisms may identify glutamatergic processes that are associated with enduring behavioral changes in general, as well as processes that are important in the vulnerability to sensitization. Additionally, this project may potentially contribute to elucidating neuroadaptations that may underlie and sustain alcoholism, leading to more effective pharmacotherapeutics targeting the glutamate system for the treatment of this disease. With this overall goal, the present project employed a largely comparative approach in order to further elucidate:

1) The role of the glutamate system in EtOH sensitization

2) How this system contributes to the variability present in the EtOH sensitization response

Specifically, our hypotheses were as follows:

**Hypothesis 1:**

Behavioural sensitization to EtOH is associated with increases in measures of glutamate neurotransmission

**Hypothesis 2:**

Individual differences in EtOH sensitization response are mediated by NMDARs
Thesis outline

We conducted several experiments to test our hypotheses and grouped them into 3 major areas of focus, organized as chapters, designed to encompass the pre-and post-synaptic components of the glutamate synapse. The first area (chapter 1) is focused on glutamate and the nucleus accumbens. Here, we examined whether HS mice would show elevated extracellular accumbal glutamate following EtOH exposure during the development and expression of sensitization.

The second area (chapter 2) addresses glutamate and NMDA receptors. In this section we examined whether differences in NMDAR subunit genes existed among HS and LS mice. Because we found that LS mice demonstrated elevated NMDAR subunit gene expression, hypothesized to be due to repeated blockade of NMDARs by EtOH, we tested whether NMDAR antagonism would prevent EtOH sensitization.

The third and final area (chapters 3-5) is focused on glutamate and markers of neuroplasticity (i.e., components of signal transduction pathways associated with NMDAR activation). In this section we conducted experiments to address whether HS mice showed elevated levels of the transcription factor phospho-cAMP response-element binding protein (pCREB), changes in accumbal spine density and morphology, and elevations in brain-derived neurotrophic factor (Bdnf), tropomyosin receptor kinase B (trkB), and activity-regulated cytoskeleton-associated protein (Arc) mRNA expression across the brain.
General methodology

1. Subjects

All procedures were approved by the Animal Care Committee at the Centre for Addiction and Mental Health and were in keeping with the guidelines and practices outlined by the Canadian Council on Animal Care. Male DBA/2 mice were obtained from Charles River (Quebec, ON). Mice were housed in polycarbonate shoebox cages (32x14x12cm) and maintained in a room controlled for temperature, humidity (21.1\(^\circ\)C, 30\% humidity), and photoperiod (12:12; lights on at either 7 or 8 am and off at 7 or 8 pm). Food and water was provided *ad libitum*. The inbred DBA strain was used for several reasons. DBA’s are known for their very robust sensitization to ethanol and the separation between high and low sensitizers is typically very clear, so that clear subgroups can always be identified. Additionally, we felt that using an inbred strain would be best because this allows us to control for as much genetic and environmental variability between individuals as possible, such that any differences we do find are more likely to be directly related to behavioural variability in sensitization rather than previously existing environmental or genotypic variations.

2. Test apparatus

Measurements of locomotor activity (LMA) were carried out in 40x40x35cm Plexiglas activity monitor chambers (MED Associates, St. Albans, VT) that automatically detect LMA by horizontal infrared beam breaks. All activity testing was performed between 10 A.M. and 3 P.M. As in previous work 15-mins sessions were used throughout. This test duration was chosen because the stimulant effects of EtOH are observed within the first 0-15 min of drug administration, after which sedative effects dominate the behavioural profile (Crabbe et al., 1982).
3. Drugs

Anhydrous ethyl alcohol (Commercial Alcohols, Brampton, ON) was diluted with physiological saline (0.9% NaCl) to a concentration of 15% w/v. Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL) during the development phase of the study. For the challenge, mice received 1.8 g/kg (i.p.) of EtOH or an equal volume of SAL. These doses were chosen for the sensitization and challenge phase of the experiment because they elicit reliable behavioural sensitization in mice in this and other laboratories (Masur and Lodder Martins dos Santos, 1988; Nona et al., 2015b; Quadros et al., 2002b).

4. EtOH sensitization protocol

Following 7 days of acclimatization to the colony room, mice underwent 3 biweekly habituation (Hab) sessions in which they were brought to the testing room and left undisturbed for 30mins. They were then placed in activity boxes for 15mins to assess baseline locomotor activity (LMA). After the third Hab session, they were equated for baseline LMA and then assigned to EtOH or saline (SAL) groups. Mice received 5-7 biweekly injections of EtOH (2.2g/kg, i.p.) or an equal volume of SAL. For odd-numbered injections (1, 3, 5, and 7), mice were placed in activity boxes to assess LMA immediately following injections. For the even-numbered injections (2, 4, and 6), they were placed in the home cage after injections and cages remained in the test room for 15minutes following the injections, after which they were returned to the colony room. Injections 1-5 correspond to the development phase of sensitization. Mice with scores in the lowest 33% of the distribution are classified as low-sensitized (LS), while those with scores in the 33% of the distribution are classified as high-sensitized (HS). To test the expression of sensitization, 2-3 weeks after the last injection mice receive a challenge dose of EtOH (1.8g/kg, i.p.) or SAL and LMA is measured for 15mins.
immediately following the injection. Figure 5 shows the typical EtOH sensitization response following this protocol.

We have chosen to separate animals into groups based on HS vs. LS status and analyze our data in this way rather than using data for all individual animals and examine the associations using a correlational approach. This is because a correlational approach assumes a linear relationship between LMA and neurobiological changes, yet we and others have noted that there is a lack of linearity between these 2 variables. Indeed, in some cases animals with the lowest locomotor response to EtOH show the greatest increases in neurobiological changes (please refer to the data in the document), whereas HS mice, who show the greatest increase in activity, show reductions in some brain measures. Therefore LS and HS mice are in fact two separate groups/populations of individuals.
Figure 5. Ethanol (EtOH) sensitization. EtOH-treated mice were classified as low or high sensitized on the basis of their locomotor activity scores for injection 7. Values reported as means ± SEM locomotor activity counts. SAL=saline; HS=high-sensitized; LS=low-sensitized **p<0.02; ***p<0.01 compared to saline controls.
5. Blood ethanol concentrations

Immediately after LMA was assessed after the last injection or on the challenge day, mice were sacrificed by cervical dislocation, brains were harvested and trunk blood was collected. Blood samples of EtOH-administered mice were immediately centrifuged and plasma was decanted and stored at -80°C until measurements of blood ethanol concentrations (BEC). BEC were determined using the BioVision Ethanol Assay Kit (Milpitas, CA).

6. In situ hybridization

Quantification of gene expression was performed using in situ hybridization. Mice were sacrificed immediately following LMA testing or following a 2-3 week withdrawal period from the last injection. Brains were removed and cut, 10µm thick, on a cryostat, thaw-mounted onto slides and stored at -80°C. On the day of processing, slides were thawed and prehybridized at room temperature. The sections were fixed in 4% paraformaldehyde for 5min, rinsed, treated with 0.1M triethanolamine HCl, acetylated and rinsed in 2X SSC. Slides were then dehydrated in graded EtOH, defatted in 100% chloroform, rehydrated, and air dried.

Hybridization was performed with [35S]-labelled radioprobes complementary to the gene of interest. RNA was extracted from mouse brain tissue and cDNA was prepared by reverse transcription primed with oligo-dT, and amplified by PCR using primers consisting of consensus promotor sequences for the desired gene products. Each radioprobe was diluted to a concentration of 18 000 cpm/µL in hybridization solution containing: 50% formamide, 35% Denhardt’s, 10% dextran sulphate, 0.1xSSC, salmon sperm DNA (300µg/mL), yeast tRNA (100µg/mL), and DDT (40µM). Slides were incubated overnight at 60°C. After hybridization, sections were rinsed with agitation as follows using decreasing concentrations of SSC.
containing 25g/mL sodium thiosulfate. Slides were rinsed 2 x 24min in 4 x SSC at 60°C then treated in RNase A solution at 45°C for 40min, followed by 2 x 24min in 2 x SSC at room temperature, 2 x 24min in 0.5 x SSC at 60°C, 24min in 0.1 x SSC at 60°C, and 24min in 0.1 x SSC at room temperature for 24min. Sections were rinsed in deionized water for 10 seconds, dehydrated in 70% EtOH for 10 seconds and air dried. Slides were exposed to Kodak BioMax film at 4°C for several days depending on the gene of interest. Details about probes used and area of DNA used to make probes for genes of interest have been described in the individual chapters.

7. Image analysis

In situ hybridization signals on film were quantified using MCID Basic 7.0 image analysis software. Standard curves obtained from calibrated radioactive standards were used to convert film optical densities to µCi/g of tissue. Brain regions were identified using the Franklin and Paxinos atlas (Franklin, 1997). Each region was sampled by a blinded observer under uniform background illumination conditions. Densitometry data for each region were averaged across brain sections for a given subject, then for all subjects in a given treatment group. Data were acquired from at least 2 sections/brain region, from 3-4 brain slices per mouse.
Chapter 1

Glutamate and the nucleus accumbens: The role of accumbens glutamate in EtOH sensitization
1. Introduction

The majority of the literature on behavioural sensitization has focused on psychostimulants and has revealed an important role of nucleus accumbens (NAc) glutamate in the expression of sensitization following a drug challenge (Tzschentke and Schmidt, 2003; Vanderschuren and Kalivas, 2000a; Wolf, 1998a; Wolf et al., 1995). This is unsurprising given that the glutamate system is a major player in neuroplasticity, primarily due to the large Ca$^{2+}$ influx and subsequent signal transduction cascades initiated when it binds to N-methyl-D-aspartate receptors (NMDARs) (Gass and Olive, 2008).

Several reports have revealed an important role for neural activity within the NAc in the expression of EtOH sensitization (Abrahao et al., 2014; Abrahao et al., 2012; Abrahao et al., 2011; Nona et al., 2015b) and a potentially crucial involvement of NAc glutamatergic neurotransmission in the expression of sensitization. For example, following protracted withdrawal from repeated EtOH, mice showing high sensitization levels have altered NAc NMDAR function compared to mice showing low sensitization or saline controls (Abrahao et al., 2013; Abrahao and Souza-Formigoni, 2012). However, studies examining the effects of an EtOH challenge on accumbal glutamate levels have reported conflicting results. Szumlinski et al. (2005, 2008) reported an increase in extracellular NAc glutamate levels, whereas Carrara-Nascimento et al. (2011) reported a decrease (Carrara-Nascimento et al., 2011a; Szumlinski et al., 2008; Szumlinski et al., 2005). These inconsistent findings in microdialysis studies may have been due at least in part to the fact that interindividual variability in EtOH sensitization was not taken into account. Indeed, an interesting observation regarding EtOH sensitization is the substantial interindividual variability present. High sensitized (HS) mice robustly sensitize, whereas low sensitized (LS) mice may only modestly sensitize, or not sensitize at all (Abrahao
et al., 2014; Abrahao et al., 2009; Masur and Lodder Martins dos Santos, 1988; Nona et al., 2013a). Therefore the first aim of the present study was to ascertain possible differences in \textit{in vivo} release of glutamate in LS vs. HS mice during the expression of EtOH sensitization. We expected that HS mice would show elevated levels of NAc glutamate following a challenge injection of EtOH.

Previous work has established that LS and HS mice do not differ in blood ethanol levels (Quadros et al., 2005) at any point and we have recently confirmed that locomotor activity does not correlate with blood ethanol levels in mice challenged during the expression phase (Nona et al., 2015b). However, EtOH levels in the brain do not seem to have been assessed in LS and HS mice. This would be important to consider, since several EtOH-metabolizing enzymes exist in the brain and it has been shown that modulating their activity alters locomotor responses to EtOH (Correa et al., 2001; Correa et al., 2009; Ledesma et al., 2014). Therefore, we also examined whether LS and HS mice differ in central EtOH concentrations.
2. Materials and methods

2.1 Subjects

Male DBA/2 mice \((n=99)\) were aged 7 weeks at the beginning of the experiment and individually housed, as described in section 1 of the General methodology.

2.2 Test apparatus

Measurements of locomotor activity (LMA) were carried out in activity monitor chambers, as described in section 2 of the General methodology.

2.3 Drugs

Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL) during the development phase of the study. For the challenge, mice received 1.8 g/kg (i.p.) of EtOH or an equal volume of SAL, as described in section 3 of the General methodology. LY354740 (Tocris, Bioscience) was dissolved in saline with dimethylsulfoxide to a 0.01% final concentration and was administered at 10mg/kg i.p. at an injection volume of 15ml/kg. This dose has previously been shown to block synaptic glutamate release (Battaglia et al., 1997; Moghaddam and Adams, 1998).

2.4 EtOH sensitization procedure

Refer to section 4 of the General methodology for more details. Challenge experiments were always performed 14 days after the last EtOH injection. A single dose of EtOH (1.8g/kg, i.p.) or saline was given and LMA was measured for 15 minutes.

2.5 Measurements of brain EtOH concentrations

Mice underwent EtOH sensitization as described. Immediately following LMA measurements on the 5th injection, mice were sacrificed by cervical dislocation and brains were
rapidly removed. For each mouse, the NAc was dissected from one hemisphere, while the other hemisphere was frozen intact. For measurements of brain EtOH concentrations, tissue was weighed and 20 µL (whole hemisphere) or 3 µL (NAc) of Tris buffer (10 mM, pH 7.4) was added; following sonication 4 x 5 sec, 10 µl (whole hemisphere) or 1 µl (NAc) of 6% perchloric acid was added to precipitate proteins. Tubes containing homogenized tissues were centrifuged at 10,000 rpm for 5 min and the clear supernatant was collected and treated with 10 µl (whole hemisphere) or 1µl (NAc) of 1.5 M potassium carbonate solution, vortex mixed and centrifuged again at 10000 rpm for 5 min. The clear 5µl supernatant was added to the Analox AM1 alcohol analyzer machine for determination of EtOH content, as described previously (Gomez and Luine, 2014). Alcohol reagent GMRD-113 was used (Analox Instruments, USA).

2.6 Microdialysis procedures

For the challenge experiment mice underwent surgery 24hr after the last sensitization injection and sample collection took place two weeks later. Under surgical isofluorane anesthesia, a guide cannula was implanted into the nucleus accumbens (A-P: +1.4, M-L: 1.3, D-V: -4.7mm, using the Franklin & Paxinos atlas (Franklin, 1997). Two weeks later, mice were lightly anesthetized with isofluorane and a microdialysis probe (MAB 10.8.2-4mm, PES membrane, Scientific Products) was inserted. The target was perfused with Ringer’s solution at a constant flow rate of 0.8µl/min using a Hamilton microinjection pump. After an equilibration period of 2 hr in the activity box, 3 baseline samples were collected at 20min intervals for 1 hour. In the challenge experiment mice were injected with EtOH and dialysate was then collected every 5 min for the first 15mins, after which samples were collected every 15mins.
For dialysis measurements during the induction phase, guide cannulae were surgically implanted one week before the first habituation session and dialysis samples were collected after injections 1 and 5. In this experiment a 6th injection was given two days later to allow for classification of mice into LS or HS groups without the possible confound of concurring dialysis measurements. Brains were removed following behavioural testing and stored at -80°C until histological processing. Dialysate samples were stored at -80°C until analysis.

2.7 HPLC analyses of microdialysis samples

Dialysate samples were analyzed using HPLC coupled to an electrochemical detection cell. O-phthalaldehyde (OPA) derivatization and OPA reagent were prepared as described previously. One μl of homo-serine (125 pmol in ACSF) was added as the internal standard to 3 μl of sample, followed by 4 μl of OPA reagent and vortexing of the sample. The mixture was incubated at room temperature for 3 min. Immediately after incubation, 5 μl of the reaction mixture was injected into the column for the analysis. The working electrode (Uniget 3 mm glassy carbon, BAS P/N MF-1003) was set at 750 mV vs. Ag/Ag/Cl reference electrode. Detection gain was 1.0 nA, filter was 0.2 Hz and detection limit was set at 100 nA. Standard neurochemicals (all from Sigma) were used to quantify and identify the peaks on the chromatographs. For this purpose, standard solutions of 1 mg per ml (in HPLC grade water) were made with pure glutamate and aspartate and diluted with artificial cerebrospinal fluid (ACSF, Harvard) accordingly to get the desired concentrations of the stock solutions for running in HPLC. The sensitivity was selected at the concentration at which we achieved a signal to noise ratio exceeding 3:1.
2.8 Blood EtOH (BEC) determinations

Blood samples from EtOH-treated mice were immediately centrifuged and plasma was decanted and stored at -80°C until measurements of blood ethanol concentrations (BEC). BEC were determined using the BioVision Ethanol Assay Kit (Milpitas, CA).

2.9 Verification of cannula placements

Harvested brains were sectioned coronally (20µm) on a Leica CM-3000 cryostat microtome at -20°C, thaw-mounted onto Fisher Scientific Positive Charge glass microscope slides (Whitby, ON, Canada) and stored at -30°C. Slides were post-fixed in 10% formalin vapour, stained with cresyl violet, and then examined at 10X magnification under a Nikon E-600 microscope. Following dialysis collections, mice were sacrificed by cervical dislocation and brains were removed, sectioned, and stained with cresyl violet to visualize microdialysis probe tracts. Only mice with probe tips in the NAc (Figure 8) were included in the analyses.

2.10 Statistical analyses

Locomotor activity data during sensitization induction as microdialysis data were analyzed with mixed model ANOVAs, with drug treatment as the between-subjects factor and time (injection day or sample collection time) as repeated measures factors. Brain EtOH concentrations were analyzed by one-way ANOVA. Bonferroni-adjusted were used for post-hoc comparisons in all cases.

3. Results

3.1 Brain EtOH concentrations

Figure 6 shows the locomotor responses of EtOH and SAL-treated mice whose brains were used for EtOH concentration measurements. ANOVA confirmed significant main effects
of test day $[F(2, 20) = 4.927, p < 0.025]$ and treatment $[F(2, 10) = 8.170, p < 0.01]$, and test day by treatment interaction $[F(4, 20) = 4.169, p < 0.025]$. Figure 7 shows EtOH concentrations in NAc (Fig. 7A) and in whole hemisphere (Fig 7B) immediately after injection 5. Following a significant ANOVA, group comparisons confirmed that EtOH levels in both LS and HS brains differed significantly from those in SAL brains ($p < 0.01$) but did not differ from one another.
Figure 6. Locomotor Activity. EtOH-treated mice were retrospectively classified as High Sensitized (HS) or Low Sensitized (LS) on the basis of their behaviour on the last EtOH injection. Values are means ± SEM locomotor activity scores. ***p<0.0125, high-vs. low-sensitized and saline controls.
Figure 7. Brain and NAc EtOH concentrations. (top) NAc EtOH concentrations did not differ between LS and HS mice, nor did brain EtOH concentrations (bottom).

***p<0.01, LS and HS vs. SAL.
Figure 8. Microdialysis probe placement. In the upper panel, filled circles indicate probes placed in the NAc. Only animals with probes placed in the core or shell of the NAc were included in the final analyses. The number at the top of each panel corresponds to the distance from bregma in mm according to the Franklin and Paxinos 1997 atlas. The lower panel shows a x 10 photomicrograph showing the tip of the microdialysis probe in the NAc (arrow).
3.2 Extracellular glutamate in the nucleus accumbens following an EtOH challenge

Figure 9 shows behavioral data for the cohort of mice that underwent dialysis during an EtOH challenge. For the development data (Figure 9A), ANOVA revealed a significant main effect of treatment \( [F(2, 7) = 18.897, p < 0.01] \), while test day and the test day by treatment interaction were not statistically significant. HS mice had higher activity scores than SAL throughout the injections \( (p < 0.05) \). For the challenge data (Figure 9B) a one-way ANOVA did not reach statistical significance \( [F(2, 5) = 3.60, p > 0.05] \), despite the large LMA counts following EtOH challenge in HS mice. It is noted that mice were connected to dialysis tubing while LMA was measured and this likely attenuated LMA counts.

Extracellular glutamate levels after the EtOH challenge are shown in Figure 10. ANOVA revealed main effects of time \( [F(9, 45) = 3.827, p < 0.002] \), treatment \( [F(2, 5) = 18.627, p < 0.005] \) and time x treatment interaction \( [F(18, 45) = 2.031, p < 0.002] \). Glu levels in HS mice were significantly higher than those in LS and/or SAL groups for 45 min after EtOH challenge. Group comparisons that survived Bonferroni adjustments included HS vs. SAL at 15min \( (p < 0.01) \), 30 min \( (p < 0.01) \) and 45 min \( (p < 0.025) \) after the EtOH challenge, as well as comparisons between HS vs. LS at 15min \( (p < 0.025) \) and 30mins \( (p < 0.025) \). Within-group comparisons indicated that in HS mice EtOH increased Glu levels at 15min \( (+67\%, p < 0.05) \) and 30mins \( (+52\%, p < 0.05) \) in comparison to baseline \( (-60\text{min}) \). There were no significant differences between LS and SAL mice at any time point. There were also no differences within each of these groups relative to their baseline values.

To verify whether the observed increase in NAc glutamate following an EtOH challenge plays a role in the expression of EtOH sensitization in HS mice, the effects of the glutamate release
blocker LY354740 were tested in a fresh cohort of 60 mice. LY354740 decreases Glu release by activating pre-synaptic mGluR 2/3 receptors; a dose of 10 mg/kg was given i.p. 20min prior to the EtOH challenge, based on demonstrations that this dose effectively blocks synaptic Glu release (Battaglia et al., 1997; Moghaddam and Adams, 1998). Figure 11 shows the development of EtOH sensitization in HS mice and the effects of LY354740 on the expression of sensitization. In the challenge experiment (Figure 11 B), following a significant one-way ANOVA \[ F (3, 20) = 24.79, p<0.01 \] Bonferroni tests indicated that the EtOH-sensitized group (EtOH) had significantly higher LMA scores than all the other groups \( (p<0.01) \) -- including the EtOH-LY35470 group. The latter group had LMA scores similar to that of saline-treated controls, suggesting that LY354740 completely blocked the expression of EtOH sensitization. Blood EtOH concentrations of mice treated with EtOH alone \( (0.48 \pm 0.15 \text{ mg EtOH/ml blood}) \) did not differ from BEC from mice receiving LY354740 prior to EtOH \( (0.47 \pm 0.14 \text{mg EtOH/ml blood}) \), confirming that this drug did not alter EtOH metabolism.
Figure 9. Behavioural sensitization to EtOH. A. Development of sensitization: EtOH-treated mice were retrospectively classified as High Sensitized or Low Sensitized on the basis of their behaviour on the last EtOH injection. Values are means ± SEM locomotor activity scores. ***p<0.0125, **p<0.025, HS vs LS mice; ###p<0.001, ##p<0.025, HS vs SAL. B. Expression of sensitization: Mice underwent in vivo microdialysis while locomotor activity following an EtOH challenge was recorded***p<0.0125, LS and HS vs SAL. Acute EtOH refers to group treated with SAL during the development and given EtOH for the first time at the challenge.
Figure 10. Extracellular glutamate levels after EtOH challenge. EtOH challenge elevated glutamate levels in HS mice in relation to their own pre-injection baseline (+67% and +52% increase; \( p < 0.05 \)) and relation to SAL and LS groups at each time point (\( ** p < 0.025 \)). Values are means ± SEM. Group Ns are indicated in parentheses. Locomotion was measured for the first 15mins after EtOH injection and mice were left in locomotor activity boxes for the full 2 hrs.
Figure 11. Effects of LY354740 on the expression of EtOH sensitization. A. Development of sensitization: EtOH-treated mice were retrospectively classified as High Sensitized or Low Sensitized on the basis of their behaviour on the last EtOH injection and only HS mice were used. Values are means ± SEM locomotor activity scores. ***p<0.0125, HS vs SAL. B. Expression of sensitization: Mice received either SAL or LY354740 20mins prior to EtOH challenge or SAL. ***p<0.0125, EtOH compared to all groups.
3.4 Extracellular glutamate in the nucleus accumbens during the sensitization induction phase

To verify whether elevated extracellular glutamate levels in response to EtOH in HS mice could also be seen during the induction phase, glutamate levels were measured following the first and fifth EtOH injection. ANOVA for LMA scores (Figure 12A) indicated a main effect of test day \([F(3,9)=14.61, p<0.01]\), but not treatment and treatment \(x\) test day interaction, which was likely due to general LMA reductions injection 1 and 5 when mice were connected to dialysis tubing.

Figure 12B and C show dialysis results for injections 1 and 5, respectively. For injection 1 (Figure 12B), ANOVAs revealed a significant effect of time \([F(4,32) = 6.44, p < 0.001]\) but no significant effects of treatment \([F(2,9) = 0.13, p < 0.88]\) or a time \(x\) treatment interaction \([F(8,36) = 0.78, p < 0.62]\). Similarly, when tests were done for each group separately (post-EtOH values compared to baseline) no significant effects were seen for any of the three groups.

For injection 5 (Figure 12C), ANOVAS revealed a significant effect of time \([F(4,36) = 4.27, p < 0.006]\) but no significant effects of treatment \([F(2,8) = 0.93, p < 0.43]\) or a time \(x\) treatment \([F(8,32) = 1.29, p < 0.282]\). When tests were done for each group separately (post-EtOH values compared to baseline) NAc glutamate levels in LS mice were significantly increased at the 5min mark (+51%, \(p<0.05\)). Increases at 10min and 15 min (Figure 12C) did not survive Bonferroni corrections. No other changes were seen at any other point for this or for the other two groups.
Figure 12. Extracellular glutamate levels during the development of sensitization. (A) EtOH-treated mice were retrospectively classified as High (HS) or Low sensitized (LS) based on scores on injection 6. (B) The 1st EtOH injection had no effect on NAc extracellular Glu level. (C) The 5th EtOH injection elevated briefly elevated glutamate levels in LS mice (***p<0.009) in comparison to their pre-injection baseline levels. Values are means ± SEM. Group Ns are indicated in parentheses.
4. Discussion

EtOH administration had different effects on extracellular NAc glutamate in LS and HS mice. Specifically, an EtOH challenge elevated glutamate levels in HS mice but had no effect in LS mice or in mice receiving EtOH for the first time. Administration of a glutamate release blocker prior to the EtOH challenge abolished the expression of sensitization in HS mice, suggesting a potentially important role for EtOH-induced synaptic release of glutamate in the expression of sensitization. When glutamate release was examined during the induction phase no effects were seen in HS or LS mice following the first EtOH injection. Surprisingly, on the 5\textsuperscript{th} injection LS mice showed a significant increase in NAc glutamate levels compared to their baseline at 5min post-EtOH injection.

It is surprising that HS mice did not demonstrate any changes in NAc glutamate during the development of sensitization, whereas LS mice showed a transient but significant increase in glutamate after the 5\textsuperscript{th} EtOH injection. We do not have a ready explanation for this observation. One speculation is that EtOH elevates glutamate release in both LS and HS mice but the effects do not appear as pronounced in HS mice because glutamate may be more efficiently removed from the extracellular space in these animals; EtOH has been reported to have divergent effects on glutamate uptake, and it is possible that LS and HS mice also differ in this regard (Griffin et al., 2015; Melendez et al., 2005). Additionally, overactivation of postsynaptic NMDARs during acute withdrawal from EtOH has been reported to increase glutamate release (Fadda and Rossetti, 1998). Conceivably by the 5\textsuperscript{th} injection, LS mice could be more sensitive than HS animals to the effects of acute EtOH withdrawal, resulting in elevated glutamate release; these mechanisms may have normalized by the time an EtOH challenge was given, or other mechanisms may be in place to normalize a potentially overactive glutamate system in this subgroup of mice.
LS and HS mice did not differ in whole-brain or accumbal EtOH concentrations, confirming that the behavioural changes are not due to differences in the metabolism of EtOH. The differential effects of an EtOH challenge on glutamate levels in LS vs. HS mice suggest the possibility that alterations in action potential generation and/or presynaptic regulation of neurotransmitter release may be one of the factors underlying the sensitized state in HS mice. Sensitizing regimens of psychostimulants and morphine result in presynaptic alterations geared towards the facilitation of transmitter release, particularly involving elevations in the expression and activity of proteins regulating neurotransmitter exocytosis (Hemby, 2004; Iwata et al., 1997a; Iwata et al., 1997b; Subramaniam et al., 2001). Future studies could examine the mechanisms underlying increased extracellular NAc glutamate, considering both proteins involved in transmitter release and NAc glutamate afferents.

The observation that LY354740 prevented the expression of sensitization in HS mice suggests that the observed elevation in NAc glutamate in this group may be required for the expression of sensitization. One limitation of the present study is that we did not measure NAc glutamate levels after when LY354740 was administered. We note however that the dose used prevented the locomotor stimulant effects of phencyclidine (PCP) and the subsequent increase in NAc and striatal glutamate release without having an effect on dopamine release (Battaglia et al., 1997; Moghaddam and Adams, 1998).

The observation that HS mice had elevated NAc glutamate during the expression but not during the induction phase of sensitization is consistent with previous evidence of a role for the NAc in the expression (Abrahao et al., 2011; Andrade et al., 2011; Nona et al., 2015b). In this regard, EtOH sensitization appears to share similar mechanisms to psychostimulant sensitization in that the NAc plays an crucial role in the expression of sensitization, and
glutamate transmission in this structure is important in the expression of a sensitized response (Tzschentke and Schmidt, 2003; Vanderschuren and Kalivas, 2000a).

A difficult question in the EtOH sensitization field is whether neural differences between LS and HS mice are pre-existing or caused by the EtOH treatment. The observation that LS and HS mice did not differ in NAc glutamate levels after injection 1 strongly suggests that there were no pre-existing differences between low- and high-sensitized mice in terms of NAc glutamate levels at baseline or in response to EtOH. The implication would be that NAc glutamate differences in the sensitized state were brought about by EtOH treatment. Similar to our findings, a previous report found that cocaine sensitized animals did not show changes in NAc glutamate levels following the first cocaine injection (Robinson et al., 1997). The neural mechanisms regulating acute drug exposure, brief drug withdrawal, and long-term drug withdrawal differ from each other and might explain why HS mice show an increase in glutamate following a challenge injection but not during the development of sensitization (Kalivas and Duffy, 1993; Segal and Kuczenski, 1992). Perhaps a different neurotransmitter and/or activity in a brain region other than the NAc may play a more prominent role in the development of EtOH sensitization. Indeed, dopamine activity in the ventral tegmental area is important in the development of psychostimulant sensitization and future studies could address whether this is the case with respect to EtOH sensitization (Vanderschuren and Kalivas, 2000a).

We aimed to target the NAc in general when measuring extracellular glutamate levels in this preliminary study, however most of our probes localized to the NAc core. It should be noted that the core and shell subdivisions of the NAc differ in projection patterns, responses to drugs of abuse, receptor densities, and neuropeptide distributions and therefore results obtained
from probes placed in the core may not necessarily be the same if we sampled from the shell (Meredith et al., 1992; Schultz, 2015). Future studies should systematically compare the effects of an EtOH injection on extracellular glutamate levels in the NA core and shell.

In summary, it was found that glutamate levels do not change in HS mice in response to EtOH in the course of sensitization, but increase robustly in response to an EtOH challenge two weeks after the last sensitizing injection. Pharmacological antagonism of glutamate release effectively blocked the expression of sensitization in HS mice. In contrast, LS mice showed an increase in NAc glutamate in response to EtOH after the 5th sensitization injection, but did not show alterations when challenged two weeks later. High- and low-sensitized mice did not differ in response to the first EtOH exposure and did not differ in brain levels of EtOH. Together these observations strongly suggest that mechanisms regulating neurotransmitter release and NAc glutamatergic afferent activity differ between HS and LS mice. It is possible that mechanisms regulating glutamate removal from the extracellular space may be different between LS and HS mice.
5. Statement of significance. In this study we found that extracellular NAc glutamate increases following an EtOH challenge in HS mice (+67%), but does not change during the development of EtOH sensitization. This increase in NAc glutamate may be necessary for the expression of sensitization since administration of a glutamate release blocker prevented the expression of sensitization. Interestingly, LS mice showed a 51% increase 5 mins after the 5th EtOH injection. It is possible that LS and HS mice differ in the mechanisms regulating extracellular glutamate uptake and LS mice may be more sensitive to the effects of repeated cycles of EtOH exposure and withdrawal on extracellular glutamate.
Chapter 2

Glutamate and NMDARs: The role of NMDARs in EtOH sensitization

Portions of this study have been published as


Statement of author contributions:

All experiments were designed by CN and JN. All experimental work and data analyses were conducted by CN. RL assisted with film reading. CN and JN wrote the manuscript.
1. Introduction

In chapter 1 we found that an EtOH challenge differentially affected NAc glutamate levels in LS and HS animals, providing the first indication that presynaptic glutamate transmission at the expression phase may be different between these mice. A major postsynaptic target of glutamate that is heavily involved in neuroplasticity is the NMDA receptor (NMDAR). Due to their large permeability to calcium and the subsequent activation of signal transduction cascades, NMDARs can promote long-lasting changes in neuronal function (Gass and Olive, 2008).

It is well known that EtOH potently inhibits N-methyl-D-aspartate receptors (NMDARs), modulating both expression and function of the receptor and its subunits after acute and chronic administration (Chandrasekar, 2013; Gonzales and Jaworski, 1997; Wirkner et al., 1999b). NMDARs are heterotetrameric protein complexes composed of two obligatory NR1 subunits and two NR2 subunits, which have four distinct isoforms (A-D) (Sanz-Clemente et al., 2013; Traynelis et al., 2010). Few NMDARs may have the NR3 subunit, which consists of two isoforms, A and B (Sanz-Clemente et al., 2013; Traynelis et al., 2010). The subunit composition of NMDARs varies both during development and across different brain regions, imparting functional diversity to the receptor complex. In the adult brain NR2A and NR2B are the predominant subunits which are sensitive to inhibition by EtOH (Chu et al., 1995a; Holmes et al., 2013; Kuner et al., 1993; Masood et al., 1994; Sanz-Clemente et al., 2013).

Since they are a primary target of EtOH, NMDARs may conceivably play an important role in the variability seen with EtOH sensitization. Emerging evidence supports this idea. For example, EtOH sensitized mice display cross sensitization to other NMDAR antagonists, have impaired NMDAR-dependent long term depression (LTD), and display decreased NMDAR function in the nucleus accumbens, while LS mice show increased NMDAR binding in some
brain areas (Abrahao et al., 2013; Abrahao et al., 2012; Quadros et al., 2002c) but see Meyer and Phillips, 2007). Such observations, which could come about by different subunit expression in distinct brain areas, may be the result of receptor alterations that either pre-existed or were induced by chronic EtOH treatment.

To date, however, it is not known whether variability in the sensitization response to EtOH both at the development and expression phases is associated with brain-wide changes in NMDAR subunit gene expression. Our first objective therefore was to test the hypothesis of subunit gene expression differences between LS and HS mice at these two time points. A second objective was to identify brain areas in which NMDARs may potentially be critical to the two sensitization phases. To this end, we mapped brain-wide changes in NR1, NR2A, and NR2B mRNA between LS, HS, and saline-treated controls at the end of the development phase and, in a separate experiment, 14 days after the last EtOH injection. At 14 days, sensitization is typically manifested as enhanced locomotor activity in response to a drug challenge and it is also a timepoint when neuroadaptations associated with EtOH sensitization are still present (Harrison and Nobrega, 2009a; Harrison and Nobrega, 2009b; Nona et al., 2013b). Our gene expression study suggested LS mice may be more sensitive to EtOH blockade of NMDARs, raising the possibility that NMDAR antagonism might prevent EtOH sensitization. Therefore, a second goal was to examine whether pharmacological blockade with the NMDAR antagonists MK-801 and CGS 19755 would prevent sensitization to EtOH in HS mice.
2. Materials and methods

2.1 Subjects

Male DBA/2 mice (n=276), aged 6 weeks at the beginning of the experiment, were housed 4 per cage, as described in section 1 of the General methodology.

2.2 Test apparatus

Measurements of locomotor activity (LMA) were carried out in activity monitor chambers, as described in section 2 of the General methodology.

2.3 Drugs

Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL) during the development phase of the study. Mice were challenged with 1.8g/kg of EtOH i.p. or an equal volume of SAL, as described in section 3 of the General methodology.

MK-801 (Sigma-Aldrich) was dissolved in physiological saline and administered i.p. at a concentration of 0.25mg/kg in a volume of 15ml/kg. This dose was chosen based on reports demonstrating its efficacy in preventing locomotor sensitization (Broadbent and Weitemier, 1999; Camarini et al., 2000a; Shen and Phillips, 1998; Wolf, 1998b).

CGS 19755 (Tocris Bioscience) was dissolved in distilled water and administered i.p. at a concentration of 10mg/kg in a volume of 15ml/kg. This dose was chosen based on reports demonstrating its efficacy in preventing locomotor sensitization (Wolf, 1998b).

2.4 EtOH sensitization procedures

For the subunits experiment, mice (n=64) were counterbalanced for baseline LMA and subsequently assigned to receive either EtOH (n=48) or SAL (n=16), as described in section 4 of the General methodology.
For NMDAR antagonist studies, MK-801 and CGS 19755 were administered 30mins prior to EtOH or SAL injections. Mice in the MK-801 experiment were divided into several groups during the development phase: SAL followed 30 mins later by SAL (SAL/SAL, n=22) or EtOH (SAL/EtOH, n=16), and MK-801 followed 30mins later by SAL (SAL/MK-801, n=17) or EtOH (MK-801/EtOH, n=16). For the expression, these mice were grouped into two categories receiving either an EtOH or SAL challenge: the SAL/SAL group from the development phase received a SAL injection followed 30mins by SAL (n=13), the SAL/EtOH group from the development received either SAL (n=10) or MK-801(n=8) followed 30mins later by an EtOH challenge, and the MK-801/EtOH group received SAL followed 30mins later by EtOH (n= 9).

Mice in the CGS 19755 experiment were divided into several groups during the development phase: SAL followed 30mins later by SAL (SAL/SAL, n=8) or EtOH (SAL/EtOH, n=24), and CGS 19755 followed 30mins later by SAL (CGS 19755/SAL, n=8) or EtOH (CGS 19755/EtOH, n=8). For the expression, these mice were grouped into two categories receiving either an EtOH or SAL challenge: the SAL/SAL group from the development phase received a SAL injection followed 30mins by SAL (n=7), the SAL/EtOH group from the development received either EtOH (n=8) or CGS 19755(n=8) followed 30mins later by an EtOH challenge, and the CGS 19755/EtOH group received SAL followed 30mins later by EtOH (n= 7).

In one experiment, CGS 19755 or SAL was administered 15mins following an injection of EtOH to examine the effects of post-EtOH NMDAR blockade on sensitization. Groups comprising the development phase included: SAL followed 15mins later by SAL (SAL/SAL, n=12), EtOH followed 15mins later by SAL (EtOH/SAL, n=9), and EtOH followed 15mins later by CGS19755 (EtOH/CGS 19755, n=8). For the expression study, these mice were
grouped into two categories receiving either an EtOH or SAL challenge: the SAL/SAL group from the development phase received a SAL injection (n=6), while the EtOH/SAL (n=9) and EtOH/CGS 19755(n=8) groups received an EtOH challenge.

Locomotor activity data during sensitization were analyzed with mixed model ANOVAs, with drug treatment as the between-subjects factor and time (injection day) as repeated measures factors. Pharmacological manipulations during EtOH challenge were analyzed by one-way ANOVA. Bonferroni-adjusted tests were used for post-hoc comparisons in all cases

2.5 In situ hybridization protocol for NR1, NR2A, and NR2B mRNA expression

Sectioning, prehybridization, and hybridizations were performed as described in section 6 of the General Methodology. Primers comprised the consensus promoter sequences for the desired gene products, according to GenBank NM_008169.2 (NR1), NM_008170.2 (NR2A), and NM_008171.3 (NR2B). Primers were complementary to bases 2884-2903 (left primer) and 3099-3080 (right) for NR1, 3617-3637 and 991-3972 for NR2A, and to bases 3518-3526 and 3732-3536 for NR2B. The slides were then exposed to Kodak BioMax film at 4 °C for 12 days.

2.6 Image Analysis

In situ hybridization signals on film were quantified using MCID Basic 7.0 image analysis software, as described in section 7 of the general methodology. Regional levels of NR1, NR2A, and NR2B were analyzed with separate one-way ANOVAs for each brain region followed Bonferroni post hoc tests where warranted.
3. Results

3.1 EtOH sensitization

Figure 13 shows the locomotor responses of EtOH and saline (SAL)-treated mice whose brains were removed immediately following the last injection (Fig 13A) or 14 days thereafter (Fig 13B). For mice sacrificed at the end of the development phase (Fig 13 A) a repeated measures ANOVA revealed a significant main effects of injection day [F (3, 60) =38.10, p<0.01], treatment group [F (2, 20) =3.97, p<0.05], the treatment x day interaction [F (6, 60) =5.36, p<0.01]. HS mice showed greater LMA scores than LS on injections day 2 (p<0.05) and 6 (p<0.01). For animals sacrificed 14 days after the last injection (Fig 13B), a repeated measures ANOVA indicated a significant main effect of treatment group [F (2,21)=23.52, p<0.01] and a significant treatment x day interaction [F(4,42)=6.67, p<0.01]. High-sensitized mice had greater LMA scores than SAL (p<0.01), and Low-sensitized mice (p<0.01) for injection 3 and 5.
Figure 13. Behavioural sensitization to EtOH. EtOH-treated mice were retrospectively classified as High Sensitized or Low Sensitized on the basis of their behaviour on the last EtOH injection. Figure A: Behaviour for brains removed after inj 6. Figure B: Behaviour for brains removed 14 days after the last injection. Values are means ± SEM locomotor activity scores. *p<0.05, difference between high-sensitized and low-sensitized, and low-sensitized and saline; **p<0.025, Low-sensitized versus saline; ***p<0.0125, high-vs. low-sensitized and saline controls.
3.2 NMDAR subunit expression at the development phase of EtOH Sensitization

NR1. One-way ANOVA indicated significant group differences in two of the brain regions analyzed, namely the nucleus accumbens core (Nacc-C) \([F(2,21)=3.77, p<0.05]\) and shell (Nacc-S) \([F(2,21)=4.24, p<0.05]\). As shown in Figure 14, low-sensitized mice had greater NR1 gene expression in the core (+11%, \(p<0.05\)) and shell (+14%, \(p<0.05\)) compared to high-sensitized mice. Low-and high-sensitized mice did not differ from saline controls in any of the other brain regions examined. See Table 1 (pg. 124) for brain region abbreviations.
Figure 14. NR1 mRNA expression. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. * p<0.05 compared to the high-sensitized group.
NR2A. Figure 15 shows the results for NR2A expression among the three treatment groups. One-way ANOVA indicated significant groups differences in the infralimbic cortex (ILA) [F(2,10)=5.39, p<0.05], the dorsomedial (CPu-Dm) [F(2,21)=4.57, p<0.025], dorsolateral (CPu-Dl) [F(2,21)=4.72, p<0.025], ventromedial (CPu-Vm)[F(2,21)=5.0, p<0.025], and ventrolateral(CPu-Vl)[F(2,21)=5.87, p<0.01] divisions of the caudate putamen, the ventral pallidum (VP) [F(2,21)= 6.02, p<0.01], and the bed nucleus of the stria terminalis (BNST) [F(2,21)=6.2, p<0.01]. Low-sensitized mice had increased NR2A gene expression compared to saline-treated controls in the ILA (+44%, p<0.05), CPu-Dm (+33%, p<0.05), CPu-Dl (+39%, p<0.025), CPu-Vm (+43%, p<0.025), CPu-Vl (+42%, p<0.01), BSNT (+60%, p<0.025), and the VP (+79%, p<0.01). High-sensitized mice showed increased NR2A mRNA expression in the BNST (+54%, p<0.025) in comparison to saline-treated mice and a 59% increase in the VP which approached statistical significance (p=0.06). See Table 1 (pg. 124) for brain region abbreviations.
Figure 15. NR2A mRNA expression. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. * p<0.05; **p<0.025; ***p<0.0125 low-sensitized compared controls; ##p<0.025, high-sensitized compared to controls.
NR2B. ANOVAs indicated significant group difference in NR2B gene expression in the CA1 hippocampal region [F (2, 21) =3.5, p<0.05]. Low-sensitized mice had greater expression of this subunit compared to saline-treated controls (+20%, p<0.05), as shown in Figure 16. Increased NR2B in LS mice compared to SAL controls approached statistical significance for the dentate gyrus (DG) (p=0.06), CA2 (p=0.08), Nacc-S (p=0.07) and BNST (p=0.09). See Table 1 (pg. 124) for brain region abbreviations.

Figure 17 shows illustrations of NR1, NR2B, and NR2A among the three groups at the development phase.
Figure 16. NR2B mRNA expression. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. * p<0.05 low-sensitized compared controls.
Figure 17. NR1, NR2A, and NR2B gene expression between the three treatment groups.
3.3 NMDAR subunit expression at the expression phase of EtOH sensitization

Gene expression results for NR1, NR2A, and NR2B are shown in Figures 18, 19, and 20, respectively. One-way ANOVAs indicated no significant group differences in expression of any of the three genes 14 days after the last drug treatment (p>0.05). Figure 21 shows illustrations of NR1, NR2B, and NR2A among the three groups at the expression phase. See Table 1 (pg. 124) for brain region abbreviations.
Figure 18. NR1 mRNA expression 14 days after the last EtOH injection. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. There were no significant differences among the three groups.
Figure 19. NR2A mRNA expression 14 days after the last EtOH injection. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. There were no significant differences among the three groups.
Figure 20. NR2B mRNA expression 14 days after the last EtOH injection. Values are means ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. There were no significant differences among the three groups.
Figure 21. *NR1*, *NR2A*, and *NR2B* gene expression in the three treatment groups 14 days after the last injection of EtOH.
3.4: Effects of NMDAR antagonists on the development and expression of EtOH sensitization

EtOH treatment in general elevated NR2A levels, which is not surprising given that this subunit contains an EtOH binding site and that chronic EtOH-induced NMDAR inhibition leads to an upregulation in expression of NMDAR subunits (Ren et al., 2012; Wirkner et al., 1999a). The increase in NR2A was more pronounced in LS mice and only during the development phase, raising the possibility that these mice may be more sensitive to NMDAR blockade by EtOH. This inhibition of NMDARs by EtOH might in turn lead to their inability to sensitize. If it is true that NMDAR inhibition may explain the failure to sensitize, we suspected that NMDAR blockers should block EtOH sensitization. Therefore, the next set of experiments tested the hypothesis that administration of NMDAR antagonists to HS mice should prevent them from demonstrating sensitization, thus attempting to mimic the hypothesized actions of EtOH on the NMDARs of LS mice.

3.4a Effects of MK-801 on the development and expression of EtOH sensitization

Mice were injected with the NMDAR receptor blocker MK-801 (0.25 mg/kg before each EtOH injection. For the development of sensitization (Figure 22A), there were significant main effects of injection day ($F_{2, 118} = 13.82, P < 0.01$), treatment ($F_{3, 59} = 25.39, P < 0.01$), and the treatment group by injection day interaction ($F_{6, 118} = 6.45, P < 0.01$). Mice receiving MK-801 prior to EtOH (MK-801/EtOH) had significantly lower scores than those receiving SAL prior to EtOH (SAL/EtOH) for injections 1, 3, 5, suggesting that NMDAR blockade prevented the development of EtOH sensitization. However, as shown in Figure 8A, MK-801 itself produced locomotor stimulant effects as the MK-801/SAL group had higher LMA than all groups for all injections.

During the expression phase, after a significant ANOVA ($F_{3, 36} = 25.79, P < 0.01$), pairwise group comparisons indicated that EtOH sensitized mice challenged with EtOH had
significantly higher LMA than the SAL control group ($P < 0.01$), the MK-801/EtOH group challenged with EtOH ($P < 0.01$), and the group receiving MK801 before EtOH challenge ($P < 0.01$). The group receiving MK-801 prior to EtOH during sensitization development (MK-801/EtOH) had greater locomotor activity than the SAL controls ($P < 0.01$). Blood EtOH concentrations of mice treated with EtOH alone ($1.08 \pm 0.04$mg EtOH/ml) did not differ from those of mice receiving MK-801 prior to EtOH ($1.10 \pm 0.04$mg EtOH/ml), thus confirming that this drug did not alter EtOH metabolism.

These results suggested that MK-801 prior to EtOH attenuates the development of sensitization by potentiating the depressant actions of EtOH; a finding not entirely surprising given that both drugs have NMDAR antagonist effects and might be expected to produce a more profound outcome when given in combination. However, one other possible interpretation is that EtOH attenuates the stimulant effects of MK-801. Furthermore, the observation that MK-801 given prior to an EtOH challenge prevented the expression of EtOH sensitization must likewise be interpreted with caution considering that in the induction experiment the first injection of MK-801 followed by EtOH had a suppressive effect on locomotor activity (Inj1, Figure 22A).
Figure 22. Effects of NMDAR blockade with MK-801 on EtOH sensitization.  A)

**Development of sensitization.** MK-801 (0.25mg/kg) was given immediately prior to EtOH or SAL.  *p<0.05, MK-801/EtOH vs. all other groups; MK-801/SAL vs. all groups.  **p<0.025, MK-801/SAL vs. all other groups; MK-801/EtOH vs. SAL/EtOH and MK-801/SAL.  ***p<0.001, SAL/EtOH vs. SAL/SAL and MK-801/EtOH; MK-801/SAL vs. MK-801/EtOH and SAL/SAL.

(B) **Effects of EtOH challenge.** X-axis shows treatment condition during the development of sensitization. The last group receiving SAL challenge served as a control group for basal activity.  ***p<0.01, EtOH group vs. MK-801/EtOH, MK-801 before EtOH challenge, and SAL groups. Values are means ± SEM. Group Ns are indicated in parentheses.
3.4b Effects of CGS 19755 on the development and expression of EtOH sensitization

Given the interpretational complications associated with the use of MK-801, a fresh experiment was conducted using a different NMDAR antagonist, CGS 19755, a competitive NMDAR blocker not known to have locomotor stimulant effects. ANOVA for LMA data during the development phase (Figure 23A), indicated a significant main effect of injection day \( (F_{2,76} = 13.38, P < 0.01) \), treatment group \( (F_{3,38} = 42.61, P < 0.01) \], and the treatment by injection day interaction \( (F_{6,76} = 6.71, P < 0.01) \]. SAL/EtOH mice had higher LMA scores than SAL/SAL \( (P < 0.01) \), CGS 19755/SAL \( (P < 0.01) \), and CGS-EtOH \( (P < 0.01) \) for injections 1, 3, and 5. SAL/SAL controls had higher LMA scores than CGS 19755/SAL \( (P < 0.05) \) and CGS 19755/EtOH \( (P < 0.05) \) for injection 1.

For challenge LMA data there were significant group differences \( (F_{3,26} = 16.16, P < 0.01) \]. As shown in Figure 23B, the EtOH sensitized group (SAL/EtOH) and the CGS 19755/EtOH groups had higher LMA levels than the group receiving CGS 19755 prior to an EtOH challenge and the group receiving SAL only (SAL/SAL)\( (P < 0.01) \). These results demonstrated that CGS 19755 had not prevented the development of EtOH sensitization, but rather masked it. However, the inhibition of sensitization expression should also be treated with caution, since the first injection of CGS 19755 followed by EtOH during the development phase also seemed to suppress basal LMA (Figure 23A, injection 1), much as was seen with MK-801. Blood EtOH concentrations of mice treated with EtOH alone (0.80 ± 0.15 mg EtOH/ml) did not differ from those receiving CGS 19755 prior to EtOH (0.71 ± 0.03 mg EtOH/ml).
Figure 23. Effects of NMDAR blockade with CGS 19755 on EtOH sensitization.

A) Development of sensitization. ***p<0.01, SAL/EtOH vs. CGS 19755/SAL, CGS 19755/EtOH, and SAL/SAL. @p<0.05, SAL/SAL vs. CGS 19755/SAL and CGS 19755/EtOH. B) Expression of sensitization. X-axis reports treatment condition during the development of sensitization. SAL challenge group serves as a control group for basal activity. ***p<0.0125, SAL/EtOH and CGS 19755/EtOH vs. CGS 19755 prior to EtOH challenge and SAL/SAL. Values are means ± SEM. Group Ns in parentheses.
3.4c Effects of post-EtOH CGS 19755 on the development and expression of EtOH sensitization

Drug administration initiates a series of molecular cascades that ultimately culminates in the enduring neurobehavioral plasticity that is called behavioral sensitization (Ron and Jurd, 2005). NMDARs play an important role in persistent and enduring changes in the CNS, like LTD and LTP, and with some drugs, sensitization (Hunt and Castillo, 2012; Landa et al., 2014). With this in mind, and in an effort to avoid potential confounds associated with acute depressant effects of CGS 19755, our next experiment set out to examine whether EtOH-initiated molecular events, and subsequently sensitization, can be inhibited by administration of an NMDAR antagonist after both EtOH administration and LMA testing.

Results are shown in Figure 24A. The ANOVA found a significant main effect of injection day \( (F_{2, 48} = 25.59, P < 0.01) \), treatment \( (F_{2, 24} = 37.03, P < 0.01) \), and the treatment by injection day interaction \( (F_{4, 48} = 8.71, P < 0.01) \). Post-hoc comparisons showed that LMA scores for EtOH-treated mice were higher than those the SAL group for injection 1 \( (P < 0.025) \), injection 3 \( (P < 0.01) \), and injection 5 \( (P < 0.01) \).

For challenge data \( (F_{2, 20} = 6.70, P < 0.01) \), both the EtOH-sensitized group (EtOH/SAL) and the EtOH/CGS 19755 group had higher LMA scores than SAL/SAL controls at all points \( (P < 0.001) \). Importantly however EtOH/SAL and EtOH/CGS 19755 groups did not differ at any point (Figure 24B), suggesting that EtOH sensitization can develop in the presence of NMDAR blockade.
Figure 24. Effects of NMDAR blockade following EtOH injection with CGS 19755 on EtOH sensitization. (A) Development of sensitization. **p<0.025, EtOH/SAL vs. SAL/SAL, ***p<0.01, EtOH/SAL and EtOH/CGS vs. SAL/SAL. B) Expression of sensitization. X-axis shows treatment condition during the development of sensitization. The last group receiving SAL challenge serves as a control group for basal activity. *p<0.05, EtOH/SAL vs. SAL/SAL, ***p<0.01, EtOH/CGS 19755 vs. SAL/SAL. Values are means ± SEM. Group Ns are shown in parentheses.
4. Discussion

The present study found that exposure to a sensitizing regimen of EtOH is associated with differential NMDAR subunit gene expression in HS and LS mice when examined at the development phase. LS mice had greater NR1 expression in the NAc compared to HS animals, and increased NR2A and NR2B levels compared to SAL controls. HS mice showed greater NR2A expression only in the bed nucleus of the stria terminalis when compared to control mice. We also found that pharmacological blockade of NMDARs did not prevent the development of EtOH sensitization.

It has been well documented that chronic EtOH exposure leads to a compensatory upregulation in NMDA receptor binding, increased subunit protein levels and gene expression, as well as hyperexcitability of the NMDAR, all of which are antagonized by NMDAR blockers (Hoffman et al., 1995; Holmes et al., 2013; Hu and Ticku, 1995; Wirkner et al., 1999a). We found a trend for EtOH to increase mRNA levels of all of the examined subunits in many of the brain regions analyzed. Interestingly, however, it was the LS mice that demonstrated the most robust increases in NMDAR subunit expression. On the other hand, we observed no group differences when examining NMDAR subunit expression 14 days after the last EtOH injection. The absence of changes in NMDAR subunit mRNA at this time point is consistent with observations that EtOH-induced upregulation of NMDAR subunit expression and function are short-lived, returning to normal levels 24-48 hours after EtOH exposure (Gulya et al., 1991; Tremwel et al., 1994). Yet, we have shown previously that the variable behavioural sensitization response seen at the development phase persists and may even be augmented when mice are challenged with EtOH after a 14 day drug-free period (Harrison and Nobrega, 2009b; Nona et al., 2013b). The present results therefore indicate that EtOH-sensitized behaviour can be expressed in the absence of any differences in NMDAR subunit expression.
The data further suggest that the differences seen at the development phase were compensatory upregulations induced by EtOH and not differences existing prior to alcohol exposure.

Our observation that LS mice showed increased NR1 mRNA expression in the core and shell of the nucleus accumbens compared to HS mice is consistent with a previously reported study which found increased MK-801 binding in the core of the accumbens in this subgroup of mice 24 hrs after the last EtOH injection (Quadros et al., 2002c). Given that NR1 is the obligatory subunit of the NMDAR and in line with increased NMDAR binding in this brain area, our results suggest that LS mice might have increased NMDAR surface expression in the nucleus accumbens. The GABAergic medium spiny neurons (MSNs) of this region project to the ventral pallidum and ventral tegmental area, areas regulating locomotor responses to drugs of abuse, including EtOH (Boehm et al., 2002; Gong et al., 1996; Heimer et al., 1991; Koob and Swerdlow, 1988; Sesack and Grace, 2010; Vanderschuren and Kalivas, 2000b). A potential upregulation of NMDARs in LS mice could indicate increased responsivity to glutamate and we speculate that glutamatergic excitation of accumbal MSNs leads to increased inhibitory tone in these output targets and subsequently a reduced locomotor response to the EtOH. Given that GABA receptor agonists infused in the ventral pallidum and ventral tegmental area have been shown to attenuate the locomotor response to drugs with psychostimulant properties, this hypothesis would seem plausible and it may warrant further investigation with regards to individual differences in the EtOH sensitization response (Boehm et al., 2002; Broadbent and Harless, 1999; Koob and Swerdlow, 1988; Leite-Morris et al., 2004). In any event our data suggest an important role for the NR1 subunit in the individual differences to EtOH sensitization. It would be important to further study the effects of altered NMDAR expression and function in EtOH sensitization by manipulating NR1 expression.
We found that both LS- and HS mice showed increased NR2A expression compared to saline controls, suggestive of a general EtOH treatment effect on the upregulation of this subunit. This would seem plausible given that the NR2A subunit contains an EtOH binding site (Ren et al., 2012). Interestingly, NR2A increases were seen in brain regions commonly implicated in addiction, reward, and drug sensitization, including the infralimbic cortex, all divisions of the caudate putamen, the hippocampus, the bed nucleus of the stria terminalis, and the ventral pallidum (Gardner, 2011; Taylor et al., 2013). These brain areas, which have remained relatively unexplored in EtOH sensitization, would seem to deserve further studies to determine their involvement in the neural circuitry underlying sensitization to EtOH.

Although both LS- and HS mice showed increased NR2A expression, the increase was more pronounced in the LS mice, who demonstrated greater expression in all the aforementioned brain areas compared to SAL controls. This is in contrast to HS mice whose NR2A expression was greater when compared to SAL controls only in the bed nucleus of the stria terminalis, although the difference in the ventral pallidum also approached statistical significance. Finally, NR2B subunit expression was increased only in the LS mice compared to controls in hippocampal CA1, although trends were seen in hippocampal CA2, the dentate gyrus, nucleus accumbens shell and the bed nucleus of the stria terminalis. Given that the NR2B and NR2A subunits have repeatedly been reported to be the NMDAR subunits most sensitive to EtOH inhibition, this increased sensitivity could potentially underlie their greater upregulation following repeated EtOH treatment, making our current findings not entirely unexpected (Chu et al., 1995b; Kuner et al., 1993; Masood et al., 1994). However, it is surprising that the upregulation appears to be restricted to LS animals and more so for the NR2A subunit, suggesting the possibility that perhaps EtOH has greater NMDAR inhibitory effects in this subgroup of mice, whereas HS mice are less sensitive to EtOH’s NMDAR
blocking activity. Indeed a recent report found that the hippocampus of LS mice shows elevated NR2A protein expression (Coune et al 2016). In LS mice, EtOH may be a more potent NMDAR antagonist due to either an increase in the number of NMDARs (inferred from increased NR1 mRNA and higher levels of receptor binding (Quadros et al., 2002c) and/or an increase in the amount of NR2A-containing NMDARs. Since both the NR2A and NR1 subunits have been previously shown to contain a binding site for EtOH, this potential increase in EtOH binding pocket availability could lead to increased EtOH inhibition of NMDARs, in turn preventing sensitization from developing (Ren et al., 2012; Smothers and Woodward, 2006). If this speculation is correct, it could explain why it is in these animals that one observes the typical effects of chronic EtOH–induced increases in NMDAR gene expression as well as increased receptor binding (Quadros et al., 2002a). NMDAR antagonism by EtOH in LS mice might function as other NMDAR blockers, which are known to block not only psychostimulant sensitization, but other forms of learning/memory and neuroplasticity in general (Rowland et al., 2005; Sison and Gerlai, 2011; van der Staay et al., 2011; Watson and Stanton, 2009; Wolf, 1998b). To test this hypothesis, we examined the effects of the NMDAR blockers on the development and expression of EtOH sensitization, with the expectation that NMDAR inhibition would prevent sensitization.

Pharmacological blockade of NMDARs failed to prevent the development of EtOH sensitization, although they appeared to potentiate the depressant actions of EtOH. Given that EtOH, MK-801, and CGS 19755 have NMDAR antagonist actions, the apparent shift in the dose-response curve toward locomotor sedation was not entirely unexpected. When MK-801 and CGS 19755 were given prior to an EtOH challenge, an apparent blocked of sensitization expression was seen. This finding, however, should be treated with caution as we and others have found that CGS 19755 and MK-801 have effects on basal locomotor activity (Liljequist,
Although our result suggests that NMDARs do not play an important role in EtOH sensitization, other studies have reported that individual variability in EtOH sensitization is associated with changes in NMDAR expression and function. For example, HS mice have reduced NMDAR subunit expression and NMDAR-dependent long-term depression in the NAc (Abrahao et al., 2013) and demonstrate cross-sensitization to intra-accumbal MK-801 administration (Abrahao and Souza-Formigoni, 2012). These findings suggest that altered NAc NMDAR functioning is associated with EtOH sensitization. Similarly, LS mice show increased NMDAR subunit gene expression across the brain and increased NR2A protein levels in the hippocampus that corresponds to a loss of synaptic plasticity in this region (Coune et al., 2016). It is also important to be aware of the limitations associated with the use of MK-801 and CGS 19755 when interpreting data obtained from use of these drugs. MK-801 is a non competitive selective open-channel blocker that binds to NMDARs which are in the open, activated state (Huettner and Bean, 1988; Sircar et al., 1987). Its affinity to NMDARs, as well as its association and dissociation rates, depend on receptor subunit composition (Bresink et al., 1995; Monaghan and Larsen, 1997). Since NMDAR number, subunit composition, and level of activity vary across brain regions, MK-801 produces differing degrees of NMDAR inhibition in different brain areas (Sanz-Clemente et al., 2013). Furthermore, MK-801 is known to bind not only to activated NMDARs, but also to acetylcholine (Ramoa et al., 1990) and dopamine (Seeman et al., 2005) receptors. Similarly, CGS 19755, a competitive NMDAR antagonist, differentially binds NMDARs depending on their subunit composition (Lehmann et al., 1988; Murphy et al., 1988). In general, these antagonists are most selective for NR2A and NR2B containing-NMDARs than they are for NR2C and NR2D containing receptors (Monaghan and Larsen, 1997). It is conceivable that NMDARs of non-targeted compositions may be crucially involved in EtOH sensitization.
In conclusion, our evidence strongly implicates NMDARs in the variability associated with the development of EtOH sensitization. In particular, LS mice showed increased gene expression of all three subunits examined in select brains regions. This upregulation was present only during the development phase and was not seen 14 days after the last drug treatment. Our findings are in line with previous work showing that LS mice appear to be more sensitive to NMDAR inhibition by EtOH and suggest a possible role for the NR1 and NR2A subunits in the resistance to EtOH sensitization seen in the low-sensitizers. The failure of NMDAR antagonism to prevent the development and expression of EtOH sensitization raises the possibility that altered NMDAR functioning and post-receptor processes may be important.
5. **Statement of significance.** In this chapter we found that LS mice showed greater NMDAR subunit gene expression following repeated EtOH exposure compared to HS and SAL mice, indicating perhaps increased sensitivity to the NMDAR-inhibitory actions of EtOH and thus a failure to sensitize. However, NMDAR blockade did not prevent the development or expression of EtOH sensitization. It is possible that altered NMDAR functioning and post-receptor processes, which are not detected at the mRNA level or by receptor antagonism, may play a role in the variability in the EtOH sensitization response.
Chapter 3
Glutamate and markers of neuroplasticity: EtOH sensitization and pCREB

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Statement of author contributions:
Experimental work and data analyses were conducted by CN and SG. Experiments were designed by JN. CN and JN wrote the manuscript.

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All experiments were designed by CN and JN. All experimental work and data analyses were conducted by CN. MK assisted with the development of DiI-coated bullets. CN and JN wrote the manuscript. AR reviewed the manuscript.
1. Introduction

We have demonstrated that HS mice show increased glutamate release in the NAc following an EtOH challenge (Chapter 1) and that LS and HS mice differ in NMDAR subunit gene expression (Chapter 2). NMDAR subunit composition plays a major role in determining post-NMDAR processes arising following receptor activation, thus suggesting that LS and HS mice may have differential activation of intracellular cascades following NMDAR stimulation (Paoletti et al., 2013).

The influx of Ca\(^{2+}\) ions following glutamate binding to NMDARs is critical to the activation of intracellular signal transduction cascades ultimately leading to the processes underlying plasticity, such as cytoskeletal remodeling, gene transcription regulation, and dendritic mRNA translation (Gass and Olive, 2008). One consequence of this Ca\(^{2+}\) influx is the phosphorylation and activation of the transcription factor pCREB (Das et al., 1997; Ghosh et al., 1994). pCREB is known for regulating the transcription of a large number of genes that alter neuronal function and regulate synaptic plasticity, and its activation in the NAc following to exposure to drugs of abuse has been well documented (Carlezon Jr et al., 2005; Nestler, 2013). A large body of literature demonstrates a critical role for pCREB activity in learning and memory and the associated long-lasting behaviours as a result of the changes in gene expression, changes which are stable and long-lasting (Carlezon Jr et al., 2005). Not surprisingly, pCREB activity has been shown to regulate behavioural responses to drugs of abuse but its role in EtOH sensitization is unknown. Therefore, our first objective was to examine whether EtOH sensitization was associated with elevated pCREB levels in the brain.

The induction of pCREB is often accompanied by structural changes. In particular, CREB activation has been shown to elevate the density of dendritic spines, small protrusions from the dendrite which receive and modulate synaptic input and are the primary sites where
excitatory neurotransmission within the CNS (Bito, 2010; Murphy and Segal, 1997; Segal and Murphy, 1998). Spine morphology and number are critical to the function of individual synapses, modulating neuron activity at the molecular, neuronal, circuit, systems, and behavioural level (Spiga et al., 2014a). Enduring behavioural changes, such as behavioural sensitization, are believed to arise from reorganization of synaptic connections and indeed have often been accompanied by persistent and stable changes in dendritic structure (Hofer and Bonhoeffer, 2010; Kasai et al., 2010).

Behavioural sensitization to various drugs of abuse has been shown to change dendritic spine density and/or morphology of nucleus accumbens (NAc) medium spiny neurons (MSNs), an effect seen across drug classes (Robinson and Kolb, 2004). However, is it not known whether behavioural sensitization to ethanol (EtOH) is also associated with structural changes in this region. Our second objective, therefore, was to examine whether there were differences in dendritic spine density and morphology between HS, LS, and saline (SAL) control mice. Spines were classified as mushroom, thin, or stubby, which reflect mature and stable, plastic, and immature synapses, respectively (Hering and Sheng, 2001; Spiga et al., 2014a). We hypothesized a greater expression of pCREB in HS mice, increased levels of the stable, mature mushroom spines, as well as a change in spine density.
2. Materials and methods

2.1 Subjects

Male DBA/2 mice (n=56) were housed 4 per cage, as described in section 1 of the General methodology.

2.2 Test apparatus

Analysis of LMA was carried out as described in section 2 of the General methodology.

2.3 Drugs

Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL). Refer to section 3 of the General methodology for further details.

2.4 EtOH sensitization procedure

Mice were assigned to receive either EtOH (n=42) or saline (SAL; n=14) treatments, as described in section 4 of the General methodology. For spine density analysis, mice were treated as described in section 4, except that following a 2-3 week drug free period after injection 7, EtOH treated mice were challenged with EtOH (1.8 g/kg) whereas control mice received a SAL injection.

2.5 Immunohistochemistry of pCREB

Sections (40 µM thick) were cut in a cryostat and stored in a cryoprotectant solution (30% ethylene glycol, 25% glycerol, 0.15M sodium phosphate) at -20°C. On the day of processing the free-floating sections were rinsed briefly in 0.1M phosphate buffered saline (PBS) containing 0.02% azide, then in 0.1M Tris buffered saline (TBS) containing 0.25% Triton X-100 for 30min, blocked in TBS with 2% BSA and 5% goat serum at room
temperature for 2hrs and incubated in anti-pCREB (Phosphosolutions, Aurora, CO) diluted 1:1500 in TBS with 2% BSA, for 72 hours at 4°C. After extensive rinsing in TBS with Elite Kit (Vectastain® Elite® ABC Peroxidase Kit, Vector Labs, Burlington, ON) following standard protocol and visualized with the DAB substrate kit (Vector Labs). Sections were then washed in PBS, mounted onto Fisher Superfrost slides, dehydrated through ethanol gradient, cleared in xylenes, and coverslipped with Eukitt® (Electron Microscopy Sciences, Hatfield, PA).

2.6 Image analysis

pCREB immunoreactivity signals were also quantified using MCID Basic 7.0 image analysis software. The image was background corrected and normalized to preset light levels to ensure consistent data collection at a magnification of 40X. All data were acquired by a researcher blind to the group condition from at least 2 sections/brain region/hemisphere/animal and averaged to obtain a single value per subject. Cell counting for each brain region was determined using measuring boxes with fixed dimensions and placed according specific anatomical reference points.

2.7 Spine density and morphology analyses

Three days after the challenge, mice were transcardially perfused with PBS and 4% PFA and coronal 100µm thick sections were cut using a vibratome. Diolistic labeling was used to randomly label neurons (Gan et al., 2000). Twenty-four hours after labeling, sections were slide-mounted and imaged by confocal microscopy by an experimenter blinded to the treatment groups. Neurons were imaged using Olympus Fluoview FV1200 software with IX83 confocal scope at 60x magnification and z-stack step size of 0.44um. For each animal 3-7 dendrites were analyzed, with images being taken from a region 20µm away from the cell soma, as
previously described (Ramsey et al., 2011; Ruddy et al., 2015). Spine morphology was determined by measurements of spine length, head diameter, and neck diameter. Image J and NeuronStudio software were used to analyze spine density and morphology, respectively. In order to have sufficient n, dendrites from both the core and shell of the nucleus accumbens were sampled.

2.8 Data analyses

Locomotor activity data were analyzed by repeated measures ANOVA, with drug treatment as a between-subjects factor and injection day as a repeated measures factor. ANOVAs were followed, where warranted, by independent t tests. Regional levels of pCREB, were analyzed with separate one-way ANOVAS for each brain region, followed where warranted by Bonferroni-adjusted group comparisons. Spine density and morphology were analyzed with one-way ANOVA followed by Bonferonni posthoc, where necessary.
3. Results

3.1 EtOH sensitization

Figure 29 (pg. 112) shows the behavioural profiles of EtOH- and SAL-treated mice. As in previous studies a clear separation was observed between high- and low-sensitized EtOH-treated groups. A repeated measures ANOVA indicated significant main effects of injection day \([F (6, 126) = 18.24, p < 0.05]\), sensitization group \([F [2, 21] = 36.13, p < 0.05]\), and a significant group x day interaction \([F [12, 126] = 11.327, p < 0.05]\).

3.2 pCREB immunoreactivity

Among the brain regions analyzed, ANOVAs indicated the presence of significant group differences only for the shell of the nucleus accumbens \([F (2, 16) = 4.62, p < 0.026]\) and the bed nucleus of the stria terminalis \([F (2, 21) = 3.92, p < 0.038]\). Compared to saline controls, brains of high-sensitized mice contained significantly more pCREB immunoreactive (IR) cells in the shell of the nucleus accumbens (+67%, \(p < 0.005\)) and in the bed nucleus of the stria terminalis (+61%, \(p < 0.04\)) (Fig 25). Values in these two regions in the high-sensitized groups were also increased in comparison with low-sensitized mice (+49%, \(p < 0.05\); and +46%, \(p < 0.024\)). No significant differences among groups were seen in any of the other brain regions examined (Fig 25). Illustrations of pCREB IR cells in HS, LS, and SAL-treated animals are shown in Figure 26. See Table 1 (pg. 125) for brain region abbreviations.
Figure 25. pCREB cell counts. Values are mean cell counts per sampling window ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. * p<0.05; *** p< 0.01 compared to Saline controls. ## p < 0.02, #p<0.05 compared to low-sensitized group.
Figure 26. Illustration of pCREB differences among groups in the NAc and bed nucleus of the stria terminalis
3.3. Spine density and morphology

For mice used in the spine analysis study repeated measures ANOVA indicated significant main effects of injection day [F (4, 60)=10.48, p<0.01], treatment [F(2, 15)=15.33, p<0.01], injection day by treatment interaction [F(2, 15)=38.88, p<0.01], demonstrating that EtOH sensitization occurred as expected (Figure 27A). One way ANOVA showed a significant effect of treatment group [F (2, 17) =11.43, p<0.01] showing that it persisted 14 days after the last injection (Figure 27B). Figure 28A shows NAc MSN dendritic spine density for all treatment groups. There were no group differences in spine density (p>0.05). In contrast, as shown in Figure 28B, morphological analysis of spines showed that LS mice presented higher levels of stubby spines (p<0.05), whereas no group differences were seen in the density of mushroom and thin spines. Figure 28C illustrates the typical appearance of NAc MSN dendrites in the 3 treatment groups.
Figure 27. Behavioural sensitization to EtOH. A: Development of EtOH sensitization: EtOH-treated mice were classified as HS or LS based on their scores for injection 7. * p<0.05; ** p<0.02, HS compared to SAL; ***p< 0.01, HS and LS compared to SAL (Bonferroni tests). B: EtOH challenge: Following a 2-3 week drug-free period, mice were challenged with EtOH or an equal volume of SAL. ***p<0.01, HS compared to LS and SAL (Bonferroni tests). In both (A) and (B), values are mean ± SEM locomotor activity scores. N= 6 per group.
Figure 28. Spine density and morphology. Three days after an EtOH challenge, brains were removed and processed. **A:** Sensitization to EtOH was not associated with changes in NAc MSN dendritic spine density. **B:** LS mice presented higher levels of stubby spine density compared to SAL animals, *p*<0.05 (Bonferroni test). **C:** Illustrations of NAc MSN dendrites among the 3 treatment groups.
4. Discussion

The main finding of this study was that compared to LS and SAL controls, HS mice had higher levels of pCREB in the nucleus accumbens (NAc) and in the bed nucleus of the stria terminalis (BNST). Contrary to our hypothesis, the elevated pCREB levels in the NAc were not associated with changes in dendritic spine density, nor with increases in the number of mushroom spines. Surprisingly, the only significant effect, namely an increase in the density of stubby spines, occurred in the EtOH-treated group that failed to sensitise.

It is interesting that HS mice had higher levels of pCREB in the shell of the NAc as well as in the BNST, since both of these brain regions have been implicated in the initiation and/or maintenance of behaviours relevant to addiction (Koob, 2009; Marin et al., 2009; Pandey et al., 1999; Vendruscolo et al., 2012). pCREB is known for regulating the transcription of a large number of genes that alter neuronal function and regulate synaptic plasticity (Carlezon Jr et al., 2005; Frank and Greenberg, 1994). It has been shown for example that sensitization to cocaine is associated with increased pCREB in the NAc, similar to our finding that HS animals have increased pCREB-IR cells in the shell of the NAc (Mattson et al., 2005). The BNST, a component of the extended amygdala, has been implicated in alcohol and drug addiction processes, thus the increased levels of pCREB in this region was not unexpected (Gardner, 2011; Jalabert et al., 2009; Koob, 2009).

It is important to note that the present study does not allow conclusions as to whether the observed increase in pCREB-IR cells seen in HS mice was induced by EtOH, rather than being a pre-existing condition. However, it should be noted that numerous studies have demonstrated that chronic treatment with cocaine and amphetamine increases pCREB levels in sensitized animals. This would strongly suggest that the observed pCREB upregulation was
induced by EtOH (Mattson et al., 2005; Turgeon et al., 1997). Future studies should examine effects of interfering with pCREB activity on EtOH sensitization.

The increase in accumbal pCREB present in HS mice in the absence of structural changes was surprising. It is possible that we did not see changes in HS mice as a result of the protracted period of withdrawal between the development and expression phases, and also following the challenge. In addition, it is also possible that the core vs shell may show differences between LS and HS mice. This is plausible given that these subregions are known to differ in projection patterns, neuropeptide distribution, receptor densities, and responses to drugs of abuse and therefore may not necessarily shown the same structural changes just as they did not demonstrate the same changes in pCREB elevation (Meredith et al., 1992; Schultz, 2015). In addition, any potential differences in core vs. shell MSNs between LS and HS mice may vary as a function of time. Time-dependent effects of withdrawal from a dependence-inducing regimen of EtOH have previously been found to be associated with differential changes in core vs shell MSNs (Peterson et al., 2015). Those results however were obtained after inducing dependence and behavioural withdrawal reactions, none of which are seen in EtOH sensitized mice. Nevertheless, future work should compare core vs. shell and examine possible time effects on MSN structure.

The lack of an association between behavioural sensitization and spine density in the NAc does not preclude the possibility that structural changes may occur elsewhere in the brain. Indeed, the role of actin dynamics, and the recruitment of the transcription factors delta FosB, and pCREB, have received considerable attention for their role in drug-induced structural plasticity and these have been associated with behavioural sensitization to EtOH in brain areas such as the ventral tegmental area, BSNT, and motor cortex (De Pauli et al., 2014; Nona et al.,
2013b; Shibasaki et al., 2012). These and other areas implicated in sensitization could also be examined for structural changes. Of note, despite the lack of structural changes in the accumbens, biochemical/neurophysiological alterations in this brain region appear to be critical to EtOH sensitization (Abrahao et al., 2013; Abrahao et al., 2011; Abrahao and Souza-Formigoni, 2012; Nona et al., 2015b).

Our observation that LS mice have increased density of immature, stubby spines was unexpected. The presence of this spine type has been associated with long-term depression and the presence of cognitive/learning deficits, both of which are present in LS mice (Coune et al., 2016; Quadros et al., 2003; Wilson and Cox, 2007; Zhou et al., 2004). Interestingly, the NR2A subunit, whose gene we found in Chapter 2 to be elevated in LS mice, is known to prevent synapse formation and maturation, and to reduce spine growth (Gambrill and Barria, 2011). It may be that elevated NR2A expression and stubby spine presence serves to reduce excitatory transmission brought about my elevated NMDAR activity in these mice. Indeed stubby spines are highly expressed in areas undergoing elevated synaptic activity in an effort to dampen activity (Halpain et al., 1998; Korkotian and Segal, 1999; Segal, 1995). It would be interesting to examine NR2A surface expression levels here and also to examine whether LS mice show increased stubby spine density in the areas showing increased NR2A gene expression.

The functional relevance of structural plasticity in behavioural sensitization has been debated. It is unclear whether structural changes lead to the sensitized response or reflect a homeostatic adaptation that compensates for additional changes caused by chronic drug exposure. In the case of EtOH sensitization, our results suggest that drug-induced changes in structural plasticity in the accumbens neurons may not be the cause of sensitized behavior.
5. **Statement of significance.** In this chapter we found that EtOH sensitization is associated with elevated pCREB levels in the NAc shell and BNST after a two week drug-free period. The increased pCREB in the NAc of HS mice was not associated with changes in spine density nor with increased density in mushroom spines. Instead, LS mice showed elevated stubby spine density. Our results suggest that in the case of EtOH sensitization, drug-induced changes in structural plasticity in NAc neurons may not be the cause of sensitized behavior.
Chapter 4

Glutamate and markers of neuroplasticity: BDNF-TrkB system in EtOH sensitization

Portions of this study have been published as:


Statement of author contributions:

Experimental work and data analyses were conducted by CN and SG. Experiments were designed by JN and CN. CN and JN wrote the manuscript.
1. Introduction

We have shown that an EtOH challenge increases NAc glutamate levels in HS mice (Chapter 1) and that these mice also demonstrate elevated pCREB levels in the NAc and BNST (Chapter 3), indicating increased activity of the glutamate system being associated with EtOH sensitization. One major way that this neurotransmitter system regulates plasticity is by interacting with the BDNF-TrkB system (Carvalho et al., 2008; Martin and Finsterwald, 2011).

BDNF promotes the development, differentiation, maintenance, and survival of neurons, and plays a significant role in synaptic plasticity (Hyman et al., 1991; Pierce and Bari, 2001). Furthermore, it has been implicated in regulating neuronal plasticity in response to drug exposure, potentially leading to psychostimulant-induced sensitization (McAllister et al., 1999; Pierce and Bari, 2001). Indeed, by binding to its receptor, tropomyosin-related kinase B (TrkB), BDNF triggers various second messenger pathways that promote cell survival, differentiation, synaptic strength and plasticity, and dendritic outgrowth (Ernfors and Bramham, 2003; Jia et al., 2008; Minichiello, 2009; Yoshii and Constantine-Paton, 2010). Furthermore, transcription of both Bdnf and trkB is regulated by pCREB, which we have found to be elevated in HS mice (Chapter 4) (Deogracias et al., 2004; Tao et al., 1998).

Because behavioural sensitization is long lasting, it has been suggested that changes in synaptic plasticity mediate this phenomenon (Pierce and Bari, 2001; Robinson and Berridge, 2000a). Since BDNF and Trkb have been implicated in mediating drug-induced behavioral and neuronal plasticity, we sought to examine whether sensitization to EtOH is associated with increased trkB and Bdnf gene expression in various brain regions, 14 days after the cessation of EtOH administration. Considering that sensitization is a long-lasting phenomenon, we chose to examine brain changes 14 days after the last EtOH injection, with the intent of avoiding acute effects of EtOH as well as potential effects of exposure to behavioural testing. We also
examined whether TrkB receptor blockade would interfere with the development and/or expression of EtOH sensitization.

2. Materials and methods

2.1 Subjects

Male DBA/2NCrl mice (n=91) were obtained at 5 weeks of age and housed 4 per cage, as described in section 1 of the General methodology.

2.2 Test apparatus

Measurements of locomotor activity (LMA) were carried out activity monitor chambers, as described in section 2 of the General methodology.

2.3 Drugs

Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL) during the development phase of the study. For the challenge, mice received 1.8 g/kg (i.p.) of EtOH or an equal volume of SAL, as described in section 3 of the General Methodology. ANA-12 (Sigma-Aldrich) was dissolved in 7% dimethyl sulfoxide (DMSO), intraperitoneally injected (15mL/kg), and used at 0.5mg/kg, 4 hours prior to EtOH injection. This is when maximal TrkB inhibition is seen (Cazorla et al., 2011; Leggio et al., 2014).

2.4 EtOH sensitization procedure

For details refer to section 4 of the General Methodology. Following habituation sessions, mice were assigned to receive either EtOH (N=24) or saline (SAL; N=8) treatments. LMA scores after the 7th injection were ranked for EtOH-treated mice in the lowest 33% of the
distribution were classified as low-sensitized, whereas those in the upper 33% were classified as high-sensitized.

2.5 TrkB receptor blockade

To examine the effects of TrkB receptor blockade on the development of EtOH sensitization, mice were divided into four treatment groups in which they received 2 i.p. injections, separated by 4hrs: those receiving 7% DMSO followed by SAL (VEH/SAL, n=15) or EtOH (VEH/EtOH, n=18), and those receiving ANA-12 followed by SAL (ANA-12/SAL, n=16) or EtOH (ANA-12/EtOH, n=10).

To examine the effects of TrkB receptor blockade on the expression of EtOH sensitization, 14 days following a drug-free period, mice from the development phase were separated into 6 treatment groups where they were challenged with either EtOH (1.8g/kg, i.p.) or an equal volume of SAL, preceded or not by ANA-12.

2.6 Data analyses

Locomotor activity data were analyzed by repeated measures ANOVA, with drug treatment as a between-subjects factor and injection day as a repeated measures factor. ANOVAs were followed, where warranted, by independent t tests. Regional levels of Bdnf and trkB were analyzed with separate one-way ANOVAs for each brain region, followed where warranted by Bonferroni-adjusted group comparisons.

2.7 In situ hybridization protocol for Bdnf and trkB mRNA expression

Two weeks after the final injection, mice were transcardially perfused with 25ml ice-cold saline containing 2µl/ml heparin, followed by 100ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer (PBS) at a rate of 6ml/min. Brains were then removed and post-fixed
with 4% paraformaldehyde in 0.1M PBS overnight at 4°C. On the following day, brains were cryoprotected by submersion in sucrose solutions of 10%, 20%, and 30% for 48hrs, then frozen in isopentane chilled in dry ice in an acetone bath. Brains were then stored at -80°C until sectioning. Sectioning, prehybridization, and hybridizations were performed as described in section 6 of the General Methodology. Primers comprised the consensus promoter sequences for the desired gene products, according to GenBank NM_012513.2 (Bdnf) and NM_012731 (trkB); primers were complementary to bases 240–259 (left primer) and 594–575 (right) for bdnf and to bases 2600–2583 and 660–680 for trkB. Slides with trkB-hybridized sections were exposed to Kodak BioMax film at 4°C for 5 days whereas those with Bdnf were exposed for 12 days.

2.8 Image analysis

In situ hybridization signals on film were quantified using MCID Basic 7.0 image analysis software, as described in section 7 of the General methodology.
3. Results

3.1 EtOH sensitization

Figure 29 shows the behavioural profiles of EtOH- and SAL-treated mice. As in previous studies a clear separation was observed between HS and LS EtOH-treated groups. A repeated measures ANOVA indicated significant main effects of injection day \([F (6, 126) = 18.24, p < 0.05]\), sensitization group \([F (2, 21) = 36.13, p < 0.05]\), and a significant group x day interaction \([F (12, 126) = 11.327, p < 0.05]\). HS mice showed higher LMA than SAL and LS mice on all injection days (p<0.05).
Figure 29. Behavioral sensitization to EtOH. EtOH-treated mice were retrospectively classified as high-sensitized (HS) or low-sensitized (LS) on the basis of their behavior on injection 7. Values are means ± SEM locomotor activity scores. **p<0.02; ***p<0.01 compared to LS and SAL control group. @@ p<0.02, LS compared to SAL.
3.3 *BDNF* mRNA expression

One way ANOVA indicated significant group differences in six of the brain regions examined, including the primary motor cortex \([F(2,21)=4.13, p<0.03]\), secondary motor cortex \([F(2,21)=4.96, p<0.017]\), insular cortex \([F(2,20)=5.05, p<0.01]\), prelimbic cortex \([F(2,20)=3.70, p<0.04]\), CA1 \([F(2,21)=3.29, p<0.05]\), and CA3 \([F(2,21)=5.97, p<0.01]\). As shown in Figure 30, LS animals had significantly less *Bdnf* mRNA expression compared to HS mice in several brain regions, including the primary and secondary motor cortices (-22%, \(p < 0.004\) and -25%, \(p < 0.01\), respectively), prelimbic cortex (-25%, \(p < 0.04\)), claustrum (-24%, \(p < 0.05\)), insular cortex (-17%, \(p < 0.05\)), CA1 (-20%, \(p < 0.03\)) and CA3 (-21%, \(p < 0.01\)). Compared to saline controls, LS mice had significantly less *Bdnf* mRNA expression in the insular cortex (-20%, \(p < 0.05\)) and CA3 (-20%, \(p < 0.01\)). HS mice did not differ from saline controls in any brain region. See Table 1 (pg. 125) for brain region abbreviations.
Figure 30. **BDNF mRNA expression.** Values are in means mRNA in µCi/gram of tissue ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. *p<0.05; ***p<0.01 compared to the high-sensitized (HS) group; ###p<0.01 compared to Saline (SAL) controls. Bonferonni comparisons.
3.4 *TrkB mRNA expression*

Results for trkB mRNA expression are shown in Figure 31. ANOVAs indicated the presence of significant group differences in all of the 27 brain regions examined (p<0.05) with the exception of the dorsal and medial raphe nuclei, for which fewer sections were available for sampling. Bonferroni-adjusted comparisons revealed a uniform pattern of results, with LS mice showing significantly less trkB mRNA expression than both HS and control mice in all 25 regions. HS mice did not differ from saline controls in any brain region. Figure 32 is an illustration showing group differences in the expression of Bdnf and trkB. See Table 1 (pg. 124) for brain region abbreviations.
Figure 31. *trkB* mRNA expression. Values are in means mRNA in µCi/gram of tissue ± SEM. Please refer to Table 1 (pg. 125) for brain region abbreviations. *** p<0.01 compared to the high-sensitized group; ### p < 0.01 compared to Saline controls.
Figure 32. Illustration of $BDNF$ and $trkB$ changes among the three groups.
3.5 *TrkB* receptor blockade

Results for TrkB blockade are shown in Figure 33. ANA-12 failed to prevent the development (Figure 33A) or the expression (Figure 33Bb) of EtOH sensitization. Repeated measures ANOVA indicated a significant main effect of injection day \( [F(2, 102)=19.08, p<0.01] \), injection day x treatment interaction \( [F(6, 102)=12.30, p<0.01] \), and main effect of treatment \( [F(3, 51)=38.77, p<0.01] \). Bonferroni post hoc tests showed that the VEH/EtOH group showed higher LMA than VEH/SAL and ANA-12/SAL (\( p<0.01 \)) for inj1, 3, 5. ANA-12/EtOH showed increased LMA than VEH/SAL and ANA-12/SAL for inj 3 and 5.

For the expression phase, one way ANOVA comparing challenge scores was significant \( [F(3, 27)=6.259, p<0.01] \). Bonferonni post hoc showed that all groups receiving EtOH challenge showed greater LMA than VEH/SAL group, demonstrating that chronic ANA-12 pretreatment during the development did not affect the locomotor response to an EtOH challenge, nor did TrkB blockade prior to EtOH prevent the expression (Figure 33B).
Figure 33. Effects of TrkB receptor blockade with ANA-12 on the development and expression of EtOH sensitization. A: Development phase. Pretreatment with ANA-12 failed to block the development of locomotor sensitization to EtOH. ***p<0.01, VEH/EtOH vs VEH/SAL and ANA-12/SAL; @@ p<0.01, ANA-12/EtOH vs VEH/SAL and ANA-12/SAL. B: Expression phase. Following a 14-day drug-free period, mice were challenged with either EtOH (1.8g/kg, i.p.) or an equal volume of SAL, preceded or not by ANA-12. ANA-12 did not affect the expression of sensitization. *** p<0.01, EtOH treated groups group compared to SAL controls.
4. Discussion

The main finding of the present study was that LS and HS mice differed in the expression of the markers of synaptic plasticity, Bdnf and trkB. Specifically, Bdnf and trkB gene expression was lower in LS mice compared to the other two groups.

The observation of decreased Bdnf and trkB restricted to LS mice is a novel and somewhat unexpected observation. As noted, our design does not permit conclusions as to whether these changes were pre-existing or whether they were induced by ethanol treatments. Of note however several studies have documented a reduction in TrkB mRNA, immunoreactivity, TrkB signaling, as well as decreased BDNF protein expression and increased cell death in various brain regions following chronic exposure to EtOH (Bhave et al., 1999b; Climent et al., 2002b; Ge et al., 2004a; Moore et al., 2004; Tapia-Arancibia et al., 2001; Walker et al., 1980; Zou and Crews, 2006). It is therefore possible that repeated EtOH treatment may have induced neuronal damage and/or impaired synaptogenesis in the subpopulation of mice that fail to sensitize. Support for this idea comes from observations of significant neuronal loss and decreased dendritic and synaptic complexity in both alcoholics and animals chronically exposed to EtOH (Abernathy et al., 2010; Kroener et al., 2012; Spiga et al., 2014b; Zhou et al., 2007). The issue would remain however as to why low expression levels of trkB and Bdnf was present in low-sensitized but not in the similarly treated Sensitized animals.

Our finding that HS and SAL mice did not differ in levels of Bdnf and trkB expression, but that LS mice had significantly lower levels of these two is inconsistent with studied demonstrating that reduced BDNF is associated with increased behavioural responsiveness to EtOH, including sensitization (McGough et al., 2004; Rueda et al., 2012). BDNF has been
implicated as a key component of a homeostatic pathway regulating responses to EtOH, where increases in its protein levels have been associated with reduced EtOH consumption, while decreases in BDNF enhances EtOH consumption and the locomotor responses to EtOH (Jeanblanc et al., 2006; Rueda et al., 2012). The fact that our HS mice did not show reduced levels of Bdnf but the LS mice did might be explained by the fact that these studies employed different sensitizing regimens and mouse strains than the current study. Strain differences appear to influence EtOH’s effect on BDNF expression. Indeed, it has been shown that Bdnf expression decreases with acute exposure to EtOH in C57BL/6 mice, but increases in the DBA strain (Kerns et al., 2005). Furthermore, these studies involved daily injections of EtOH at a lower dose than that used in the current study (1.8g/kg and 2g/kg, versus 2.2g/kg) (McGough et al., 2004; Rueda et al., 2012). It is possible that the repeated EtOH exposure and withdrawal cycles in our study might have influenced Bdnf expression. Additionally, we assessed mRNA levels 2 weeks after the last EtOH injection and it is plausible that Bdnf mRNA levels returned to control levels during this time period only in HS mice. Interestingly, 5 days of withdrawal from a sensitizing regimen of cocaine induced a brain-specific decrease in trkB, similar to what we have seen in LS mice (Filip et al., 2006). It is possible that the LS mice are more susceptible to EtOH withdrawal-induced neurochemical changes than are HS mice. Decreased neurotrophic levels might indicate chronic EtOH-induced neurodegeneration, again suggesting that EtOH may have greater neurotoxic effects in LS than HS mice. However, it is important to be cognizant of the fact that the relationship between BDNF and EtOH remains unclear and, as the results of the present study show, inconsistent (Davis, 2008).

The reduced trkB levels in mice suggested the possibility that signaling through TrkB receptors is necessary for EtOH sensitization. However, pharmacological blockade of TrkB failed to prevent the development or expression of EtOH sensitization and suggests that brain-
wide suppression of TrkB signaling may not be critical to this behavior. Although there is existing evidence demonstrating that the ANA-12 regimen we employed in this study reduced TrkB receptor activity, our conclusions should be verified with other methods of TrkB antagonism (Cazorla et al., 2011; Leggio et al., 2014).

Irrespective of mechanisms involved, the results of the current study indicate that variability in the sensitization response to EtOH is differentially associated with trkB and Bdnf expression in various brain regions. Specifically, LS mice showed significantly lower mRNA expression levels of trkB in nearly all brain regions analyzed, while the reduction in Bdnf gene expression was present in select brain regions. These results demonstrate that variability in the EtOH sensitization response is associated with differences in synaptic plasticity and suggest the possibility that EtOH may have neurotoxic effects in a subpopulation of mice, which might in turn prevent the development of behavioural sensitization.
5. **Statement of significance.** Here, we show that LS mice have reduced trkB and Bdnf gene expression in the brain compared to HS and SAL mice. However, blockade of BDNF-TrkB signaling by receptor antagonism did not prevent the development and expression of EtOH sensitization. The observed decrease in gene expression in LS mice suggests the possibility that EtOH may have neurotoxic effects in a subpopulation of mice, which in turn may prevent the development of behavioural sensitization. The lack of difference in Bdnf and trkB expression between HS and SAL mice suggests that the mechanisms in EtOH sensitization may be different from those mediating sensitization to other psychostimulants when it comes to BDNF-TrkB involvement.
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<thead>
<tr>
<th>Brain region</th>
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<tr>
<td>Anterior cingulate cortex</td>
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<td>Ventromedial caudate putamen</td>
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**Table 1.** Brain region abbreviations used in NMDAR, pCREB, trkB, and Bdnf projects
Chapter 5

Glutamate and markers of neuroplasticity: EtOH sensitization and Arc gene expression

This study has been published as:


Statement of author contributions:

All experiments were designed by CN and JN. All experimental work and data analyses were conducted by CN. ML assisted with film reading. CN and JN wrote the manuscript.
1. Introduction

Glutamate receptor activity has been shown to regulate the expression of activity-regulated cytoskeleton-associated protein (Arc), an immediate early gene (IEG) whose expression is elevated following exposure to drugs of abuse (Hearing et al., 2008; Li et al., 2015; Pei et al., 2004; Schochet et al., 2005; Ujike et al., 2002).

IEG levels are transiently increased shortly after a stimulus in the brain areas which process that stimulus (Okuno, 2011). They are considered to be markers of neuronal activity which translate extracellular stimuli into altered patterns of neuronal gene expression and long-term changes in cellular functioning. Therefore drug-induced IEG expression can be seen as an important first step in mediating the molecular cascades underlying drug experience-dependent plasticity. Arc is an effector IEG that encodes a protein directly affecting neuronal plasticity. Specifically, the gene is targeted to synaptic sites undergoing strong activity, where its protein binds to actin filaments, influencing their dynamics and thus regulating spine plasticity (Bramham et al., 2008; Ujike et al., 2002). ARC protein plays an important role in the processes underlying the formation of long-term changes in neuron function and synaptic modifications, and is required for other forms of synaptic plasticity such as long-term potentiation (LTP), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor endocytosis, and cell migration (Bramham et al., 2008). Thus, Arc expression serves as an excellent marker of drug-induced neuronal activity leading to long-term changes in neuron function. The goal of the present study was to examine differences in Arc mRNA expression in LS, HS, and saline (SAL) mice after EtOH exposure in order to identify brain areas where neuroplastic changes may contribute to the development and expression of EtOH sensitization. We hypothesized that HS mice would show greater brain levels of Arc expression in compared to LS and SAL mice.
2. Materials and Methods

2.1 Subjects

Male DBA/2 mice (n=85), Mice were housed 4 per cage as described in section 1 of the General methodology.

2.2 Test apparatus

Measurements of LMA were carried out as described in section 2 of the General methodology.

2.3 Drugs

Mice received 2.2g/kg of EtOH i.p. (15mL/kg), or an equal volume of saline (SAL) during the development phase of the study. For the expression phase, mice were challenged with 1.8g/kg, i.p., of EtOH. For more information please refer to section 3 of the General methodology.

2.4 EtOH sensitization procedures

Details on the EtOH sensitization procedure are provided in section 4 of the General methodology. To examine Arc expression during the development, mice were counterbalanced for baseline LMA and subsequently assigned to receive either EtOH (n=29) or SAL (n=8). An identical sensitization protocol was used in a separate cohort designed to test persistent sensitization effects. After the 5th injection, a 14 day drug-free period was allowed and then LS (n=16), HS (n=16) and SAL (n=16) mice assigned to receive either an EtOH (1.8 g/kg) or a SAL challenge prior to a final LMA test and sacrifice. For both cohorts, brains were removed following behavioural testing and stored at -80°C until sectioning and processing for in situ hybridization analyses.

2.5 In situ hybridization protocol for ARC mRNA expression
Sectioning, prehybridization, and hybridizations were performed as described in section 6 of the General methodology. Primers comprised the consensus promoter sequences for the arc gene, according to Genbank # NM_001276684.1. Primers were complementary to bases 1442-1462 (left primer) and 1995-1976 (right primer). Sections were exposed to Kodak BioMax film at 4°C for 21 days.

2.6 Image analysis

In situ hybridization signals on film were quantified using MCID Basic 7.0 image analysis software, as described in section 7 of the General methodology.

2.7 Statistical analyses

Behavioural data were analyzed by repeated measures ANOVA, with Group (HS, LS, and SAL) as the between-subjects factor and injection day as the repeated measures factor. Regional Arc levels for the development phase groups were analyzed with one way ANOVAs, followed by Bonferroni-adjusted within-group and between-group comparisons, where warranted. For the challenge experiment Arc levels were analyzed with 2-way ANOVAs with prior sensitization status (HS, LS, and SAL) and challenge drug (EtOH and SAL) as factors; Arc levels in each EtOH group were compared to the corresponding SAL group using Bonferroni-adjusted tests. Finally, the magnitude of EtOH-SAL differences were contrasted among the 3 groups (SAL, LS, HS) using one-way ANOVAs, followed by Bonferroni post hoc tests where warranted.
3. Results

3.1 EtOH sensitization

Figure 34 shows the LMA profiles of EtOH- and saline (SAL)-treated mice whose brains were removed following the last injection during the development phase (Fig 34A) or after the challenge (Fig 34B). A repeated measures ANOVA for mice sacrificed after injection 5 (Fig 34A) showed significant main effects of injection day \([F (2, 40) = 32.81, p<0.01]\) and treatment \([F (2, 20) = 22.58, p<0.01]\) and their interaction \([F (4, 40) = 16.391, p<0.01]\). Bonferroni-adjusted within-group comparisons indicated that HS scores at inj 5 and inj 3 were higher than scores at all preceding points (all \(p<0.01\) or less). LS scores at inj 3 were significantly higher than at inj 1 (\(p<0.004\)) but did not differ from those at inj 5 (\(p>0.15\)). In addition, Bonferroni-adjusted between-group comparisons indicated that LS and HS scores were greater than SAL mice for inj 3 (\(p<0.002\) and \(p<0.0004\) respectively) and at inj 5 (\(p<0.0001\) in both cases). At inj 5 HS scores were significantly higher than LS scores (+97%, \(p<0.0001\)).

For animals sacrificed after a challenge injection of SAL or EtOH (Fig 34B), there was a significant main effect of test day \([F (2, 66) = 28.42, p<0.0001]\), a main effect of treatment \([F (2, 33) = 25.88, p<0.0001]\) and a day by treatment group interaction \([F (4, 66) = 12.34, p<0.0001]\). Bonferroni-adjusted within group comparisons indicated that HS scores at inj 5 were higher than at inj 3 (\(p<0.02\)) and at inj 1 (\(p<0.0001\)) and that inj 3 scores were higher than inj 1 scores (\(p<0.01\)). LS scores did not significantly differ among the three injection points. In addition, Bonferroni-adjusted between-group comparisons indicated that LS and HS scores were greater than SAL scores at inj 3 (\(p<0.003\) and \(p<0.0001\) respectively) and inj 5 (\(p<0.0001\) in both cases). At inj 5 HS scores were significantly higher than LS scores (+1%)\%, \(p<0.0001\)). During the challenge, LMA scores did not differ between EtOH and SAL within each of the three
sensitization groups, suggesting that locomotor activation was primarily a function of prior sensitization regimen (Fig 34B). This was confirmed by an ANOVA for the challenge data which indicated a main effect of prior sensitization status (SAL, HS or LS) \( [F(2,42) = 45.2, \ p < 0.01] \), no main effect of challenge (EtOH or SAL)\( [F(1,42) = 0.595] \) and no interaction \( [F(2,42) = 0.096] \).
Figure 34. Behavioural sensitization to EtOH. EtOH-treated mice were retrospectively classified as High Sensitized or Low Sensitized on the basis of their behaviour on the last EtOH injection (inj5) for both the development (panel A) and expression of EtOH sensitization (panel B). For the challenge groups filled black symbols represent EtOH challenges and open white symbols represent SAL challenges. Values are means ± SEM locomotor activity scores. ### p<0.01 or lower, compared to all previous injection points for that group; *** p<0.001, compared to saline controls; @@@ p<0.001, compared to low-sensitized mice; &&& p<0.001, high-sensitized (EtOH and SAL challenged) compared to LS. All p values represent Bonferroni-adjusted values. N=8 for all groups.
3.2 Arc gene expression during the development of sensitization

As shown in Figure 35, EtOH treatment resulted in a generalized decrease in Arc levels relative to SAL controls, notable exceptions being subnuclei of the central amygdala (CeA), where positive albeit non-significant changes were observed. Separate one-way ANOVAs indicated significant group differences in 23 regions of the 43 regions examined (see Table 2 for brain region abbreviations): OTu, IL, PrL, CgCtx, MotCx, CpuA, CpuDL, CpuDm, CpuV, CpuVL, CpuP, LSD, MS, PirCtx, CA1, CA2, DG, SubD, SubV, BLA, LaDL, Pn, and VTA (all F values with 2,20 dfs, all p < 0.05). Bonferroni-adjusted comparisons indicated that in most cases (13 regions) significant decreases occurred both in LS and HS groups, as shown in Figure 35. These areas included the OTu, IL, CpuA, CpuDl, CpuDm, CpuV, CpuVl, CA1, CA2, DG, SubD, and the SubV (p <0.05). In some cases large percent changes (e.g. ventral pallidum, -53% and -62% in HS and LS groups, respectively) did not achieve statistical significance due to high variability within the SAL control group (not depicted in the Figure). Of note, the only brain region where Bonferroni tests indicated significant differences between LS and HS mice was the VTA (p <0.05).
**Figure 35.** *Arc* mRNA expression during the development phase. Values are means ± SEM % changes in *Arc* levels relative to the control mean.  

* * p < 0.05, HS vs. SAL;  

• * p < 0.05 , LS vs. SAL;  

• • * p < 0.05, HS vs. LS (Bonferroni comparisons following one-way ANOVAS). See Table 2 (pg. 144) for brain region abbreviations.
3.3 Arc gene expression after an EtOH challenge

In animals previously treated with SAL and receiving EtOH for the first time, Arc expression was generally decreased in comparison with their similarly SAL-pretreated counterparts challenged with SAL (Figure 36). Although changes were significant in 28 out of 48 brain regions examined, only 11 of these survived Bonferroni corrections for 48 comparisons. As shown in Fig. 36, these included decreases in caudate-putamen, ventral pallidum, hippocampus and amygdala nuclei. In addition, however, Arc levels were significantly increased in central amygdala (CeA) subnuclei (capsular, median and lateral), increases ranging from 233% to 395% (Figure 36). A 73% increase in the bed nucleus of the stria terminalis did not reach statistical significance in Bonferroni-adjusted comparisons. See Table 2 for brain region abbreviations.
Figure 36. *Arc* mRNA expression in SAL mice after an EtOH challenge. Values are means ± SEM relative to SAL mice challenged with SAL. *p < 0.05, Bonferroni-adjusted comparisons. See Table 2 (pg. 144) for brain region abbreviations.
Results for LS mice are shown in Figure 37. In comparison to LS mice challenged with SAL, LS mice challenged with EtOH showed widespread reductions, 9 of which survived Bonferroni adjustments. These included decreases in prefrontal and cingulate cortices, caudate-putamen and hippocampal subregions. In addition, pronounced increases in ARC mRNA levels were seen in the medial (+57%) and lateral aspects (+65%) of the central amygdala, as well as the medial (+54%) and lateral septum (+30%) and the BNST (45%). Except for the medial septum, statistical significance in these regions was lost after Bonferroni adjustments for 48 comparisons. An illustrative example of increased Arc expression in the CeA is shown in Figure 39.
Figure 37. *Arc* mRNA expression in LS mice after an EtOH challenge. Values are means ± SEM relative to SAL mice challenged with SAL. *p < 0.05, Bonferroni-adjusted comparisons.

See Table 2 (pg. 144) for brain region abbreviations.
Figure 38 shows results for HS mice. In comparison to HS mice challenged with SAL, HS mice challenged with EtOH showed reductions in Arc expression throughout the brain, with the prelimbic and cingulate cortices, CA1 and CA2 and basomedial amygdala surviving Bonferroni adjustments for statistical significance. Significant increases in Arc expression were not observed in any brain region, although positive trends were seen in 8 of the 48 regions examined. See Table 2 for brain region abbreviations.
Figure 38. *Arc* mRNA expression in HS mice after an EtOH challenge. Values are means ± SEM relative to SAL mice challenged with SAL. *p* < 0.05, Bonferroni-adjusted comparisons. See Table 2 (pg. 144) for brain region abbreviations.
Figure 39. Illustrations of Arc mRNA expression in the central amygdala (arrow) of mice challenged with EtOH (left) versus SAL (right).
Figure 40 compares the *degree* to which an EtOH challenge suppressed or increased *arc* expression in LS, HS, and SAL (i.e., acute EtOH) groups in brain regions appearing to differ greatly among the three groups. Separate one-way ANOVAs revealed significant differences in 10 of the 15 brain regions analyzed. Bonferroni corrected t-tests found that in 8 of these regions there were significant differences in the magnitude of the EtOH signal between mice receiving EtOH for the first time (SAL group) and mice classified as LS or HS. The LSD, MS, BNST, and BLP differed between LS and HS mice.
Figure 40. Magnitude of arc mRNA expression changes in SAL, LS, and HS mice challenged with EtOH. Values are means ± SEM % changes in arc levels relative to the respective control means. *p<0.05, LS vs SAL; ≥p<0.05 HS vs SAL; ≥p<0.05, LS vs HS. See Table 2 (pg. 144) for brain region abbreviations.
<table>
<thead>
<tr>
<th>Brain region</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Infralimbic cortex</td>
<td>IL</td>
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<tr>
<td>Prelimbic cortex</td>
<td>PrL</td>
</tr>
<tr>
<td>Nucleus accumbens core</td>
<td>NAc core</td>
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<tr>
<td>Nucleus accumbens shell</td>
<td>NAc Shell</td>
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<tr>
<td>Lateral accumbal shell</td>
<td>NAc LAbSh</td>
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<tr>
<td>Ventral tegmental area</td>
<td>VTA</td>
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<tr>
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<td>CA1</td>
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<tr>
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<td>CA2</td>
</tr>
<tr>
<td>Hippocampal CA3</td>
<td>CA3</td>
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<tr>
<td>Dentate gyrus</td>
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<td>Basolateral amygdala anterior</td>
<td>BLA</td>
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<td>CeC</td>
</tr>
<tr>
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<td>CeM</td>
</tr>
<tr>
<td>Central amygdaloid nucleus, lateral division</td>
<td>CeL</td>
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<tr>
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<tr>
<td>Basolateral amygdaloid nucleus, ventral part</td>
<td>BLV</td>
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<tr>
<td>Lateral amygdaloid nucleus, dorsolateral part</td>
<td>LaDL</td>
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<tr>
<td>Lateral amygdaloid nucleus, ventrolateral part</td>
<td>LAVL</td>
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<tr>
<td>Lateral amygdaloid nucleus, ventromedial part</td>
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<td>Basomedial amygdaloid nucleus, posterior part</td>
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<td>Basolateral amygdala posterior</td>
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<td>Island of callea</td>
<td>iCj</td>
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<td>Dorsal subiculum</td>
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<tr>
<td>Dorsal entorhinal cortex</td>
<td>DEnt</td>
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<tr>
<td>Lateral septal nucleus, dorsal part</td>
<td>LSD</td>
</tr>
<tr>
<td>Lateral septal nucleus, ventral part</td>
<td>LSV</td>
</tr>
<tr>
<td>Medial septal nucleus</td>
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<td>BNST</td>
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<tr>
<td>Piriform cortex</td>
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<td>EndoPIR</td>
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<tr>
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</tr>
<tr>
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<td>Cpu VL</td>
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<tr>
<td>Ventral pallidum</td>
<td>VP</td>
</tr>
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</table>

**Table 2.** Brain region abbreviations used for Arc project
4. Discussion

EtOH administration was found to be generally associated with reduced Arc mRNA levels in most brain regions, irrespective of treatment group or phase of sensitization. When brains were analyzed following the 5th sensitization injection, the only brain regions showing trends toward increased Arc expression were central amygdaloid nuclei. When brains were examined after an EtOH challenge following a two-week drug-free period Arc levels were again found to be generally decreased in most brain areas in all treatment groups. Surprisingly, however, in mice receiving EtOH for the first time Arc expression was strongly increased in the central amygdala, with strong albeit non-significant increases in the BNST. Central amygdala increases were also seen in LS mice, but were not significant. There was also a trend for increased expression in the septum and BNST of these mice. In contrast, in HS mice no brain regions showed statistically significant increases in Arc expression when EtOH and SAL challenge values were compared. When comparing the magnitude of Arc expression changes among the three groups following an EtOH challenge, LS mice demonstrated increased expression in the septum, basolateral amygdala, and BSNT compared to HS mice.

4.1 Development of EtOH sensitization

Surprisingly, no brain areas showed increased Arc levels following the final EtOH injection in HS mice. This is contrary to what has been reported with sensitization to other drugs (Moro et al., 2007; Samaha et al., 2004; Samaha et al., 2005; Ujike et al., 2002). Since Arc was used as a marker of neural activity to identify brain areas undergoing plasticity induced by EtOH, it was expected that its levels would be elevated upon EtOH exposure in sensitized mice, given that sensitization is a form of drug-induced neurobehavioural plasticity (Chen et al., 2009).
It is conceivable that the absence of Arc increases in HS mice may relate to a desensitization of Arc-inducing effects as a result of repeated drug exposure. Indeed, chronic EtOH exposure has been shown to desensitize expression of the IEG products c-Fos and Egr-1, an effect also seen with chronic administration of psychostimulants (Chang et al., 1995; Faria et al., 2008; Vilpoux et al., 2009). It would be interesting to track the changes in neural activity induced by EtOH to assess whether differences between high- and low-sensitized mice in EtOH-induced neural activity can be detected throughout the course of sensitization development.

Although there was a general EtOH effect in Arc reduction throughout the brain, some areas showing reductions were unique to LS or HS mice and could potentially explain variability in the sensitization response. Areas where Arc decreases were significant only in LS mice were largely areas of the neocortex (e.g., prelimbic, motor, cingulate) and lateral septum. In contrast, areas showing decreases only in HS mice included the VTA. Although the functional role of Arc reduction and presumably regional inactivation in these areas is unclear, it seems safe to conclude that at this phase differences in Arc expression between LS and HS mice are mostly differences of degree rather than overall direction of changes.

4.2 Expression of EtOH sensitization

In SAL treated animals acutely challenged with EtOH Arc expression was significantly reduced in caudate-putamen and hippocampal areas. In addition, there was a dramatic increase in Arc gene expression in the central amygdala (medial, capsular and lateral subdivisions; 313%, 234%, 395%, respectively) compared to SAL mice challenged with SAL. The BNST also showed elevated Arc following an EtOH challenge (71% increase). In LS mice, an EtOH challenge had a very similar effect, except that the increases in the central amygdala and BNST were not as pronounced, and was not statistically significant, as in the first-time treated animals.
(57% in the CeM, 65% in the CeL, and 46% in the BNST). Furthermore, in LS mice the septum also showed increased gene expression (54% in the medial septum). In contrast to both LS and SAL mice, no brain areas in HS mice showed significantly increased levels, despite trends in the VTA, CeA and ventral pallidum.

The fact that HS mice did not show significant increases in Arc expression upon an EtOH challenge goes against our original hypothesis and is inconsistent with the sensitization literature demonstrating that a challenge in sensitized animals leads to increased levels of other IEGs (Faria et al., 2008; Hope et al., 2006; Jedynak et al., 2012; Mattson et al., 2007). Perhaps examining expression of another IEG in addition to Arc might provide further information as to the brain areas important in EtOH sensitization, since the level of expression can vary depending on the IEG and the dose of EtOH used (Vilpoux et al., 2009). Nonetheless, these results suggest the possibility that increased tolerance both to Arc-inducing and Arc-suppressing effects of EtOH may translate into high behavioral sensitization.

Dopamine (DA) D1 receptor stimulation has been shown to increase the expression of the Arc gene, whose transcription is regulated by pCREB (Berke et al., 1998; Cadet et al., 2010; Kawashima et al., 2009). It is interesting that HS mice, despite having elevated accumbal pCREB and hypersensitive DA D1Rs, do not show increased Arc expression in this area (Abrahao et al., 2014; Abrahao et al., 2011; Nona et al., 2013b). While this discrepancy should be examined further, it is possible that the lack of accumbal Arc effects may be at least partially due to opposing effects of released DA on D1 and D2Rs following an EtOH challenge. Abrahao and colleagues have shown that sensitized mice have hyper responsive DA D1Rs as well as hyper responsive DA D2Rs (Abrahao et al., 2014; Abrahao et al., 2012; Abrahao et al., 2011). Of interest, in the accumbens D1R agonists increase whereas D2R
agonists decrease Arc expression (Waters et al., 2014). It is conceivable that these effects might partially cancel each other out, resulting in a net lack of change in the accumbens.

Many reports show that the CeA and BNST are EtOH-sensitive regions (for review see Vilpoux et al., 2009). Because these regions play a role in behavioural responses to environmental and internal stressors, which themselves increase IEG expression, one may speculate that increases in Arc may be a stress effect caused by intraperitoneal irritation following an EtOH injection (Smith and Aston-Jones, 2008). However, in those studies mice were chronically handled and injected prior to the challenge; in addition, EtOH has been shown to induce IEG expression in these areas when different routes of administration are employed, such as vapour or intragastric administration (Ryabinin et al., 1997; Vilpoux et al., 2009). Therefore, we are confident that increased Arc expression in these areas is reflective of the pharmacological actions of EtOH in the brain.

The functional role of Arc activation in the sensitization response is unclear. However, the increase in EtOH-induced Arc induction in the septum, basolateral amygdala and BNST of LS compared to HS mice suggests the possibility that neural activity in these area might serve to inhibit behavioural sensitization to EtOH. This is in contrast to what has been reported previously where sensitivity to the locomotor stimulant effects of EtOH was associated with increased Fos expression in the CeA (Demarest et al., 1999; Hitzemann and Hitzemann, 1997). Indeed, future studies could examine whether transient tissue inactivation of these regions leads to EtOH-induced stimulation of locomotor activity. Interestingly, the septum of LS mice was an unexpected region that showed sensitivity to the Arc-inducing effects of EtOH. The septum sends projections to the VTA and thus regulating the activity of dopamine (DA) neurons projecting to the accumbens (Louilot et al., 1989). Indeed, inhibition of septal neurons increases accumbal DA levels, whereas excitation restrains them (Sheehan et al., 2004). As a
challenge injection of EtOH in sensitized animals has been shown to increase extracellular accumbal dopamine levels, it is plausible that increased Arc in septum of LS mice indicates a failure of EtOH to elevate accumbal dopamine levels and thus a failure to express sensitization (Szumlinski et al., 2005 but see Zapata et al., 2006). Future studies could further probe into the mechanisms by which the septum and its connectivity patterns may be involved in the EtOH sensitization response.

The overall reduction in Arc expression as a consequence of EtOH exposure would make sense given that EtOH inhibits the functioning of NMDARs, one factor regulating arc gene expression. Perhaps the increase present in the septum, basolateral amygdala, and BNST of LS mice and the CeA of SAL mice may indicate a reduced ability of EtOH to block NMDARs and/or a reduction in the amount of NMDARs in these brain regions. It is important to note, however, that receptors other than NMDARs are involved in the regulation of Arc gene expression which may account for elevated Arc in these regions (Korb and Finkbeiner, 2011).

It was very surprising to see that sensitized mice challenged with SAL showed locomotor responses very similar to the sensitized mice challenged with EtOH (Figure 34B). Such conditioned locomotion indicates that the expression of EtOH sensitization is at least in part due to associative learning mechanisms, an observation noted in the psychostimulant sensitization literature (Bell et al., 2000; Robinson et al., 1998; Vezina and Stewart, 1990). Future studies should aim to parse out the contribution of conditioned locomotion to EtOH versus pharmacological sensitization in the behavioural and neurobiological effects of repeated EtOH exposure.

In summary, EtOH treatment resulted in widespread decreases in Arc expression throughout the brain. Contrary to our hypothesis, EtOH injection did not elevate Arc levels in
HS mice. Instead, localized increases were seen in SAL (central amygdala) and LS (septal regions, basolateral amygdala, BNST) mice after an EtOH challenge. These findings raise the possibility that inhibiting neural activity in these regions may result in increased susceptibility to EtOH-induced stimulation and sensitization.
5. **Statement of significance.** Here we show that EtOH induces a generalized decrease in *arc* expression throughout the brain after the final injection, a pattern similar to that seen following an EtOH challenge two weeks after the last sensitization injection. However in this cohort, *Arc* expression was significantly *increased* in the central amygdala (CeA) of SAL mice receiving EtOH for the first time and in the septum, basolateral amygdala, and BNST of LS mice. No significant increases in *Arc* expression were seen in brains of sensitized (HS) animals. The dramatic increases in *Arc* expression in the CeA, septal regions, and BNST in the absence of behavioural stimulation in LS and SAL mice suggests that neural activity in these regions may serve to inhibit the stimulant effects of EtOH. The observation that HS mice do not show significant increases in *Arc* expression following an EtOH injection suggests the possibility that increased tolerance to the *Arc*-inducing effects of EtOH may be a factor in behavioural sensitization.
General discussion

Repeated, intermittent exposure to drugs of abuse produces an enduring enhancement of their psychomotor-activating effects upon subsequent exposure to the same or lower dose (Robinson and Berridge, 1993; Stewart and Badiani, 1993; Vanderschuren et al., 2001). This long-lasting phenomenon is referred to as behavioural sensitization and is hypothesized to play an important role in addiction and to induce neurobiological changes that model the changes taking place during the addiction process (Robinson and Berridge, 2000a; Steketee and Kalivas, 2011).

Neurobiological processes underlying sensitization have been conceptualized into two temporally distinct phases: a development phase referring to short-term neuroadapations induced by intermittent drug exposure, and an expression phase involving more persistent changes mediating supersensitivity to later drug exposure (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000b).

Given that the long-lasting nature of sensitization indicates neuroplastic changes, it is not surprising that a significant amount of evidence points to a critical role for glutamate neurotransmission, whose prominent role in neuroplasticity has been well established (Kalivas, 2009; Rao and Finkbeiner, 2007; Wolf, 1998b). Although such studies have been extremely insightful in further elucidating the mechanisms of addiction and drug action, they have focused primarily on the psychostimulants cocaine and amphetamine, with few probing into the mechanisms underlying EtOH sensitization. This is an important gap in the literature that needs to be addressed because elucidating the role of glutamatergic neurotransmission in EtOH sensitization may hold tremendous therapeutic potential as well as providing further insight into the mechanisms underlying long-term behavioural changes.
In this project, we examined glutamatergic mechanisms underlying EtOH sensitization and the interindividual variability present in this phenomenon. We hypothesized that behavioural sensitization to EtOH is associated with increases in measures of glutamate neurotransmission and that individual differences in EtOH sensitization response would be mediated by NMDARs. To test these hypotheses, we examined the involvement of accumbal extracellular glutamate neurotransmission during the development and expression of sensitization. This was followed by a series of experiments addressing the role of NMDARs in behavioural sensitization to EtOH. Finally, we looked at 4 markers of plasticity—pCREB, Bdnf, trkB, and Arc—and their association with EtOH sensitization and interindividual variability.

1. Summary of experimental work

In this section we provide a summary and conclusion of our findings. For a summary table of results, please refer to Table 3 (pg. 154).

1.1. Theme 1: Glutamate and the nucleus accumbens

In chapter 1, we confirmed that brain and accumbal EtOH concentrations of LS and HS mice were not significantly different. We also examined whether variability in the EtOH sensitization response is differentially associated with changes in extracellular accumbal glutamate levels during the development of EtOH sensitization and after an EtOH challenge. *In vivo* microdialysis experiments showed that the 1st and 5th injection of EtOH did not change extracellular glutamate levels in the NAc of HS mice, whereas LS mice showed a 51% increase in glutamate 5mins after the 5th injection. In contrast, an EtOH challenge elevated glutamate levels in the NAc of HS mice by 67%. To determine whether this enhanced glutamate activity was an important contributor to the expression of sensitization, we stimulated mGluR2/3
autoreceptors with LY354740, known to suppress synaptic glutamate release (Battaglia et al., 1997; Moghaddam and Adams, 1998), and found that the expression of sensitization in HS mice was prevented. These results indicate that there are no pre-existing differences in the NAc glutamate response of LS and HS mice after the first EtOH injection and that the repeated EtOH treatment induces a change in HS mice such that EtOH-induced glutamate release in the NAc may be required for the expression of sensitization.

1.2. Theme 2: Glutamate and NMDA receptors

In chapter 2 we examined NMDAR subunit expression and receptor function in the EtOH sensitization response. We found that LS and HS mice differed in the expression of subunit genes when brains were removed immediately after the development phase but not when brains were removed during the expression phase. LS mice demonstrated elevated expression of NR1, NR2A, and NR2B gene expression in some brain regions. Despite these changes, NMDAR blockade with CGS 19755 and MK-801 did not prevent the development of EtOH sensitization. These findings suggest the possibility that, although their expression is altered during the course of repeated EtOH treatment, key neuroadaptations during the development of sensitization may not be mediated by NMDARs.

1.3. Theme 3: Glutamate and markers of neuroplasticity

In chapter 3, we examined whether EtOH sensitization was associated with increased pCREB in the brain. We found that HS mice had elevated pCREB levels in the NAc and BNST, which confirmed our hypothesis that sensitized animals would show increased pCREB expression. Although elevated pCREB levels are often associated with structural changes, we did not find any changes in NAc MSN spine density or morphology in HS mice. Surprisingly, LS mice had elevated stubby spine density. Our results imply a potentially important role for
pCREB in the expression of EtOH sensitization which should be further explored and suggest that, in the case of EtOH sensitization, drug-induced changes in the structural plasticity of NAc neurons may not be the cause of sensitized behavior.

Another way that synaptic glutamate promotes changes leading to neuroplasticity is by its interactions with the BDNF-TrkB system (Carvalho et al., 2008; Martin and Finsterwald, 2011). Therefore, in chapter 4 we examined the role of BDNF-TrkB signaling in the EtOH sensitization response. Contrary to our hypothesis that HS mice would show increased trkB and Bdnf gene expression, they did not differ from controls. Instead, LS mice showed reduced expression of these genes throughout the brain. Furthermore, we found that blockade of TrkB receptors with ANA-12 did not prevent the development or expression of sensitization. We concluded that the BDNF-TrkB system does not appear to play a crucial role in EtOH sensitization.

In chapter 5 we examined the expression of the immediate early gene, Arc, whose transcription is elevated following glutamate receptor activation (Li et al., 2015; Pei et al., 2004). Arc mRNA was examined across the brain after an EtOH injection during the development and expression phases of sensitization in order to identify areas in which neuroplastic changes might contribute to sensitization. Surprisingly, we found that HS mice did not show elevated Arc expression in any of the brain regions examined. After an EtOH challenge, Arc levels were significantly higher in the LSD, MS, BNST, and BLP of LS mice compared to HS mice. The differential response in EtOH-induced Arc expression between LS and HS mice may account for the behavioural differences in their propensity to sensitize and suggests the possibility that neural activity in the aforementioned structures might explain the failure to sensitize.
<table>
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| 1: NAc glutamate in EtOH sensitization | - NAc glutamate levels in HS mice remain unchanged during the induction of sensitization but are elevated following EtOH challenge.  
- The increases following EtOH challenge appears necessary for the expression of sensitization |
| 2: Role of NMDARs in EtOH sensitization | - LS mice demonstrate increased NMDAR subunit gene expression in the brain during the induction phase  
- No changes in subunit expression were evident when brains were analyzed following a 14 day drug-free period  
- Pharmacological blockade of NMDARs failed to prevent the development of EtOH sensitization |
| 3. EtOH sensitization and pCREB | - HS mice have increased pCREB levels in the NAc  
- HS mice do not show increased accumbal spine density or changes in spine morphology  
- LS mice have increased accumbal stubby spine density |
| 4. BDNF-TrkB system in EtOH sensitization | - LS mice have reduced Bdnf and trkB mRNA expression throughout the brain  
- Pharmacological blockade of TrkB did not block the induction or expression of EtOH sensitization |
| 5. EtOH sensitization and Arc gene expression | - EtOH reduces Arc gene expression throughout the brain  
- Following an EtOH challenge, Arc levels are increased in the central amygdala of SAL mice and the septum of LS mice  
- LS mice have increased Arc mRNA expression in the septum, bed nucleus of the stria terminalis, and basolateral amygdala compared to HS mice. |

Table 3. Summary of experimental results obtained in this thesis
2. Analysis of findings and future directions

This project has demonstrated the existence of glutamatergic differences between LS and HS mice. Results of this thesis may provide new insights into the underlying neurobiological mechanisms mediating EtOH-induced neuroplasticity and enduring behaviours in general. Although the majority of our findings have been descriptive in nature, they have laid the foundation for a further exploration of the glutamate system in susceptibility and resistance to EtOH sensitization.

One surprising and consistent finding from these experiments is that, despite their failure to sensitize, LS mice demonstrate the most neural changes following repeated EtOH treatment compared to HS mice. This was unexpected given that HS mice show the greatest behavioural plasticity, and this was contrary to our hypothesis that HS mice would demonstrate increases in the glutamatergic measures assessed in this project. What could account for such diverse findings among individuals of an inbred strain whose DNA sequence is identical and whose environment can be assumed constant? We speculate that epigenetic factors are at play. Recent evidence has shown that HS and LS DBA mice show different epigenetic changes following repeated EtOH exposure, changes which are crucial to the expression of sensitization (Botia et al., 2012; Legastelois et al., 2013). This line of research should be further pursued.

What follows is a discussion of the potential neuronal mechanisms that might explain some of our observations, as well as the factors that might contribute to susceptibility and resistance to developing EtOH sensitization.
2.1. Low-sensitized mice: Could enhanced EtOH-inhibition of NMDARs explain the failure to sensitize?

One hypothesis that might account for LS mice failing to sensitize is that compared to HS mice, their NMDARs may be more strongly inhibited by EtOH. Differential EtOH inhibition of NMDARs is associated with variability in the response to EtOH’s stimulant actions. For example, Tamara Phillips’ group showed that the NMDARs of mice resistant to the locomotor stimulant effects of EtOH are more strongly antagonized by EtOH than those of mice sensitive to EtOH’s stimulant properties (Daniell and Phillips, 1994). Some of our data supports the possibility that this may be occurring in LS mice. One adaptation of repeated NMDAR blockade by EtOH is an upregulation of receptor expression and function (Chandrasekar, 2013). We found that LS mice demonstrated an upregulation of all the NMDAR subunits we examined, and Quadros and colleagues reported significant elevations in NMDAR binding in the NAc and prefrontal cortex of LS mice compared to HS and control animals (Quadros et al., 2002c). Quadros’ study also showed that LS mice demonstrated elevated binding in all the other brain regions examined. Collectively, these results are in favour of the hypothesis that LS mice may have stronger EtOH-induced NMDAR inhibition compared to HS animals. This would mean elevated NMDAR expression, and likely functioning, following cessation of EtOH administration.

Although our NMDAR antagonist studies suggested the possibility that NMDARs might not be crucial to the EtOH sensitization response, it is possible that secondary effects as a result of increased NMDAR activity may lead to the failure to sensitization. For example, the increase in NR1 mRNA and NMDAR binding in the NAc of LS mice may indicate a supersensitivity of NAc MSNs to glutamate, which has implications for NAc efferents. NAc
GABAergic MSNs project to the ventral pallidum (VP) and ventral tegmental area (VTA), regions regulating locomotor responses to drugs of abuse, including EtOH (Sesack and Grace, 2010; Vanderschuren and Kalivas, 2000b). Glutamatergic excitation of NAc MSNs might lead to increased inhibitory tone in the VP and VTA and subsequently a reduced locomotor response to EtOH. GABA receptor stimulation in these areas has been shown to attenuate the locomotor response to psychostimulants and future studies can examine GABA activity in these areas in the context of EtOH sensitization (Boehm et al., 2002; Koob and Swerdlow, 1988; Leite-Morris et al., 2004).

Our hypothesis of increased EtOH inhibiton of NMDARs in LS mice could account for several of our observations. First, it might explain the increase in NAc glutamate following injection 5; one consequence of NMDAR upregulation following withdrawal from EtOH exposure is an increase in Ca\(^{2+}\)-mediated synthesis and release of retrograde messengers that enhance glutamate release and impair glutamate uptake (Fadda and Rossetti, 1998). Second, it may account for our observation that LS mice have elevated stubby spine density in the NAc, as this spine type is often present under conditions of excessive glutamatergic activity to compensate for elevated synaptic activity (Halpain et al., 1998; Korkotian and Segal, 1999; Segal, 1995). Although the hypothesis of excessive glutamatergic activity in the NAc of LS mice may initially appear inconsistent with our finding that an EtOH challenge does not alter accumbal extracellular glutamate in this subgroup, it is important to note that extracellular glutamate cannot provide any evidence regarding glutamate receptor sensitivity. Also, it is possible that there are mechanisms in place to keep an otherwise enhanced glutamatergic tone at a minimum which we were not able to detect via in vivo microdialysis. For example, the cerebrospinal fluid of alcohol-dependent patients has elevated levels of N-acetylaspartylglutamate (NAAG), a peptide transmitter that inhibits NMDARs and glutamate
release. This may serve as a protective mechanism against enhanced glutamate neurotransmission (Tsai et al., 1998; Vornov et al., 1999). Future work can address whether LS mice may have enhanced levels of NAAG which prevents the observation of increased accumbal glutamate following an EtOH challenge.

Increased activity of the glutamate system can be toxic to neurons and one way EtOH results in neuronal degeneration is by enhancing glutamate transmission through NMDARs as a consequence of repeated receptor blockade (Fadda and Rossetti, 1998). Neurotrophic factors are protective against EtOH-mediated glutamate toxicity (Climent et al., 2002a; Heaton et al., 2000). A third observation accounted for by the hypothesis of increased EtOH-inhibition of NMDARs in LS mice is the reduction in Bdnf and trkB gene expression across their brain. Several studies report reduced trkB mRNA, immunoreactivity, trkB signaling, decreased BDNF protein expression, and increased cell death in various brain regions following repeated EtOH exposure (Bhave et al., 1999a; Climent et al., 2002a; Walker et al., 1980). It is therefore possible that repeated administration and withdrawal cycles of EtOH in LS mice may have neurotoxic effects and in turn interfere with the development of behavioural sensitization. Indeed, BECs obtained in our experiments have been found to reach a maximum of approximately 24mM, a concentration which has been shown previously to increase the production of reactive oxygen species and secretion of prostaglandins in astrocytes, changes that are known to promote neuroinflammation and excitotoxicity (Haorah et al., 2008; Tilleux and Hermans, 2007). Future work should examine neuronal damage in the context of EtOH sensitization.

A fourth observation accounted for by the hypothesis of stronger EtOH inhibition of NMDARs in LS mice would be the increase in Arc mRNA expression present in low-
sensitizers compared to the high-sensitizers; Arc transcription is elevated following NMDAR activity (Bramham and Messaoudi, 2005; Steward and Worley, 2001). The lateral septal nucleus, medial septal nucleus, and the basolateral amygdala, all components of the mesocorticolimbic DA system which are important in sensitization, demonstrated increased Arc in LS compared to HS mice. Since Arc might indicate elevated neural activity in these brain areas, it is possible that activity here serves to inhibit the development of sensitization in LS mice. The septum projects to the VTA and thus regulates DA neurons projecting to NAc (Louilot et al., 1989). Inhibition of activity in the septum elevates NAc DA levels, whereas excitation restrains them (Luo et al., 2011; Sheehan et al., 2004). Since an EtOH challenge elevates accumbal dopamine levels in sensitized mice, increased Arc in the septum of LS mice might indicate a lack of EtOH-induced elevation of NAc DA and the inability to sensitize (Szumlinski et al., 2005).

2.2 High-sensitized mice: Discussion and assimilation of findings

Against our hypothesis, HS mice did not demonstrate increases in all measures of glutamate neurotransmission examined in this project. They did, however, show increased glutamate release in the NAc following an EtOH challenge and elevated accumbal pCREB levels.

The NAc receives glutamatergic input from the frontal cortex, hippocampus, thalamus, and amygdala (Carnes et al., 1990; Groenewegen et al., 1999). Determining which of these inputs may be causing the increased glutamate release following an EtOH challenge is important to further elucidate the circuitry mediating EtOH sensitization. One way to do this would be to measure IEG levels after an EtOH challenge in regions providing glutamate input to the NAc, as IEG expression has often been used as an indication of neural activity (Okuno,
However, we did not see elevated Arc expression in these brain regions. Desensitization of IEG-inducing effects as a result of repeated drug exposure has previously been reported and may account for this (Chang et al., 1995; Faria et al., 2008; Vilpoux et al., 2009). Perhaps examining expression of another IEG might provide further information as to the brain areas important in EtOH sensitization, since the level of expression can vary depending on the IEG and dose of EtOH used (Vilpoux et al., 2009). It is also possible that more obvious changes/indicators of activity occur in the nerve terminal and involve mechanisms of neurotransmitter release which would not be detected at the IEG level.

Sensitizing regimens of psychostimulants and morphine result in presynaptic alterations that facilitate transmitter release. For example, increases in the mRNA, protein, and phosphorylation of synapsin in the NAc have been reported in sensitized animals, as well as elevated neuromodulin levels (Iwata et al., 1997a; Iwata et al., 1997b; Subramaniam et al., 2001). Both these proteins play a role in neurotransmitter release and it is possible that a similar observation may be occurring in HS mice that might account for the increased glutamate release following an EtOH challenge.

Why might HS mice show elevated NAc glutamate following an EtOH challenge, but fail to show any changes in glutamate levels during the induction of sensitization? Behavioural sensitization has been conceptualized into two distinct phases, the induction and expression phases, mediated by different neural changes and time sensitivities to drug exposure (Vanderschuren and Kalivas, 2000b). One possibility is that brain regions other than the NAc may be changing during the induction of sensitization. We have shown previously that high-frequency stimulation of the NAc prevents the expression but not development of EtOH sensitization, suggesting that activity in the NAc may be more important for the expression
rather than the development of sensitization (Nona et al., 2015b). Another possibility is that the 45mins sampling time frame may not be sufficient to detect any potential changes in NAc glutamate following EtOH injection during the induction of sensitization. Changes in the time course of glutamate efflux in response to drug have been reported previously (Amitai et al., 2012; Pierce et al., 1996; Robinson et al., 1995).

It is interesting that despite increases in extracellular glutamate levels and pCREB in the NAc of HS mice, there were no changes in gene expression or spine density and morphology. One explanation for this may be that the convergence of different signaling pathways that act in concert to regulate the pattern of the expressed target genes, such as that initiated by activation of different receptors, might have opposing effects. For example, HS mice have hyperresponsive D1 and D2 receptors in the NAc and stimulation of either receptor induces opposite effects on Arc expression such that stimulation of both simultaneously would cancel out eachother’s effects. A similar phenomenon may be occurring with the other genes (Abrahao et al., 2014; Abrahao et al., 2012; Abrahao et al., 2011; Waters et al., 2014).

An additional factor to consider is the activity of gene expression repressors, which might antagonize the actions of pCREB, and factors regulating mRNA stability. Neuron-restrictive silencer factor (NRSF) is a transcriptional repressor which inhibits the transcription of multiple neuronal genes to regulate the proper timing of gene expression during neurogenesis (Thiel et al., 1999). EtOH treatment enhances the binding activity of NRSF and it is possible that this occurs in the brains of HS mice, counteracting the effects of pCREB and thereby preventing an increase in gene expression (Tateno and Saito, 2008; Tateno et al., 2006). Additionally, EtOH has been shown to alter mRNA stability, either increasing or decreasing it depending on the gene, and absolute measures of gene expression as carried out
in this project provide no indication of the in/stability of these transcripts (Anji and Kumari, 2006; Pietrykowski et al., 2008). All in all, these speculations indicate that levels of pCREB alone may not be indicative of gene expression status.

The presence of elevated pCREB in the NAc of HS mice may be a negative feedback mechanism regulating MSN reactivity to increased glutamate levels, as glutamate increases the firing of NAc neurons (Hu and White, 1996; O'Donnell and Grace, 1995). In cocaine sensitization, subsensitivity of NAc neurons to glutamate has been reported and may arise from increased pCREB expression in this area (Carlezon Jr et al., 2005; Dong et al., 2006). Specifically, increased pCREB expression leads to increases in cell excitability and in doing so counteracts cocaine’s behavioural effects (Carlezon Jr et al., 2005; Dong et al., 2006; Fasano et al., 2009; Huang et al., 2008). Perhaps a similar mechanism in HS mice may be taking place and future studies should address the casual role of pCREB in EtOH sensitization and MSN excitability.

3. Comparing EtOH and psychostimulant sensitization

Since EtOH sensitization differs from psychostimulant sensitization in many respects, it is conceivable that the mechanisms underlying sensitization to EtOH might differ from those regulating the psychostimulants. EtOH’s stimulant effects are only observed within 0-15mins after drug administration and only within a narrow dose range (1.8-2.5g/kg). It is most reliably demonstrated in mice, with some mouse strains more robustly sensitizing than others (Crabbe et al., 1982; Masur and Boerngen, 1980a; Phillips et al., 1994). This project has shown that EtOH sensitization has some similarities with psychostimulant sensitization. The importance of the NAc in the expression but not development of EtOH sensitization (Nona et al., 2015b) is also observed with the psychostimulants, as is the elevation in glutamate levels following a
challenge, and increased pCREB expression (Mattson et al., 2005; Shi and McGinty, 2007; Tzschentke and Schmidt, 2003). However, in psychostimulant sensitization, and in contrast to EtOH sensitization, we often see an important role for BDNF-TrkB and NMDAR signaling, increased \textit{arc} expression, and elevated spine density in NAc MSNs (Bahi et al., 2008; Filip et al., 2006; Klebaur et al., 2002; Kodama et al., 1998; Robinson and Kolb, 2004; Wolf, 1998b). Although methodological/procedural factors might partially account for these discrepancies between EtOH and psychostimulant sensitization, they suggest that neural differences exist in the mechanisms underlying EtOH sensitization compared to those of the psychostimulants.

4. Variability in the EtOH sensitization response

4.1. Brain EtOH concentrations

One factor that may explain the observed interindividual variability in the EtOH sensitization response is differences in EtOH metabolism. LS and HS mice do not differ in BEC, nor does BEC correlate with LMA (Nona et al., 2015b), ruling out differential peripheral metabolism of EtOH as a factor in behavioural variability (Quadros et al., 2005). Furthermore, we have shown in chapter 1 that NAc and total brain EtOH levels do not differ between LS and HS mice, indicating this is not a variable that contributes to differences in EtOH response.

4.2. Can we predict sensitization based on baseline locomotor activity?

Previous studies have shown that individual variability in the locomotor response to novelty predicts the locomotor stimulant response to drugs, the sensitization response, and the reinforcing effects of these drugs (Bevins and Peterson, 2004; Hooks et al., 1992a; Hooks et al., 1992b; Hooks et al., 1991; Hoshaw and Lewis, 2001). However, it is unknown if this is the case for EtOH. To assess whether the locomotor response to novelty might be a predictor of the
acute stimulant effects of EtOH and/or the sensitization response, we performed correlations between activity for the first habituation session and activity following injection 1 and the final injection during the development phase for EtOH-treated mice. As shown in Appendix 1A (pg. 196), there was no significant correlation between habituation 1 scores and the locomotor response to EtOH injection 1. Similarly, there was no correlation between locomotor scores for habituation 1 and those of the last EtOH injection (Appendix 1B, pg. 197). Since some studies reported a correlation between EtOH injection 1 and the sensitization response (Abrahao et al., 2013; Nona et al., 2013b), we performed a correlation analysis between activity scores for injection 1 and the last injection. Again, the correlation was not significant (Appendix 1C, pg. 198). Therefore, with respect to EtOH sensitization, it appears that locomotor response to novelty does not predict the acute EtOH response, or the sensitization response. In addition, the acute locomotor response to EtOH also fails to predict the sensitization response.

4.3. Pre-existing differences between LS and HS mice versus differences caused by EtOH treatment

One important question is whether the observed neural differences in LS and HS mice are caused by EtOH treatment or whether they existed prior to treatment. Since most of our studies were descriptive in nature, it is difficult to answer such a question. However, various inferences can be made that might aid in solving this mystery. In our microdialysis study, we failed to observe any major differences in NAc glutamate during the development of sensitization between LS and HS mice, but we found a robust difference following an EtOH challenge. These results suggest that there are no pre-existing differences in NAc glutamate following the 1st EtOH injection and, further, that withdrawal from repeated EtOH differentially restructures the brains of LS and HS mice such that their NAc glutamate response
to an EtOH challenge is dissimilar. It is highly likely that the different glutamate response following EtOH challenge between LS and HS mice is caused by EtOH treatment and it is one factor likely to be responsible for the different sensitization responses of LS and HS mice.

A similar conclusion can be drawn from our Arc data. Here, the most robust differences between LS and HS mice were present following a 2 week drug-free period and after an EtOH challenge, as opposed to the development. Although this indicates that the differences are caused by EtOH treatment, it remains to be established whether they are responsible for the differential sensitization responses or merely a pharmacological effect of EtOH that has no effect on sensitization. Since IEGs are often used as indicators of neural activity, an ideal experiment to address whether the observed differences in Arc expression between LS and HS mice is responsible for the different sensitization responses would be to inactive the brain regions of interest, using the local anesthetic muscimol. Although we have attempted this, we unfortunately are left with inconclusive results due to complications with intracerebral injections using the sensitization protocol in DBA mice (refer to the Project Limitations section on pg. 170).

When examining the NMDAR subunits, pCREB, Bdnf, trkB, and spine density data, it seems that the observed differences were caused by intermittent EtOH exposure rather than a withdrawal from repeated treatment. LS mice demonstrated elevated NMDAR subunit gene expression when brains were removed shortly after injection 6 but not after a 14 day drug-free period. It is known that repeated EtOH treatment elevates NMDAR subunit expression, with levels returning to normal 24-48hr after EtOH exposure (Gulya et al., 1991; Tremwel et al., 1994). Therefore, the absence of mRNA changes after this 14 day withdrawal period suggests that the differences seen during the development were likely induced by EtOH. However, why
this is seen only in LS mice is unclear and may indicate group differences in NMDARs that existed prior to EtOH treatment. When we administered NMDAR antagonists, we found them to be ineffective in preventing the sensitization response. Therefore, it is most plausible that LS and HS mice demonstrate pre-existing differences in NMDAR subunit composition, which in itself may not be responsible for their different sensitization responses but the secondary effects/consequences of this difference may (as described in section 2.1 on pg. 155).

Our pCREB data showed that HS mice had higher levels of pCREB-immunoreactive cells than both LS and SAL mice 14 days after a drug-free period. Because activated transcription factors are known to return to normal levels shortly following stimulation by the inducing stimulus (Michael et al., 2000), it may be tempting to speculate that the increased pCREB in HS mice was a pre-existing condition since EtOH, the proposed inducing stimulus, was not on board for 14 days. It is more likely that this increase in pCREB was caused by intermittent EtOH exposure when sensitization was developing. We speculate that repeated EtOH induced persistent neuroadaptations in HS mice, rendering intracellular cascades activating CREB hyperactive. Indeed HS mice have hyperresponsive accumbal dopamine D1 receptors, receptors which are known to promote CREB phosphorylation (Dudman et al., 2003). It is important to keep in mind that although these differences exist between LS and HS mice, it remains to be elucidated whether they are the cause of the differential response to sensitization or merely an effect of chronic EtOH exposure that does not have a role in the sensitization response. It would also be interesting to examine what makes HS mice susceptible to this pCREB-inducing effect of EtOH.

Comparing Bdnf and trkB mRNA expression between LS and HS mice 14 days after the last injection, we note a dramatic reduction in the expression of these genes in the brains of
LS mice. Because the effects of EtOH exposure on the expression of neurotrophic factors and their receptors are time sensitive and may be short-lived, it would be expected that EtOH-induced changes in gene expression as seen in LS mice would have normalized following withdrawal from EtOH treatment, as may have been the case with HS mice (Ge et al., 2004b; Heaton et al., 2003). However, changes in BDNF expression have persisted 90 days following repeated cocaine treatment, thus our observations may not be entirely surprising (Grimm et al., 2003). Based on our finding that TrkB receptor blockade with ANA-12 failed to prevent EtOH sensitization, it is likely that these gene expression changes are merely pharmacological effects of EtOH which LS mice are more sensitive to and which have no bearing on the sensitization response.

When we looked at spine density in the NAc, we found that LS mice had higher stubby spine density compared to SAL controls shortly following an EtOH challenge. It is unclear why EtOH induced a significant increase in stubby spine density in LS mice, while HS mice appeared resistant to this effect, or, if there were any changes, they returned to control levels sooner in these mice. As discussed earlier, the increase in stubby spine density may be a compensation for elevated excitatory transmission caused by chronic EtOH and future studies could examined whether these differences are the cause of resistance to sensitization present in LS mice.

4.4. Does susceptibility to EtOH sensitization depend on dopamine?

Substantial evidence supports a crucial role for dopamine transmission in behavioural sensitization to psychostimulants. Increased responses of the dopamine system that develop in parallel to behavioural sensitization include elevated dopamine release in the NAc (Fukakusa et al., 2008), supersensitivity of accumbal D1 receptors, and D2 autoreceptor subsensitivity in
the ventral tegmental area (Henry et al., 1998; Pierce et al., 1995). Dopamine mediates locomotor behavior (Amalric and Koob, 1993; Hooks and Kalivas, 1995) and enhanced dopamine release into the NAc in response to drugs of abuse is associated with their locomotor stimulant effects (Di Chiara and Imperato, 1988; Wise and Bozarth, 1987a). EtOH has been shown to act in the ventral tegmental area to increase dopamine neuron activity and to promote its release in the NAc (Brodie et al., 1990; Xiao et al., 2007). In addition, metabolism of EtOH forms the dopamine condensation product, salsolinol, which increases the excitability and firing activity of dopamine neurons in the ventral tegmental area (Xie et al., 2013). Given the link between dopamine and locomotor stimulation, it is plausible that HS mice may be very sensitive to the dopamine-stimulating effects of EtOH and this might explain their susceptibility to sensitization. In support of this hypothesis, a crucial role for dopamine in EtOH sensitization has been well established. For example, D1 (Abrahao et al., 2011) and D2 (Abrahao et al., 2012) receptor blockade prevents the expression of EtOH sensitization. Sensitized mice present hyperresponsive NAc D1Rs (Abrahao et al., 2014) and show enhanced dopamine release in the accumbens following EtOH challenge (Kapasova and Szumlinski, 2008; Szumlinski et al., 2005 but see Zapata et al., 2006). Furthermore, rats demonstrate locomotor sensitization to salsolinol when it is repeatedly injected in the VTA (Hipolito et al., 2010). That salsolinol, a dopamine product of EtOH metabolism, was able to produce sensitization in EtOH sensitization-resistant rats suggests that the dopamine-stimulating effects of EtOH could potentially explain the susceptibility to develop EtOH sensitization.

Our finding for a requirement of NAc glutamate in the expression of EtOH sensitization may initially appear to contradict our hypothesis for a crucial role of dopamine in EtOH sensitization. It should be noted, however, that dopamine and glutamate interact in the NAc to regulate information flow to and from the accumbens (Floresco, 2007). For example,
dopamine and glutamate terminals synapse on the same accumbal dendritic spines (Surmeier et al., 2007), dopamine D1 receptors interact with and modulate NMDAR function (Lee et al., 2002), dopamine neurons projecting to the NAc co-releasing glutamate (Stuber et al., 2010), cocaine-induced increases in ventral tegmental area glutamate depends on D1 receptor stimulation (Kalivas and Duffy, 1995), and finally, glutamate transmission has been shown to have a facilitatory role on presynaptic dopamine release in the NAc (Carter et al., 1988; Cheramy et al., 1986). Therefore, the importance of NAc glutamate in EtOH sensitization expression may arise from its interaction with dopamine.

5. The neural mechanisms of enduring behavioural changes

Simply put, neuroplasticity can be described as long-term changes in brain functioning as a result of experience. Therefore, one would expect enduring behavioural changes to be accompanied by changes in the brain. It was therefore expected that HS mice would show increases in the measures of glutamate neurotransmission that were measured in this thesis. Against our hypotheses, HS mice only showed two increases, namely increased synaptic release of glutamate in the NAc following EtOH challenge and elevated pCREB levels. Interestingly, these changes are consistent with several reports demonstrating that the neural basis mediating long-lasting phenomena primarily involve increases in neurotransmitter release and transcription factor activity (Castellucci and Kandel, 1976; Richter-Levin and Segal, 1996; Silva et al., 1998; Tanaka, 1999). In addition, despite several differences in the mechanisms underlying EtOH, psychostimulant, and opiate sensitization, behavioural sensitization to these drugs shares in common the presence of increased neurotransmitter release following a drug challenge as well as increased transcription factor expression (Farahmandfar et al., 2011; Kaplan et al., 2011; Nakagawa et al., 2011; Soares-Simi et al., 2013; Szumlinski et al., 2008; Szumlinski et al., 2005; Turgeon et al., 1997; Winstanley et al., 2009). These observations
again highlight that transmitter release and transcription factor activity are two crucial properties in the neurobiology underlying enduring behaviours, the specific neural mechanisms of which may differ depending on the behavior and brain region in question.

Also against our hypothesis were the findings that LS mice demonstrated most of the brain changes in response to repeated EtOH treatment; they demonstrated the most neural plasticity in the absence of behavioural changes. This highlights an interesting observation noted previously in the sensitization literature—that neurochemical changes associated with repeated drug treatment may not be associated with behavioural changes and vice versa. For example, tetrodotoxin-induced inhibition of glutamate release into the NAc did not prevent the manifestation of cocaine sensitization in rats (Robinson et al., 1997). Additionally, repeated administration of EtOH in rats sensitizes electrically-evoked dopamine release from NAc slices, yet rats are known not to display behavioural sensitization to EtOH (Brodie et al., 1990; Masur et al., 1986).

6. Project limitations

One limitation of the current project was the use of a single dose of MK-801 (0.25 mg/kg), CGS 19755 (10 mg/kg), and ANA-12 (0.5 mg/kg) chosen based on extensive literature demonstrating their efficacy at a given concentration (refer to chapter for further details). Although in our hands these doses failed to prevent behavioural sensitization to EtOH, it is possible that a different dose would have been effective and this should be kept in mind when interpreting data from these projects.

A further limitation of this project is that it is largely associational. Although our findings may provide impetus for future work, there are limitations in regards to the conclusions that
can be made. In what follows I describe some difficulties encountered when we attempted to move beyond correlation and into causation.

The results from our NMDAR antagonist experiments suggest that key neuroadaptations during sensitization development may not be mediated by NMDARs. To confirm this conclusion it would be ideal to examine the sensitization response in mice lacking NMDARs. If indeed NMDARs were not required in EtOH sensitization, we would expect to see sensitization in these animals. We therefore examined the sensitization response in NR1-knockdown (NR1-KD) mice, transgenic animals which have a 90% reduction in NMDAR expression (reviewed in Ramsey, 2009). Unfortunately, however, we were unable to come to any conclusion as we found the wildtype mice (WT) failed to sensitize (Appendix 2 on pg. 199). We speculate that the failure of WT mice to sensitize may be due to the C57BL/6J x 129lSv/J background strain used, as mouse strain is known to play an important role in the susceptibility to EtOH sensitization (Hitzemann and Hitzemann, 1997; Phillips et al., 1994; Phillips et al., 1995a). Future work can address the role of brain-region and subunit-specific NMDARs in EtOH sensitization as well as employing other methods to decrease NMDAR expression levels.

Another difficulty we encountered was in performing in vivo brain manipulations using the sensitization protocol in DBA/2J mice. In particular, we noted that in several of our studies a saline infusion in the brain reduces the LMA of mice and in this way compromises the integrity of our results. Knowing that the NAc and its output targets are important in drug-induced locomotor stimulation and general motor activity (Hooks and Kalivas, 1995; Kelsey et al., 2009), coupled with our work showing elevated pCREB and glutamate release in the NAc of HS mice, we performed tissue inactivation studies using the GABA_A receptor agonist,
muscimol, in order to determine whether neural activity in the NAc and its output target, the ventral pallidum (VP), was required for the expression of behavioural sensitization to EtOH (refer to Appendix 3A on pg. 200 for treatment groups used in these studies). In our first experiment, we examined the effects of muscimol infusion in the VP prior to an EtOH challenge (Appendix 3B on pg. 201). We noted that a SAL infusion dramatically reduced the LMA of mice and speculated it might have been caused by a short time interval between infusion and i.p. injection with subsequent LMA measurements (1min). Therefore, we repeated the experiment this time with a 10min period between completion of the infusion and i.p. injection of SAL or EtOH (Appendix 3C on pg. 202). Again, we saw that a SAL infusion dramatically reduced the LMA of mice. We were unsure if this effect of intra-VP SAL infusion on LMA was specific to the VP, so we repeated the experiment this time administering muscimol in the NAc. As shown in Appendix 3D (on pg. 203), a SAL infusion in this area also suppressed LMA.

Why a SAL infusion suppresses LMA in these mice is unclear. It is unlikely that the infusion volume/rate accounts for this as we followed previous reports in which baseline LMA was not affected (Feja et al., 2014; Shimura et al., 2006). Although the mice appeared healthy after the surgeries, it is possible that postsurgical complications not readily visible might explain this, since such complications in DBA mice have been reported previously (Gremel and Cunningham, 2008; Nona et al., 2015b). A further possibility may simply be that the NAc and VP are areas regulating LMA and having any liquid in these structures might interfere with basal activity (Hooks and Kalivas, 1995). Future studies using DBA mice can aim to identify the optimal experimental manipulation that will allow for the determination of brain regions crucial to the expression of EtOH sensitization.
7. Conclusions

In closing, this project has identified several components of the glutamate system that, due to differential responses to EtOH, may lead to interindividual variability in the EtOH sensitization response. Although we looked at various indicators of glutamate involvement in EtOH sensitization, not all should receive equal weight primarily because the expression of several of our measures can be regulated by transmitter systems other than glutamate. For this reason, the NMDAR subunits, NMDAR antagonists, and microdialysis experiments are perhaps the most directly related to the glutamate system and therefore are considered to be the most informative when it comes to reaching a final conclusion regarding the role of this transmitter in EtOH sensitization. Based on these data, an important role for EtOH-induced presynaptic alterations in the NAc may be responsible for the expression of sensitization. Different neurotransmitter systems and/or different brain regions may regulate the development of EtOH sensitization. With respect to the low sensitizers, we found that they demonstrated the most robust changes in the brain following repeated EtOH treatment. We suspect that enhanced NMDAR inhibition by EtOH may be at the heart of their failure to sensitize. Our findings highlight the complex nature of neuroadaptations underlying and sustaining repeated alcohol treatment, as well as demonstrating that individual differences in the glutamate system may potentially explain variability in long-lasting behavioural changes.
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Appendix 1A: Correlation analysis between habituation 1 and EtOH injection 1

Figure 41. Habituation 1 and injection 1 locomotor activity scores. There was no significant correlation between locomotor response to novelty (habituation 1) and the locomotor response to acute EtOH (n=58).
Appendix 1B: Correlation analysis between habituation 1 and the final EtOH injection during the development phase

Figure 42. Habituation 1 and final EtOH injection locomotor activity scores.
There was no significant correlation between locomotor response to novelty (habituation 1) and the locomotor response to the last EtOH injection of the development phase (n=57).
Appendix 1C: Correlation analysis between EtOH injection 1 and the final EtOH injection during the development phase

$r = 0.12\ (p=0.21)$

Figure 43. Injection 1 and final EtOH injection locomotor activity scores.
There was no significant correlation between the locomotor response to acute EtOH and the locomotor response to the last EtOH injection of the development phase (n=58).
Appendix 2: EtOH sensitization in NR1-KD mice

Figure 4. EtOH sensitization response in NR1-KD mice. Wildtype (WT) and NR1-knockdown (NR1-KD) mice were either given EtOH (WT-EtOH and KD-EtOH) or SAL (WT-SAL and KD-SAL) during the development and expression of sensitization. WT mice failed to show behavioural sensitization to EtOH. NR1-KD mice have a 90% reduction in NMDAR expression (Ramsey, 2009). NR1-KD mice had higher LMA than all other groups (significance stars removed for simplicity, p<0.05).
## Appendix 3A: Treatment groups used in tissue inactivation studies

<table>
<thead>
<tr>
<th>Challenge group (treatment during development/treatment during challenge)</th>
<th>Rationale for group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (SAL)/SAL + intracerebral (intra-)-SAL</td>
<td>Baseline locomotion</td>
</tr>
<tr>
<td>SAL/SAL + intra-muscimol</td>
<td>Effects of muscimol on baseline locomotion</td>
</tr>
<tr>
<td>SAL/ethanol (EtOH) + intra-SAL</td>
<td>Acute effects of EtOH</td>
</tr>
<tr>
<td>SAL/EtOH + intra-muscimol</td>
<td>Effects of muscimol on acute EtOH response</td>
</tr>
<tr>
<td>EtOH/EtOH + intra-SAL</td>
<td>Baseline expression of sensitization</td>
</tr>
<tr>
<td>EtOH/EtOH + intra-muscimol</td>
<td>Effects of muscimol on baseline expression of sensitization</td>
</tr>
<tr>
<td>SAL/EtOH + bilateral cannula (no infusion)</td>
<td>Effect of handling procedure and cannulae on acute EtOH response</td>
</tr>
<tr>
<td>EtOH/EtOH + bilateral cannula (no infusion)</td>
<td>Effect of handling procedure and cannulae on EtOH sensitized response</td>
</tr>
</tbody>
</table>

**Table 4.** Treatment groups used in intracerebral muscimol studies.
Figure 45. Intra-VP muscimol. Two weeks following the development of sensitization, mice were challenged with either EtOH (1.8g/kg, i.p.) or an equal volume of SAL 1 min after either a muscimol or SAL intra-VP infusion. Groups are designated by injection 5/challenge treatment. Intra-VP SAL infusion depressed LMA. Infusions were performed at 200ng/0.5ul/hemisphere over a 1min infusion. SAL infusion suppressed LMA, as shown in the red box.
Figure 46. Intra-VP muscimol repeated study. Two weeks following the development of sensitization, mice were challenged with either EtOH (1.8g/kg, i.p.) or an equal volume of SAL 10 mins after either a muscimol or SAL intra-VP infusion. Groups are designated by injection 5/challenge treatment. Intra-VP SAL infusion depressed LMA. Infusions were performed at 200ng/0.5ul/hemisphere infused over 1 min. SAL infusion suppressed LMA, as shown in the red box.
Figure 47. Intra-NAc muscimol. Two weeks following the development of sensitization, mice were challenged with either EtOH (1.8g/kg, i.p.) or an equal volume of SAL 10 mins after either a muscimol or SAL intra-NAc infusion. Groups are designated by injection 5/challenge treatment. Intra-NAc SAL infusion depressed LMA. Infusions were performed at 0.05ug/0.3ul per hemisphere at a rate of 0.1ul/30s over 1.5mins. SAL infusion suppressed LMA, as shown in the red box.