

The Effects of Ethanol on Bidirectional Synaptic Plasticity in the Hippocampus

by

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B.Sc., University of Toronto, 2006

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Supervisory Committee

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Abstract

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The effects of ethanol on the brain are far reaching. However, the effect of ethanol on synaptic plasticity has not yet been fully explored. Although it has been established that ethanol inhibits long-term potentiation (LTP) in the CA1 of the hippocampus, before our study, it was unclear whether long-term depression (LTD) was attenuated or enhanced by ethanol. We found that different concentrations of ethanol attenuated LTD in the CA1 (CA1-LTD) at younger ages. At more mature ages, CA1-LTD was completely blocked by ethanol. In contrast to this, LTD in the dentate gyrus (DG-LTD) was not significantly affected by ethanol concentrations up to 100 mM at any age group tested. These results suggest there are mechanistic differences in LTD induction in the DG and CA1, using the same induction protocol and that CA1-LTD is ethanol sensitive but DG-LTD is not.

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List of Abbreviations

- 5-HT – 5-hydroxytryptamine
AC – associational commissural fibres
aCSF – artificial cerebral spinal fluid
AMPA – α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
APV – D-2-amino-phosphonopentanoate
CA1 – Cornu ammon's region 1
CA2 – Cornu ammon's region 2
CA3 – Cornu ammon's region 3
CaMKII – calcium/calmodulin dependent protein kinase II
CNS – central nervous system
DAG – diacyl glycerol
DCG-IV – 2-(2,3-dicarboxycyclopropyl) glycine
DG – dentate gyrus
DHPG – 3,5-dihydroxyphenylglycine
EC – entorhinal cortex
EPSP – excitatory postsynaptic potential
EtOH – ethanol
fEPSP – field excitatory postsynaptic potential
GABA – γ -aminobutyric acid
GIRK – G protein gated inward-rectifying K⁺
H.M. – Henry Molaise
HFS – high frequency stimulation
I1 – inhibitor 1
IP₃ – inositol-1.4.5-triphosphate
IPSP – inhibitory postsynaptic potential
KCC2 – K⁺/Cl⁻ co-transporter 2
L-AP4 – 2-amino-4-phosphonobutyrate
LFS – low frequency stimulation

LPP – lateral perforant path
LTD – long-term depression
LTP – long-term potentiation
MAP – mitogen-associated protein
MCPG – (+)- α -methyl-4-carboxyphenylglycine
MF – mossy fibers
mGluR – metabotropic glutamate receptor
MPP – medial perforant path
nAChR – nicotinic acetylcholine receptor
NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoline-2,3-dione
NMDA – N-methyl-D-aspartate
P14 – postnatal day 14
P21 – postnatal day 21
P28 – postnatal day 28
PLC – phospholipase C
PP – perforant path
PP1 – protein phosphatase 1
SC – Schaffer collateral
SEM – standard error of the mean
STP – short-term potentiation
SUB – subiculum

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1. Introduction

Introductory Statement

Ethanol, the active drug in alcoholic beverages, has a number of effects in humans. Some of its short term effects include: intoxication, dehydration, alcohol poisoning (which can be fatal) and memory loss. In the longer term alcohol affects heart function, liver metabolism and brain function. When taken during pregnancy, excessive ethanol consumption can also damage the developing fetus (Battaglia et al. 1996; Berman and Hannigan 2000; Jones et al. 1973). Alcoholic beverages are one of the earliest drug used by humans, dating as far back as 10,000 BC (Patrick 1952). Today, alcohol is one of the most widely used drugs and in the recent years its use has increased from 40 to 51% by adults in the United States (King et al. 2009). Despite its long history and common use, the biological effects of ethanol on the brain are still not completely known. Our study focuses on the effects of alcohol on synaptic plasticity in the hippocampus, a region of the brain with a major role in memory formation (Burgess et al. 2002; O'Keefe and Dostrovsky 1971; Scoville and Milner 1957).

Hippocampus

The hippocampus, one of the most thoroughly studied areas of the brain, has a very minor role in processing olfactory function, fear, and attention control. The most important role of the hippocampus, however, is in memory formation. The most famous patient providing direct evidence for hippocampal involvement in memory is Henry Molaison, commonly referred to as H.M. Due to severe seizures H.M. had a bilateral removal of medial aspects of the temporal lobe (where the hippocampus is located),

removing two thirds of his hippocampus and leaving a non-functional and atrophied third (Scoville and Milner 1957). Although H.M.'s seizures were reduced, after the surgery he had slight retrograde amnesia (minor loss of memory of events prior to the surgery) and severe anterograde amnesia (losing the ability to form and retain any new memories subsequent to the surgery). This amnesia most notably impairs declarative memory formation, the ability to form memories that store facts and can be consciously discussed. In animals, declarative memory is not easily measured but nonetheless, evidence has shown the hippocampus is crucial to spatial memory tasks (Burgess et al. 2002; O'Keefe and Dostrovsky 1971).

A useful feature of the hippocampus is that its basic layout of cells and fiber pathways are much the same in all mammals, making it valuable as a neural system for comparative studies. The hippocampus is organized such that the projections follow a unidirectional pathway. Starting from the medial and lateral entorhinal cortex (EC), axons project to dendrites of granule cells in the dentate gyrus (DG) via the medial and lateral perforant paths (MPP and LPP, respectively). From the DG, granule cells with axonal terminations called mossy fibers (MF) synapse with pyramidal cells in Cornu ammon's region 3 (CA3). These cells then send Schaffer collateral (SC) axons to pyramidal cells located in Cornu ammon's region 1 (CA1), which send axonal projections to the subiculum (SUB) and EC. This hippocampal circuit is preserved (in the rodent) when the brain is sectioned along the transverse plane and is easily visible by light microscopy in slice preparations (Figure 1). The simple unidirectional organization and visually recognizable regions make the hippocampus a straightforward area to study.

In the present study focus was primarily on the CA1 and DG subfields of the hippocampus. These regions are not only distinct in their anatomy and location within the hippocampal circuit, but also have different excitatory and inhibitory properties. In the CA1 the inhibitory component is less pronounced than in the DG, and some estimates predict granule cells in the DG establish 10 times as many connections with inhibitory interneurons than CA1 pyramidal cells (Knowles and Schwartzkroin 1981; Scharfman et al. 1990). To exacerbate this difference, there is greater excitatory activity in the CA1 (as well as in the CA3) due to recurrent excitatory axon collaterals (Buhl et al. 1994; Deuchars and Thomson 1996). In contrast to this the dentate does not establish recurrent feedback onto itself. Additionally, interconnectivity, due to recurrent excitatory collaterals, is greater in the CA3 than the CA1. In addition to having different inhibitory and excitatory properties, a substantial difference between the DG and CA1 is that adult neurogenesis occurs in the DG but not in the CA1. Although once a long standing dogma, that adult brains do not form new neurons, it is now widely accepted that the DG is one of two areas in the brain (the other being the subventricular zone) that is neurogenic, i.e. undergoes neurogenesis. In rats, the DG adds thousands of neurons daily throughout adulthood (Cameron and McKay 2001) a portion of which are functionally integrated into the hippocampal circuit (van Praag et al. 2002).

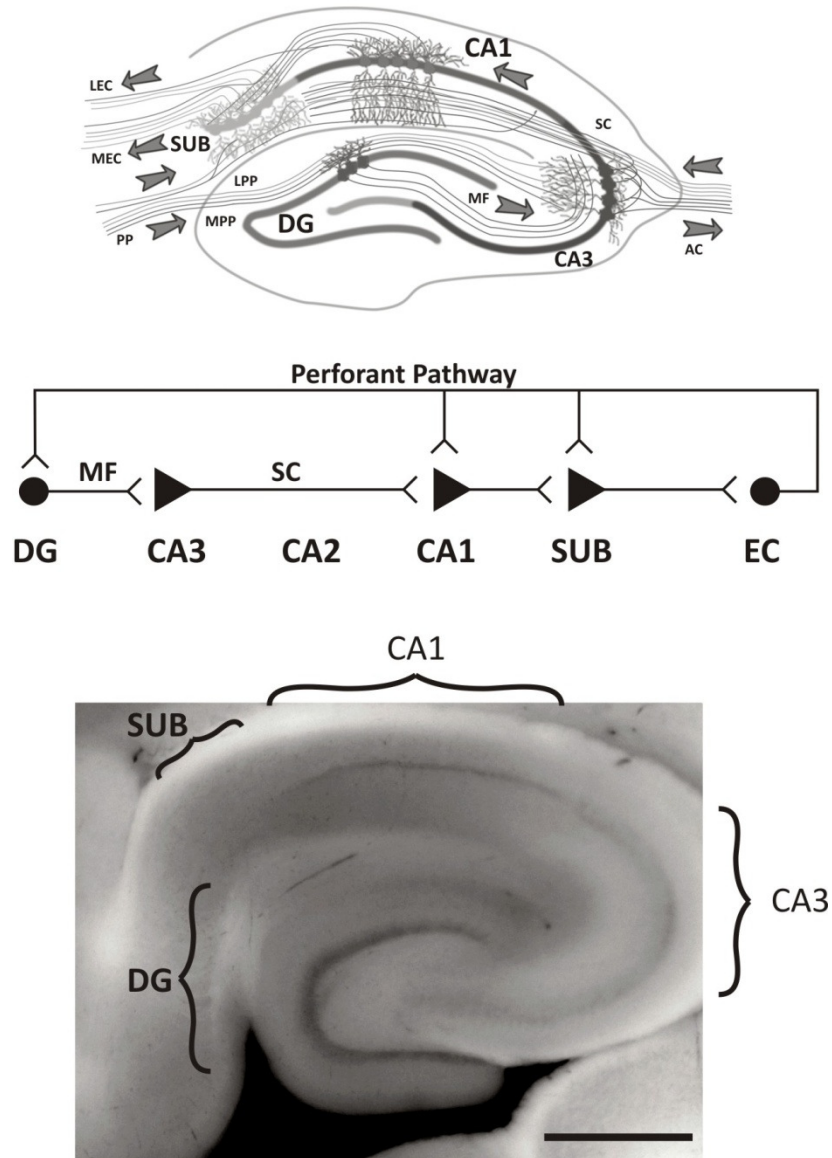


Figure 1. The hippocampus is organized in a unidirectional pathway within the transverse plane. The upper panel depicts the hippocampus with excitatory pathways and connections of the DG, CA3, CA1 and SUB. Afferent pathways of the perforant path (PP) and associational commissural path (AC) are depicted. Efferent paths from AC and medial and lateral entorhinal cortex (MEC and LEC, respectively) are also shown. The middle panel shows a simplified diagram of the primary excitatory pathways between principal neurons in the hippocampus. The lower panel shows a picture taken of a hippocampal slice using a dissecting microscope. The scale bar represents 500 μm . (The upper panel was modified with permission from <http://www.bristol.ac.uk/synaptic/info/pathway/figs/hippocampus.gif>).

Glutamatergic Transmission

The major excitatory neurotransmitter in the hippocampus is L-glutamate. Release of glutamate activates metabotropic glutamate receptors (mGluRs), which are G protein coupled. Metabotropic glutamate receptors modulate excitability by inhibiting or exciting, depending on the receptor subtype or cell context (De Blasi et al. 2001). There are up to 8 known mGluRs divided into 3 groups based on their pharmacological and functional properties. Group I mGluRs, consisting of mGluR1 and mGluR5, are activated by 3,5-dihydroxyphenylglycine (DHPG) and function to enhance excitability. Group II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7 and mGluR8) are selectively activated by 2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV) and 2-amino-4-phosphonobutyrate (L-AP4), respectively and decrease neural excitability.

Glutamate also activates ionotropic glutamate receptors, which conduct ions to produce an excitatory postsynaptic potential (EPSP). There are 3 broad classes of ionotropic glutamate receptors, which were designated after the corresponding relatively selective agonists that activate them. These are N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and kainate receptors (which show much closer homology to AMPA receptors than NMDA receptors) (Ozawa et al. 1998). Although kainate receptors are abundant in the hippocampus, their role remains largely unknown, and synaptic responses mediated by them are very small and generally require trains of high frequency stimulation (Cossart et al. 2002). NMDA receptors, however, have been widely studied due to their importance to several forms of synaptic plasticity. NMDA receptors exist as heteromultimers and are made up of a combination of two obligatory NR1 subunits as well a combination of two

NR2A-D subunits (Cull-Candy and Leszkiewicz 2004). Functional characteristics of NMDA receptors are strongly influenced by the identity of the NR2 subunit present (Cull-Candy and Leszkiewicz 2004). In general, NMDA receptors have slower kinetics than AMPA receptors. NMDA receptors require three conditions to become active: For activation, NMDA receptors need the binding of agonist, glutamate, as well as activation of the receptor by co-agonist, glycine. It was generally thought that under normal conditions extracellular levels of glycine tonically activate the co-agonist site of NMDA receptors. However, this view has been challenged and seems to depend on regional differences in receptor subtype expression and the expression of specific glycine transporters (Parsons et al. 1998; Wood 1995). Finally, there must also be sufficient post-synaptic depolarization, usually provided by activation of AMPA and kainate receptors, to remove Mg^{+2} ions from the pore of NMDA receptors so ions can transit through them (Nowak et al. 1984). Upon opening, NMDA receptors are highly permeable to Ca^{+2} and monovalent cations (Ascher and Nowak 1988). Even though NMDA receptors are ionotropic, Ca^{+2} signalling gives them additional metabotropic-like qualities, since Ca^{+2} acts as a second messenger. It's this quality that makes NMDA receptors an important mediator of synaptic plasticity.

In the hippocampus, subpopulations of NMDA receptors containing NR2A and NR2B subunits dominate and the expression of NR2C and NR2D containing NMDA receptors are much less prevalent (Monyer et al. 1994; Zhong et al. 1995). The expression of NR2A and NR2B subpopulations of NMDA receptors is not static but changes developmentally. Neonatal rats express NR2B containing NMDA receptors at high levels in the hippocampus and this expression remains into adulthood. However, at birth there is

very little presence of NR2A containing NMDA receptors and this expression steadily increases until it plateaus at about the third postnatal week (Dumas 2005; Monyer et al. 1994; Zhong et al. 1995). The increasing NR2A expression matches NR2B expression at approximately the second postnatal week and exceeds NR2B expression after that point. NR2A expression remains higher than NR2B throughout adulthood. These studies are in agreement with measured protein levels of NR2A and NR2B (Wenzel et al. 1997). The time course and change of expression seems to be similar or the same in all regions of the hippocampus, including the DG and CA1 (Ritter et al. 2002).

AMPA receptors, which are also activated by presynaptic release of glutamate, are comprised of GluR1-4 subunits (Hollmann and Heinemann 1994; Wisden and Seeburg 1993). Different combinations of these subunits form functional tetramers. In the hippocampus the majority of these are thought to be GluR1-2 containing and GluR2-3 containing AMPA receptors (Ozawa et al. 1998; Wenthold et al. 1996). AMPA receptors containing a GluR2 subunit are impermeable to Ca^{+2} , as is the case in the hippocampus (Geiger et al. 1995). Although AMPA receptors do not conduct Ca^{+2} in the hippocampus, they are responsible for contributing to a large portion of the EPSP. Due to voltage dependent block by Mg^{+2} (MacDonald and Wojtowicz 1980), which is maintained by γ -aminobutyric acid (GABA) mediated inhibition, NMDA receptors can play a minor role in mediating the EPSP (Behr et al. 2000; Collingridge et al. 1983; Collingridge et al. 1992; Neagu et al. 2008). Because AMPA receptors are large contributors to the EPSP, they are a key target to modulate synaptic plasticity. Furthermore, opening probability and conductance of these receptors can be modulated by phosphorylation (Banke et al. 2000; Derkach et al. 1999).

GABAergic Transmission

Whereas excitatory synaptic transmission is mainly glutamatergic, the inhibitory component is mainly γ -aminobutyric acid (GABA) mediated. GABA receptors are divided into ionotropic (GABA_A) and metabotropic (GABA_B) receptors (Bormann 2000). GABA_A receptors are functional pentamers, and in the hippocampus are composed of two α subunits two β subunits and either a γ or δ subunit (Chang et al. 1996). GABA_A receptors are only permeable to anions, the majority of which are Cl^- but also HCO_3^- (Kaila 1994). Due to this, functions of GABA_A receptors are dependent on the chloride reversal potential, which changes during neonatal development. An interesting consequence of this is that, although in the mature central nervous system (CNS) GABA_A receptors are normally hyperpolarizing and inhibitory, in neonatal rats activation of GABA_A receptors leads to (excitatory) depolarization (Ben-Ari et al. 1989; Mueller et al. 1984). This developmental phenomenon is due to a depolarizing reversal potential of Cl^- , which is then switched to hyperpolarizing in mature neurons. This is mainly attributed to an increase in expression of K^+/Cl^- co-transporter 2 (KCC2) (Hubner et al. 2001; Rivera et al. 1999). In neonatal neurons intracellular concentrations of Cl^- are high due to insufficient extrusion of Cl^- , and GABA_A receptor activation leads to depolarization. After developmental up-regulation of KCC2, the chloride concentration gradient is reversed by the end of the second postnatal week, causing GABA_A receptor activation to produce hyperpolarizing inhibitory currents (Rivera et al. 1999; Zhang et al. 1991). This chloride reversal potential is then maintained in the mature CNS. Activation of GABA_B receptors, however, lead to the opening of G protein gated inward-rectifying K^+ (GIRK) channels (Andrade et al. 1986; Misgeld et al. 1995). Thus the function of GABA_B

receptors is independent of the chloride reversal potential and the developmental changes in intracellular chloride concentrations. A functional difference between excitatory synapses and inhibitory synapses is that GABA released from presynaptic terminals bind to extrasynaptic receptors as well as post synaptic receptors, whereas spill over from glutamatergic synapses is minimal. Functionally, GABAergic transmission contributes to a level of tonic (Buzsaki 1989; Nusser and Mody 2002) as well as activated inhibition in both CA1 and DG subfields of the hippocampus. This has significant implications on synaptic plasticity.

Long-Term Potentiation

Both excitatory and inhibitory networks play a large role in synaptic plasticity. One form of synaptic plasticity, long-term potentiation (LTP) is a strong candidate as a cellular mechanism contributing to learning and memory formation. Hebb's rule, first postulated by Canadian psychologist Donald Hebb in 1949, stated that when one neuron continuously fires on another neuron, changes occur to increase the efficiency between those neurons (Hebb and Konzett 1949). Decades later, LTP, a process that had similar qualities described by Hebb's rule was discovered in the hippocampus by Bliss and Lomo (Bliss and Lomo 1973). Furthermore, LTP has many characteristics shared by learning and memory. Like memories, LTP can be rapidly induced, and also, LTP is long lasting, with accounts of LTP lasting for months (Abraham et al. 2002). To further support this, Whitlock and colleagues showed that learning has led to LTP (Whitlock et al. 2006). The hippocampus seems particularly relevant in the context of learning and memory with

studies that suggest 80-90% of synapses on excitatory hippocampal pyramidal cells exhibit long term synaptic plasticity (Debanne et al. 1999; Petersen et al. 1998).

NMDA receptors are essential for the induction of particular forms of LTP. The dual (ligand and voltage gated) properties of NMDA receptors make them an ideal coincidence detector, which provides a mechanism for processes postulated by Hebb. Because of this dual property, postsynaptic NMDA receptors are able to detect presynaptic release of glutamate in coincidence with postsynaptic depolarization. Upon sufficient depolarization and activation by glutamate, Ca^{+2} entry through NMDA receptors is able to initiate signalling cascades leading to LTP (Collingridge et al. 1983; Collingridge et al. 1992; Harvey and Collingridge 1992; Malenka et al. 1992). Upon Ca^{+2} entry a number of different kinases may be activated including: protein kinase C (Lovinger et al. 1987), calcium/calmodulin dependent protein kinase II (CaMKII) (Malinow et al. 1989), tyrosine kinases (O'Dell et al. 1991) several mitogen-associated protein (MAP) kinase cascades (English and Sweatt 1997; Opazo et al. 2003; Zhu et al. 2002), amongst others. Needless to say, LTP involves multiple parallel kinase signalling cascades. The cascades lead to modification of existing synaptic AMPA receptor kinetics allowing greater conductance (Barria et al. 1997a; Barria et al. 1997b; Benke et al. 1998; Poncer et al. 2002) and, importantly, LTP leads to insertion of AMPA receptors to potentiated synapses (Lu et al. 2001; Man et al. 2003; Pickard et al. 2001; Shi et al. 1999). Through these mechanisms LTP is able to increase the efficacy of signalling at synapses, or often termed synaptic weight. Thus, LTP is dependent on protein synthesis and protein kinase activity. Short-term potentiation (STP) however, the increase in synaptic efficacy occurring prior to LTP, is not dependent on protein synthesis or kinase

activity as it is not blocked by kinase or protein synthesis inhibitors (Frey et al. 1988; Lisman et al. 2002; Lovinger et al. 1987). LTP occurs after an hour of induction, whereas STP is the potentiation prior to LTP.

An important property of LTP induction is cooperativity, whereupon stimulation of two paths on their own is insufficient to induce LTP, but when activated together results in LTP (McNaughton et al. 1978). In other words, activity at one input has the potential to promote LTP at another input (Levy and Steward 1979). This has significant implications on the role of GABAergic networks on LTP. When afferent pathways are activated to evoke EPSPs, GABAergic interneurons are activated to mediate feedforward inhibition. Release of GABA leads to a biphasic inhibitory postsynaptic potential (IPSP). The first fast phase is mediated by GABA_A receptors and a slow component mediated by GABA_B receptors. It's the fast GABA_A component that coincides with NMDA receptor mediated conductance. The IPSP intensifies Mg⁺² mediated block of NMDA receptors by hyperpolarizing postsynaptic potentials (Dingledine et al. 1986; Herron et al. 1985). During LTP induction paradigms, GABA release is sufficiently reduced due to activation of GABA_B autoreceptors (Davies et al. 1991). In other words, GABA released in response to afferent stimulation activates GABA_B receptors leading to inhibition of subsequent GABA release.

Long-Term Depression

LTP would not be an effective means to create and store memories if an opposite process did not also exist. Since it's possible to saturate LTP, if an opposite process did not exist all connections would eventually lead to maximal saturation, leaving no

capacity for further plasticity (or learning). To efficiently create and store memories, the brain needs a system where strength of connections may both increase and decrease in a use-dependent manner (Christie et al. 1994). These increases and decreases correspond nicely with LTP and long-term depression (LTD), respectively. Hence, LTD is the decrease in synaptic weight.

Like LTP, LTD also has NMDA receptor dependent forms (Christie and Abraham 1992a; Dudek and Bear 1992). Similar to LTP, NMDA receptor dependent LTD is dependent on Ca^{+2} entry (Cummings et al. 1996; Mulkey and Malenka 1992). However, unlike LTP, LTD induction leads to activation of protein phosphatases instead of protein kinases. To account for the same signalling to activate both LTP and LTD, it was proposed that protein phosphatase (in LTD) induction had higher affinity than protein kinases, and therefore, would activate at lower concentrations of Ca^{+2} (Lisman 1989). It was discovered that low levels of Ca^{+2} activate calcineurin (also known as protein phosphatase 2B) (Mulkey et al. 1994). In turn calcineurin dephosphorylates inhibitor 1 (I1), which, when phosphorylated inhibits protein phosphatase 1 (PP1). Thus PP1 is activated by disinhibition and dephosphorylates CaMKII (Blitzer et al. 1998; Lisman 1989; Mulkey et al. 1994; Mulkey et al. 1993). This leads to the internalization of AMPA receptors (Beattie et al. 2000). Since AMPA receptor internalization occurs at extrasynaptic sites, lateral diffusion of AMPA receptors occurs before internalization (Ashby et al. 2004). It is an over simplification to state that LTP is induced through phosphorylation, LTD is induced through dephosphorylation. For instance phosphorylation of GluR2 is required for the internalization of AMPA receptors leading to LTD (Ahmadian et al. 2004; Fox et al. 2007). However, it seems safe to state that

lower concentrations of Ca^{+2} through NMDA receptors lead to LTD and higher concentrations of Ca^{+2} lead to LTP (Cho et al. 2001; Wu et al. 2001). Some forms of LTD have also been shown to be dependent on mGluR activation (O'Mara et al. 1995; Poschel and Manahan-Vaughan 2007). Although there is less research on the topic, the proposed mechanism of mGluR dependent LTD is an activation of mGluRs leading to G_q -mediated activation of phospholipase C (PLC). PLC goes on to hydrolyze inositol-1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). This causes a release of Ca^{+2} from intracellular stores (Poschel and Stanton 2007). As in NMDA receptor LTD, it has been shown that mGluR dependent LTD also results in the removal of AMPA receptors (Camodeca et al. 1999; Zhang et al. 2006)

Ethanol

Despite alcohol use being ingrained in cultures around the world, and knowing excessive ethanol consumption promotes a form of amnesia termed blackout (Ryback 1970), it is still unclear exactly how ethanol affects different forms of synaptic plasticity, particularly LTD. Ethanol has a myriad of effects on the brain including dysgenesis of serotonergic system, interference with cell-cell adherence, oxidative stress and free radical damage, disruption of growth factor signalling, disruption of metabolizing enzymes, and disruption of the blood brain barrier (Goodlett et al. 2005; Haorah et al. 2007; Pan et al. 2008). Furthermore, ethanol has many known effects on receptor function. Ethanol has been observed to inhibit NMDA receptor function (Lovinger et al. 1989), and L-type Ca^{+2} channels (Wang et al. 1994). Ethanol also enhances GABA_A receptors (Harris et al. 1997), 5-hydroxytryptamine (5-HT) receptors (Lovinger 1999), nicotinic acetylcholine receptors (nAChRs) (Narahashi et al. 1999), and GIRK channels

(Kobayashi et al. 1999; Lewohl et al. 1999). With such a plethora of targets in the brain, it is not surprising that ethanol has wide ranging effects. It has been well established that acute exposure to ethanol inhibits the induction of LTP (Blitzer et al. 1990; Durand and Carlen 1984; Izumi et al. 2005; Morrisett and Swartzwelder 1993; Mulkeen et al. 1987; Sinclair and Lo 1986). However, the effects of ethanol on LTD are not as clearly established. One group has reported enhancement of LTD by ethanol exposure (Hendricson et al. 2002), and another group reported inhibition of LTD by similar exposure (Izumi et al. 2005). The purpose of this study is to clearly elucidate the effects of ethanol on synaptic plasticity, and to reconcile the differences between conflicting studies. To study this we employed *in vitro* electrophysiology on hippocampal slices.

2. Materials and Methods

Animals

Sprague Dawley rats were ordered from Charles River and bred on site in the University of Victoria animal care facilities. Female Sprague Dawley rats (280-320 grams) were paired with male Sprague Dawley rats (350-450 grams) for 7 ± 2 days. The animals were separated after mating and then approximately 21 days after pairing, pregnant dams gave birth and their pups were culled on postnatal day 2 ± 1 , leaving 5-9 male pups in the litter. All experiments were performed on male pups aged P14-P32 (postnatal day 14 to 32). For age specific experiments: pups aged P14 and P15 were used in the P14 age group, pups aged P20 and P21 were used in the P21 age group, and pups aged P27-P28 were used in the P28 age group. Left over rats from litters aged more than P28 rats were used in paired pulse experiments. All experimental animals were group housed at room temperature ($21 \pm 1^\circ\text{C}$) in transparent, $59 \times 38 \times 20$ cm, polycarbonate cages. The colonies were kept in a regular 12/12 hour light/dark cycle and electrophysiology experiments were performed during the light cycle. Animals had *ad libitum* access to food and water. In total, 179 slices were recorded from 63 animals in the study. Experiments were performed with accordance to University of Victoria and Canadian standards for animal care. Attention was taken to minimize animal suffering.

In Vitro Electrophysiology

Standard *in vitro* electrophysiology was employed to obtain field excitatory post synaptic potentials (fEPSPs) from the DG and CA1 regions of hippocampal slices as described previously (Christie et al. 2006; Froc et al. 2003; Vasuta et al. 2007). To prepare

hippocampal slices, rats were swiftly decapitated and the brain was excised. Four hundred micron transverse slices were then obtained using a cooling vibratome 1500 sectioning system from Ted Pella, Inc., (Redding, CA, USA). All sections were made in oxygenated artificial cerebral spinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaHPO₄, 25 NaHCO₄, 2 CaCl₂, 1.3 MgCl₂, and 10 dextrose (305 mOsm; pH of 7.3). While sectioning with the vibratome, the brain was cooled to approximately 2°C. Hippocampal slices remained in 30°C aCSF for 30 minutes, and then at room temperature aCSF for an additional 30 minutes, so that slices sat for at least 1 hour prior to recording.

Field potentials were recorded using glass microelectrodes (1-4 MΩ) filled with aCSF in hippocampal slices constantly perfused with oxygenated aCSF at approximately 1.5 mL/min. Glass microelectrodes were made by pulling fire polished borosilicate glass capillary tubes with Flaming/Brown micropipette puller Model P-87 (Sutter Instrument, Novato, CA, USA). Post synaptic potentials were elicited using a concentric bipolar stimulating electrode (FHC, Bowdoin, ME, USA), to stimulate Schaffer collaterals or the medial perforant path in CA1 and DG recordings, respectively (Figure 2). To ensure the MPP was being stimulated and recorded from, electrodes were placed in the middle third of the molecular layer (Figure 2). Prior to establishing a steady 15 minute baseline, an input output curve was measured to determine the maximum fEPSP size. The stimulus strength was then adjusted to produce fEPSPs approximately 40-60% of the max fEPSP. During LTD experiments a steady 15 minute baseline at this stimulus strength (40-60% of max fEPSP) was recorded before applying a low frequency stimulus (LFS) protocol (900 pulses at 1 Hz). Likewise, for LTP experiments a 15 minute steady baseline was

recorded before applying a high frequency stimulus (HFS) protocol (50 pulses at 100 Hz, 4 times with a 30 second interval) as previously used (van Praag et al. 1999). During paired-pulse protocols, paired stimuli were applied with an inter stimulus interval of 50 ms to produce paired pulse facilitation. Paired stimuli were induced every 15 seconds. Paired pulse ratios were determined by averaging 6 consecutive paired pulse sweeps in each treatment groups. From these averages, the second pulse was then divided by the first to give a ratio value. Recordings with population spikes interfering with the fEPSP waveform at 40-60% of max fEPSP were discarded due to population spike interference with initial slope. Thus a potential data analysis error was avoided. During drug applications, oxygenated aCSF containing ethanol (50 or 100 mM); ifenprodil (3 μ M) (Sigma-Aldrich); or bicuculline-methiodide (5 μ M) (Sigma-Aldrich) was washed over the slices at the same flow rate (1.5ml/minute) as control aCSF. Ifenprodil is an NMDA receptor antagonist with 400 fold higher affinity for NR2B subunits than NR2A (Williams 1993). Bicuculline-methiodide is a GABA_A receptor antagonist.

Data Analysis and Statistics

Data was acquired using pClamp 10.2 and analyzed using clampfit 10.2 (MDS analytical technologies). To quantify the excitatory synaptic response the initial slope of the fEPSP waveform was measured. The slope was normalized with the average slope of the 15 minute baseline and expressed as a percentage. To determine changes in synaptic efficacy (LTP and LTD), the normalized slope of the fEPSP was measured at 55-60 minutes post LFS and compared with the average of the fEPSP slope obtaining prior to the application of the conditioning stimulus. All data are expressed as mean \pm SEM (standard error of the

mean). Statistical significance was established by Student's t -test. Statistical significance is defined as $P < 0.05$.

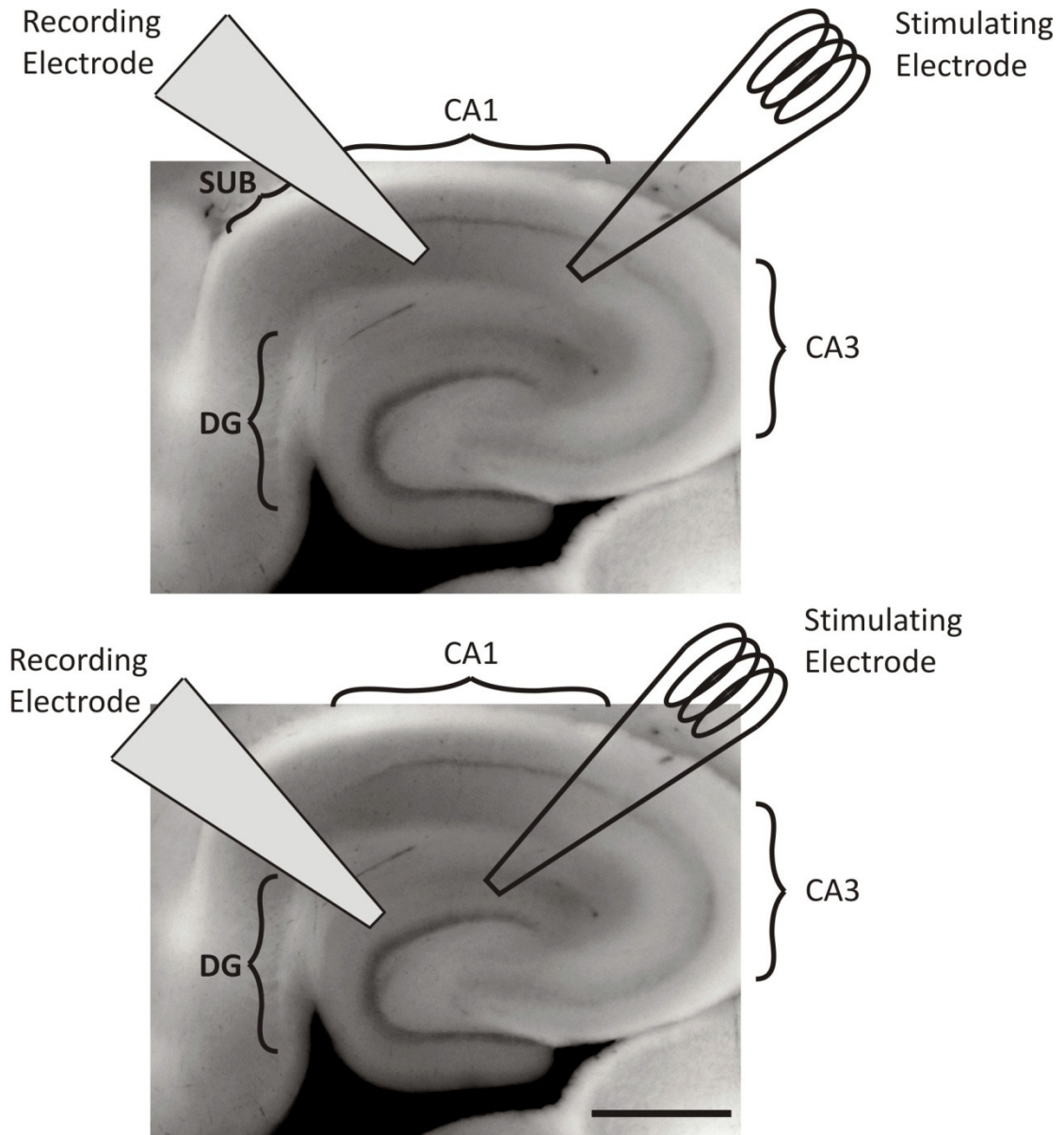


Figure 2. Recording and stimulating electrode placements are depicted for the CA1 (in the top panel), and for the DG (in the bottom panel). The scale bar represents 500 μm .

3. Results

Ethanol Effects on Long-Term Depression in the CA1

Before testing the effects of ethanol (EtOH) on LTD, we first needed to ensure reliable LTD was inducible. Due to previously reported significant differences of LTD in young animals, rats were separated into 3 age groups (Dudek and Bear 1993). The age groups chosen for our study were P14, P21 and P28. These ages were chosen to closely match the age of the animals used in the contradictory studies by Hendricson and colleagues in 2002 and Izumi and colleagues in 2005, in which P12-20 and P30-32 rats were used, respectively. We were able to reliably induce LTD in the CA1 (CA1-LTD) by applying a protocol of 900 pulses delivered at 1 Hz (or 15 minutes) (Figure 3). CA1-LTD was greatest in the youngest animals tested showing $-51.24 \pm 4.87\%$ change in fEPSP compared to baseline. Animals older than P14 showed significantly reduced CA1-LTD with $-12.91 \pm 4.61\%$ and $-22.24 \pm 6.44\%$ change of fEPSP in P21 and P28 animals, respectively (Figure 3, 4 and 7). Upon parsing out the age effects on CA1-LTD, we tested the effects of ethanol on LTD of these 3 age groups (Figure 4). We discovered that ethanol significantly attenuated CA1-LTD at the youngest age group tested, producing only $-26.36 \pm 7.47\%$ ($n = 6$; $t_{(10)} = 3.0567$; $P < 0.05$) and $-33.10 \pm 6.84\%$ ($n = 7$; $t_{(11)} = 2.2662$; $P < 0.05$) change of fEPSP in animals treated with 50 and 100 mM EtOH, respectively. This is significantly reduced compared to the $-51.24 \pm 4.87\%$ ($n = 6$) change measured in control animals. CA1-LTD in rats aged P21, however, were not significantly affected by either 50 mM EtOH ($0.48 \pm 7.53\%$; $n = 7$; $t_{(12)} = 1.6375$; $P = 0.1275$) or 100 mM EtOH ($-16.88 \pm 4.86\%$; $n = 7$; $t_{(12)} = 0.6534$; $P = 0.5258$). The most dramatic effect

of EtOH was observed in the oldest animals tested (P28), with CA1-LTD completely inhibited by both 50 mM EtOH ($3.02 \pm 3.75\%$; $n = 5$; $t_{(14)} = 2.5607$; $P < 0.05$) and 100 mM EtOH ($2.51 \pm 5.00\%$; $n = 5$; $t_{(14)} = 2.5468$; $P < 0.05$).

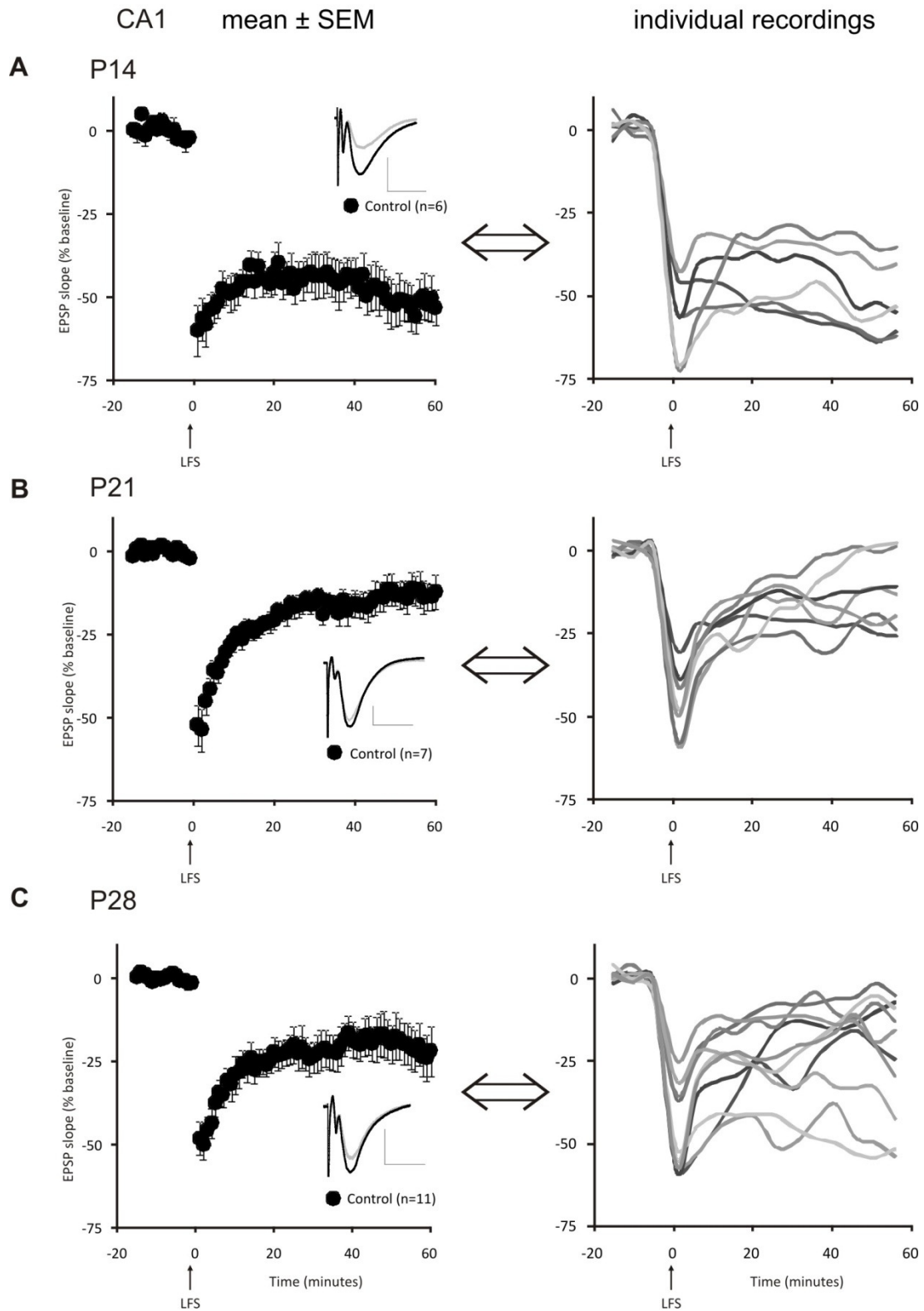


Figure 3. Mean control CA1-LTD recordings were compared with individual time course plots. Left side shows mean \pm SEM of time course plots from individual recordings, which are shown on the right side in shades of grey. Representative traces averaged from the baseline (black) and 60 minute post-LFS (grey)

are depicted as an inset in each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively. Recordings for P14, P21 and P28 rats are shown in panels A, B and C, respectively.

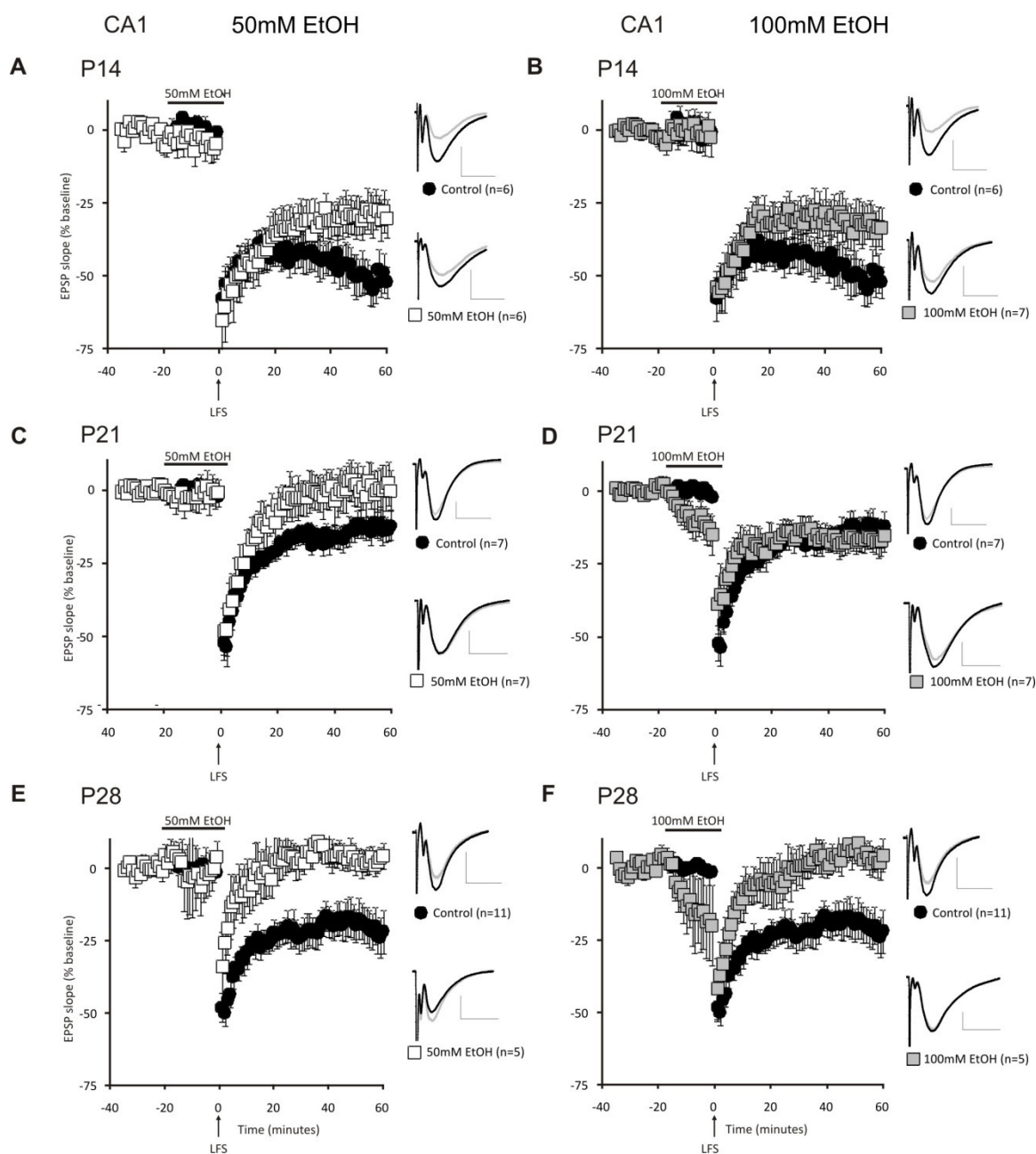


Figure 4. Ethanol has age dependent inhibitory effects on CA1-LTD. LFS, as denoted by the arrow (\uparrow) at time zero, elicited significant CA1-LTD in control slices (\bullet) of all age groups: P14, P21 and P28 ($P < 0.001$, $P < 0.05$ and $P < 0.01$, respectively). A) Slices treated with 50mM EtOH (\square), denoted by a solid bar (—), significantly attenuated CA1-LTD when compared to untreated control slices of P14 rats ($P < 0.05$). B) Likewise, slices treated with 100mM EtOH (\blacksquare), denoted by a solid bar, also significantly impaired the amount of CA1-LTD induction in P14 rats ($P < 0.05$). C) and D) However, in slightly older P21 animals, there is no significant difference in CA1-LTD between groups treated with 50mM or 100mM ethanol and untreated control groups ($p = 0.133$ and $p = 0.526$, respectively). It should be noted, however, that P21 slices treated with 50mM EtOH did not have significant CA1-LTD when compared to baseline

responses (prior to LFS). In contrast to this control slices and slices treated with 100mM EtOH were significantly depressed 60 minutes post-LFS ($P < 0.05$ and $P < 0.01$, respectively). E) In P28 animals, CA1-LTD was completely blocked in slices treated with either 50 mM EtOH or F) 100 mM EtOH. This was significantly less CA1-LTD than measured in control animals ($P < 0.01$ for both 50 and 100 mM groups). Representative traces averaged from the baseline (black) and 60 minute post-LFS (grey) are depicted on the right side of each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively.

Ethanol Effects on Long-Term Depression in the Dentate Gyrus

Having characterized the effects of EtOH on CA1-LTD, we were interested if these effects were common to other areas of the hippocampus like the DG. LTD was reliably inducible in the DG in all age groups tested, with all age groups showing a significant reduction in fEPSP at 55-60 min after applying LFS protocol ($P < 0.05$) (Figure 5).

However, we found that EtOH was unable to significantly alter LTD in the DG (DG-LTD) at 50 mM ($-16.44 \pm 6.85\%$; $n = 5$; $t_{(9)} = 0.8507$; $P = 0.4170$) or 100 mM ($-16.37 \pm 6.88\%$; $n = 5$; $t_{(9)} = 0.8588$; $P = 0.4128$) in P14 animals. Similarly, in P21 animals 50 mM EtOH ($-12.82 \pm 6.11\%$; $n = 5$; $t_{(8)} = 0.1379$; $P = 0.8937$) and 100 mM ($-15.30 \pm 10.84\%$; $n = 5$; $t_{(8)} = 0.1467$; $P = 0.8870$) did not significantly alter DG-LTD, nor did 50 mM ($-21.64 \pm 9.37\%$; $n = 5$; $t_{(9)} = 0.9830$; $P = 0.3513$) or 100 mM ($-12.23 \pm 12.05\%$; $n = 6$; $t_{(10)} = 0.0871$; $P = 0.9323$) EtOH in P28 animals. There was significantly less DG-LTD ($-21.46 \pm 2.06\%$; $n = 6$; $t_{(10)} = 6.1660$; $P < 0.001$) than CA1-LTD at P14. However, at all other time points, there was no significant difference between CA1-LTD and DG-LTD (Figure 5 and 7). Looking at the different ages tested, in the absence of EtOH there was no significant difference in DG-LTD between ages P14, P21 and P28, which showed $-21.46 \pm 2.07\%$, $-13.76 \pm 4.54\%$, and $-13.24 \pm 3.94\%$ change in fEPSP, respectively. Although EtOH treatments did not significantly change the amount of DG-LTD, it was noticed that at the two oldest time points (P21 and P28), 100 mM EtOH caused much

greater variability with SEM of 10.84% and 12.05% compared to 4.54% and 3.98% in control slices of P21 and P28 animals, respectively (Table 1 and Figure 6). This increase in variability was also noted for 50 mM EtOH treatment (SEM of 9.37%) at P28, but was not as dramatic at other age groups and treatments.

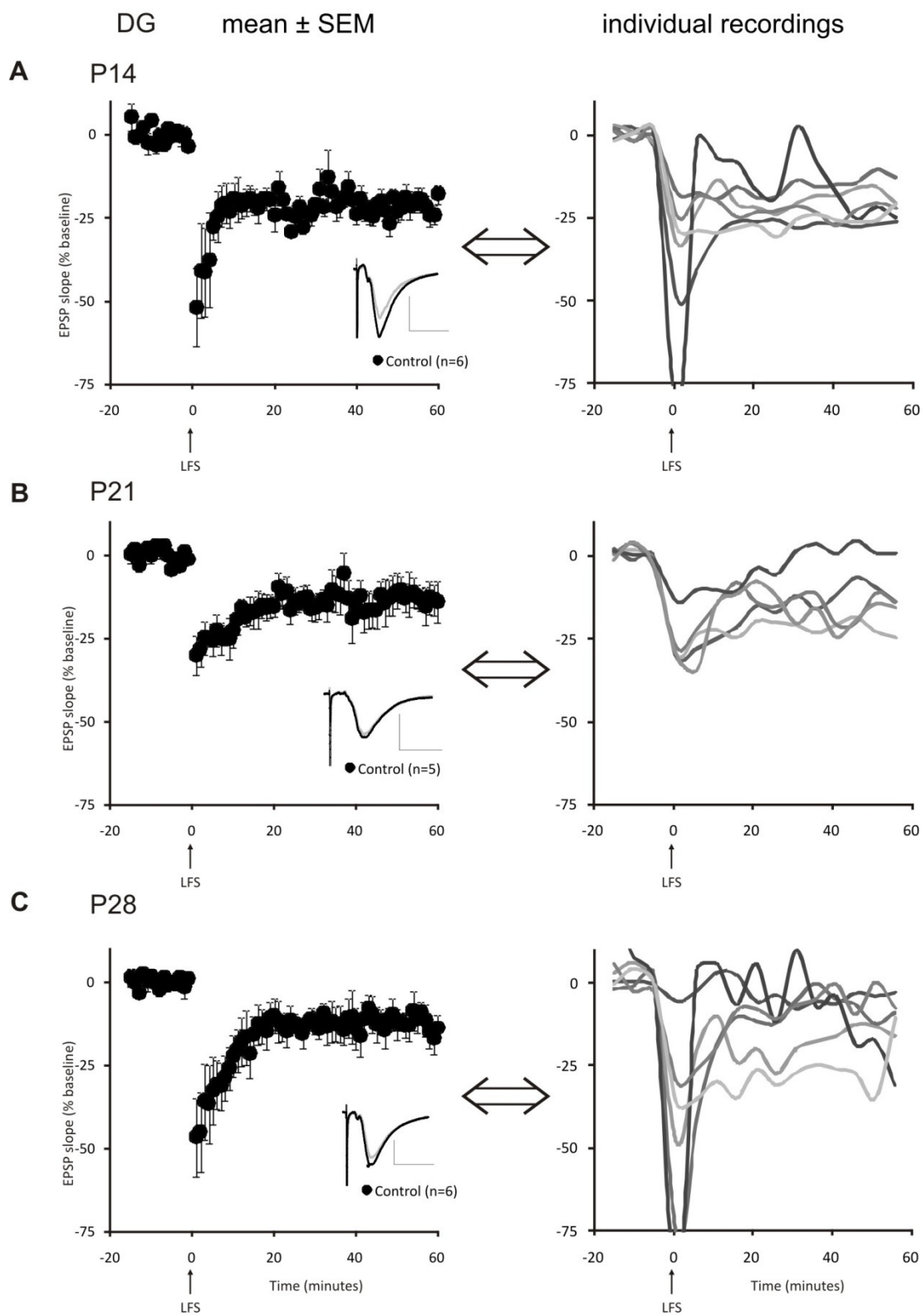


Figure 5. Mean control DG-LTD recordings were compared with individual time course plots. Left side shows mean \pm SEM from corresponding individual time course plots, which are shown on the right side. Representative traces averaged from the baseline (black) and 60 minute post-LFS (grey) are depicted

as an inset in each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively.

Recordings for P14, P21 and P28 rats are shown in A, B and C, respectively.

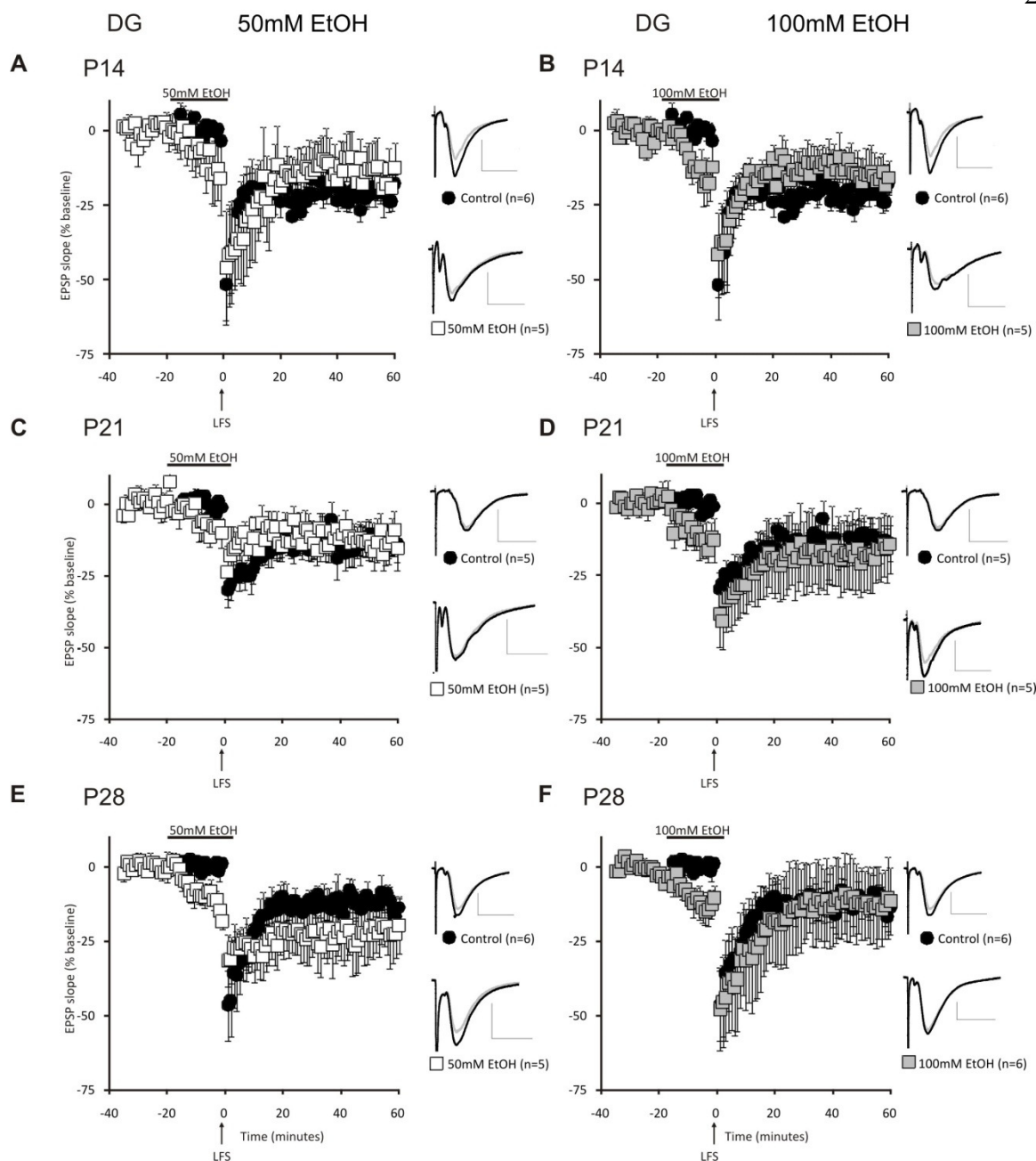


Figure 6. Ethanol does not have significant effects on DG-LTD. LFS, as denoted by the arrow (\uparrow) at time zero, elicited significant DG-LTD in control slices (\bullet) of all age groups: P14, P21 and P28 ($P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively). A) and B) Application of either 50 mM EtOH (\square) or 100 mM EtOH (\blacksquare), as denoted by the solid bar (—), did not significantly affect the amount of DG-LTD compared to control slices in P14 rats. C), D), E) and F) Similarly, 50 mM and 100 mM EtOH was insufficient to significantly attenuate (or augment) DG-LTD in P21 and P28 rats. Representative traces averaged from the baseline (black) and 60 minute post-LFS (grey) are depicted on the right side of each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively.

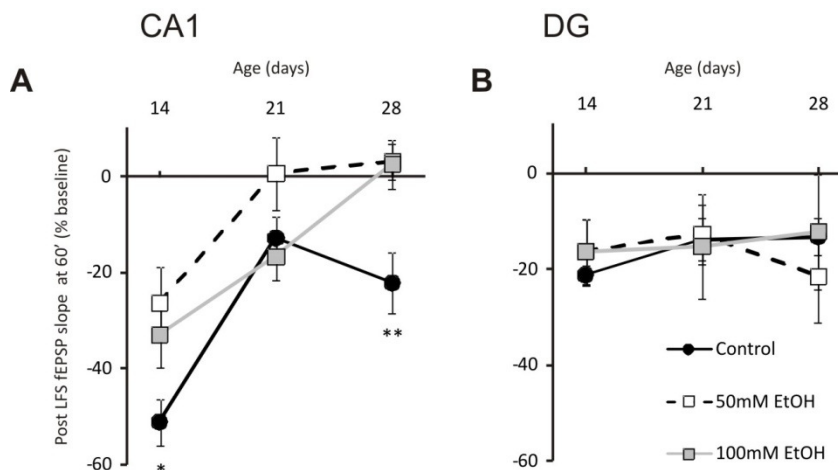
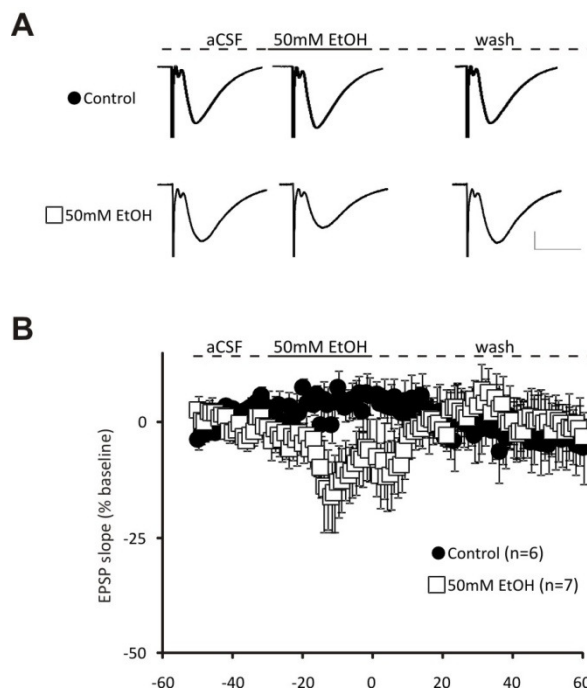


Figure 7. Ethanol has inhibitory effects on CA1-LTD not DG-LTD. The mean fEPSP slope (% baseline) of the LTD recordings at 60 minutes (average of the final 5 minutes) are plotted against age (in days). A) The magnitude of CA1-LTD is age dependent. As well, in the CA1, the amount of inhibition from EtOH is age dependent. B) DG-LTD does not show strong age dependence, nor does EtOH exhibit inhibitory effects on DG-LTD. (* denotes significance at $P < 0.05$, and ** denotes significance at $P < 0.01$, compared with controls at the same age group).

Effects of Ethanol

Upon discovering the inhibitory effect of ethanol on LTD in CA1, we became interested in discovering possible mechanisms that ethanol may be affecting LTD. We tested whether EtOH alone, without any LFS or additional drugs, significantly affected the fEPSP. In the CA1 we washed on 50 mM EtOH for 35 minutes (the same amount of time EtOH washed over the slices during LFS experiments; 20 minutes prior to LFS and 15 minutes during LFS). Ethanol significantly reduced the strength of the fEPSP and at maximal reduction showed $-16.19 \pm 7.74\%$ ($n = 7$; $t_{(11)} = 2,1792$; $P < 0.05$) change of baseline fEPSP (Figure 8). The fEPSP fully recovered upon wash. Testing the effect of EtOH with paired pulse stimulation gave us insight into whether EtOH had its effect through any presynaptic mechanisms. We observed no significant difference in the ratio of paired pulse before, during (1.48 ± 0.08 ; $n = 4$; $t_{(3)} = 1.6891$; $P = 0.1898$) or after a 35 minute ethanol wash (1.43 ± 0.05 ; $n = 4$; $t_{(3)} = 1.5576$; $P = 0.2172$) (the same duration of



EtOH exposure during LFS experiments) as seen in Figure 9. We did notice, however, that although EtOH did not affect the overall

Figure 8. Ethanol significantly attenuated fEPSP in the CA1 of P28 rats. A) Representative traces averaged from consecutive stimuli are shown for control and 50 mM EtOH treated slices. Dashed lines (- - -) represent slice perfusion with normal aCSF and solid bar (—) represents traces recorded from EtOH perfused slices. Control slices (●) were only perfused with normal aCSF. Vertical and horizontal scale bar represent 0.05 mV and 10 ms, respectively. B) Time course of recordings show EtOH attenuates fEPSP and fEPSP is recovered after wash with control aCSF.

paired-pulse ratio, the overall size of the fEPSPs of both pulses decreased during the 35 minute exposure as shown in trace 2 of Figure 9. This is consistent with our data showing that EtOH alone has depressant effects on fEPSP (Figure 8). Lastly, in an attempt to mimic the effects of EtOH on CA1-LTD, we applied 3 μ M ifenprodil prior to and during LFS due to previous reports of similarities between the effects of ifenprodil and EtOH (Hendricson et al. 2002; Izumi et al. 2005; Lovinger 1995). We found that ifenprodil did not mimic the effects of EtOH, and there was no significant difference in CA1-LTD

between ifenprodil treated slices ($-27.96 \pm 7.51\%$, $n=9$; $t_{(18)} = 0.6130$; $P = 0.5476$) and control slices ($-22.24 \pm 6.44\%$, $n=11$) (Figure 10).

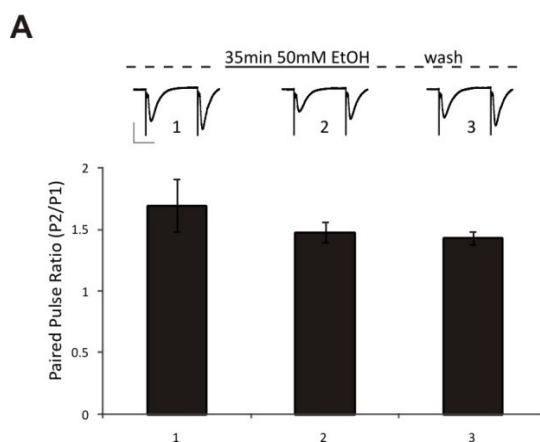


Figure 9. Paired pulse enhancement is not significantly affected by EtOH. Representative traces show paired pulse prior to, during and after EtOH wash, as denoted by dashed (---) and solid lines (—). Vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively. Corresponding bar chart shows paired pulse ratio did not significantly change during and after EtOH wash ($n = 4$, $P > 0.05$).

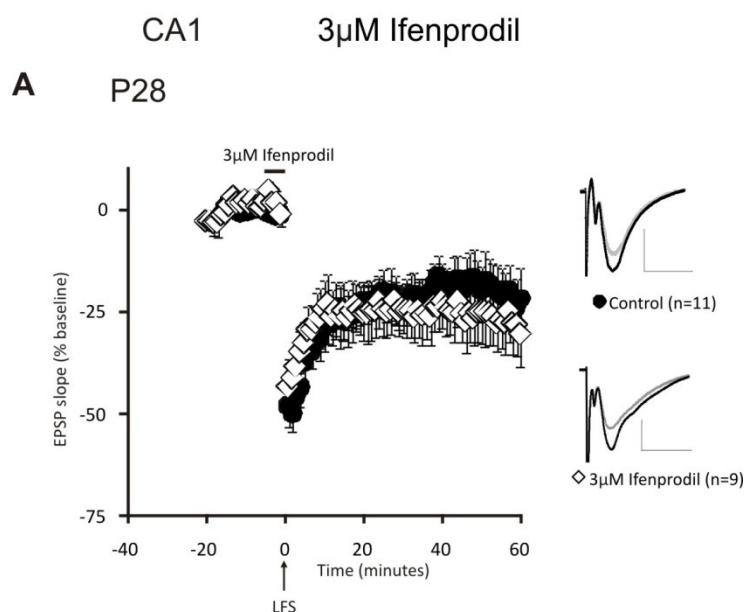


Figure 10. Ifenprodil does not mimic the effects of ethanol on CA1-LTD of P28 rats. Slices treated with 3 μ M ifenprodil (\diamond), as denoted by the solid bar (—), did not have significantly different CA1-LTD than untreated control slices (\bullet). Representative traces averaged from the baseline (black) and 60 minute post-LFS (grey) are depicted on the right side of each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively.

The Effect of Ethanol on Long-Term Potentiation

Upon characterizing some of the effects of EtOH on LTD, we attempted to reproduce acute EtOH inhibition of LTP (Blitzer et al. 1990; Izumi et al. 2005; Morrisett and Swartzwelder 1993; Schummers et al. 1997; Sinclair and Lo 1986). Using a high frequency stimulus protocol; 4 \times 100 Hz trains for 500 μ s, with an inter-train interval of 30 seconds (as in van Praag et al., 1999), we were able to induce LTP (Figure 11 and 12). Although we did significantly reduce LTP in the CA1 (CA1-LTP) of P28 rats with 50 mM EtOH ($13.04 \pm 7.94\%$) compared to control LTP ($43.03 \pm 11.79\%$), the control slices in these recordings were prone to seizures (Figure 11A and 12B). It is known that in the hippocampus CA3 pyramidal cells are most prone to epileptiform activity (Chesnut and Swann 1988; Jensen and Yaari 1988; Miles and Wong 1986). Severing the CA1-CA3 connection, as depicted in Figure 11E, abolishes CA1 epileptiform activity

(Schwartzkroin and Prince 1978). Similarly, in our rats, severing the CA1-CA3 connection reduced seizure activity (Figure 12C). However, with the CA1-CA3 connection severed, less CA1-LTP was induced ($30.36 \pm 5.52\%$) compared to the intact preparation ($43.03 \pm 11.79\%$), although this was not a statistically significant decrease. In the severed CA1-CA3 preparation we found that ethanol still significantly reduced CA1-LTP, exhibiting only a $5.58 \pm 9.61\%$ increase in fEPSP when treated with 50 mM EtOH (Figure 11C and Table 1). We then attempted to induce inhibition of LTP in the DG (DG-LTP) by ethanol. To reliably induce robust DG-LTP from *in vitro* slice preparations, a blockade of the inhibitory circuit is required (Wigstrom and Gustafsson 1983). To do this we applied 5 μ M bicuculline-methiodide, which is a GABA_A receptor antagonist. We were able to induce reliable DG-LTP ($32.11 \pm 13.63\%$) in the presence of bicuculline-methiodide. Application of 50 mM EtOH was insufficient to significantly change the amount of LTP produced, showing $29.74 \pm 13.12\%$ change in fEPSP, as depicted in Figure 11B. In an effort to determine whether the lack of DG-LTP inhibition by EtOH is due to disinhibition by bicuculline-methiodide or due to regional differences between the DG and CA1, we tested whether LTP was inhibited in the CA1 by EtOH in the presence of bicuculline-methiodide. With the knowledge that bicuculline-methiodide would exacerbate epileptiform activity in the CA1 (Tancredi et al. 1990), we induced LTP in the severed CA1-CA3 preparation. In the presence of bicuculline-methiodide and ethanol we were unable to significantly impair the amount of CA1-LTP, showing $18.28 \pm 8.23\%$ when treated with 50 mM EtOH and $29.48 \pm 17.69\%$ when untreated (Figure 11D). In these experiments epileptiform activity decreased but was not completely abolished in the

untreated control slices (Figure 12D). It should be noted however, in all non-ethanol treated CA1-LTP control slices, recordings were exceptionally variable.

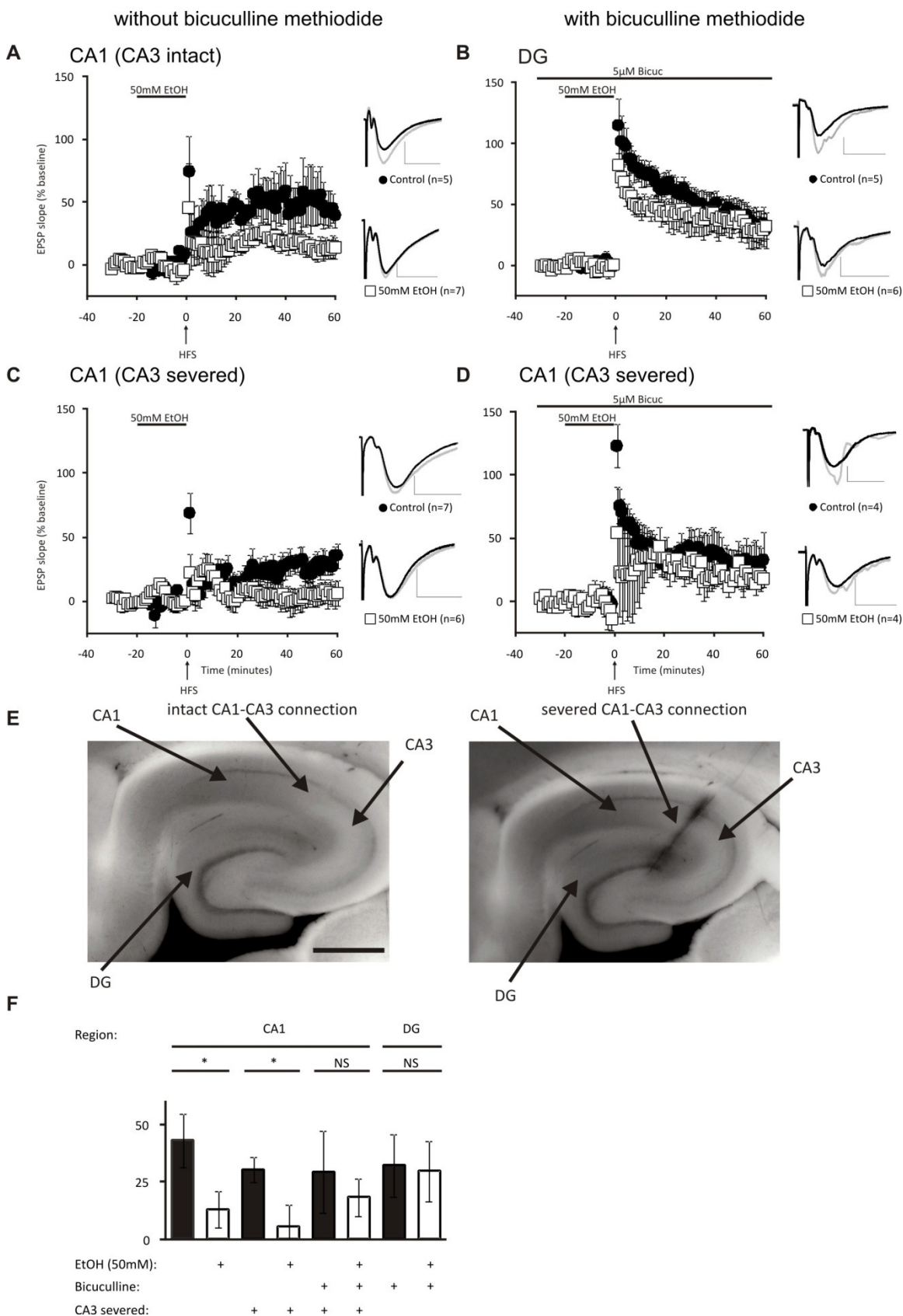


Figure 11. Ethanol does not attenuate LTP in the presence of bicuculline-methiodide. HFS was used to

elicit LTP, as denoted by arrow (\uparrow) at time zero. A) Slices treated with 50 mM EtOH (\square) had significantly reduced CA1-LTP than control slices (\bullet). C) As well, in slices with the CA1-CA3 connection severed, CA1-LTP was significantly reduced at 60 minutes post-HFS. However, in slices recorded in the presence of bicuculline-methiodide there was no significant difference between control slices and ethanol treated slices in both the DG (B) and CA1 (D). Representative traces averaged from the baseline and 60 minute post-HFS are depicted on the right side of each panel A-D. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively. E) Photographs of representative slices show intact and severed CA1-CA3 connection. Scale bar represents 500 μm . F) EtOH significantly reduces CA1-LTP unless in the presence of bicuculline-methiodide. Plus sign (+) denotes presence of EtOH (50 mM), bicuculline-methiodide, or a severed CA1-CA3 connection. An asterisk (*) denotes statistical significance ($P < 0.05$). NS denotes not statistically significant differences between groups. To determine significance, control groups (black) were compared with EtOH treated groups (white).

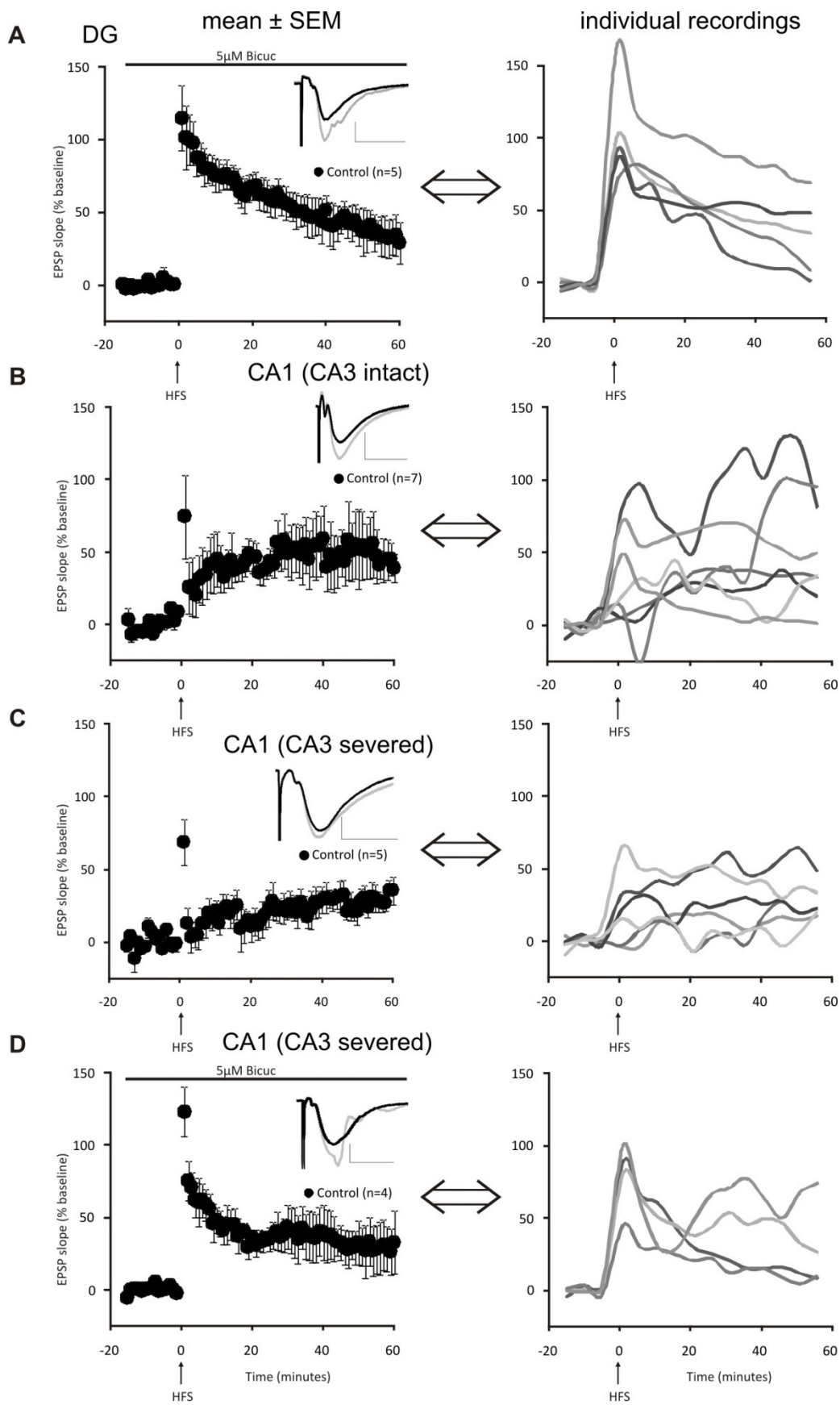


Figure 12. Mean control CA1-LTP and DG-LTP recordings were compared with corresponding

individual time course plots. Shown on the left are mean \pm SEM time course plots taken as the average of corresponding individual time course plots (shown on the right). In each panel, representative traces averaged from the baseline (black) and 60 minute post-LFS (grey) are depicted as an inset in each panel. All vertical and horizontal scale bars represent 0.05 mV and 10 ms, respectively. A) Control DG-LTP recordings were recorded in the presence of bicuculline-methiodide as indicated by the solid bar (—). B) CA1-LTP with axons projecting from the CA3 intact show seizure activity in individual time course plots. C) Control CA1-LTP recordings with axons projecting from the CA3 severed show less seizure activity in individual time course plots (shown on right side). D) CA1-LTP recordings with CA3 connection severed recorded in the presence of bicuculline-methiodide showed slightly more seizure activity.

Summary of % change at 60 minutes post LFS/HFS

Condition		n	% change
LTD (1 Hz×15 min)			MEAN ± SEM
CA1			
P14	Control	6	-51.24 ± 4.87
	50mM EtOH	6	-26.36 ± 7.47 *
	100mM EtOH	7	-33.10 ± 6.84 *
P21	Control	7	-12.91 ± 4.61
	50mM EtOH	7	0.48 ± 7.53
	100mM EtOH	7	-16.88 ± 4.68
P28	Control	11	-22.24 ± 6.44
	50mM EtOH	5	3.02 ± 3.75 **
	100mM EtOH	5	2.51 ± 5.00 **
	Ifenprodil (3μM)	9	-27.96 ± 7.51
DG			
P14	Control	6	-21.46 ± 2.07
	50mM EtOH	5	-16.44 ± 6.85
	100mM EtOH	5	-16.37 ± 6.88
P21	Control	5	-13.76 ± 4.54
	50mM EtOH	5	-12.82 ± 6.11
	100mM EtOH	5	-15.30 ± 10.84
P28	Control	6	-13.24 ± 3.94
	50mM EtOH	5	-21.64 ± 9.37
	100mM EtOH	6	-12.23 ± 12.05
LTP (100 Hz×0.5 sec × 4 times)			
CA1			
	Control	5	43.03 ± 11.79
	50mM EtOH	7	13.04 ± 7.94 *
CA3 severed	Control	7	30.36 ± 5.52
	50mM EtOH	6	5.58 ± 9.61 *
CA3 severed	Control + Bicuculline	4	29.48 ± 17.69
	50mM EtOH + Bicuculline	4	18.28 ± 8.23
DG			
	Control + Bicuculline	5	32.11 ± 13.63
	50mM EtOH + Bicuculline	6	29.74 ± 13.12

* denotes significance at P<0.05

** denotes significance at P<0.01

4. Discussion

Age Dependence of Long-Term Depression

A goal of this study was to clarify differences between conflicting studies reporting the effects of ethanol on CA1-LTD (Hendricson et al. 2002; Izumi et al. 2005). In the present study, it was found that CA1-LTD was either significantly attenuated or completely blocked, depending on the age (Figure 4). These results corresponded well with the report by Izumi and colleagues in 2005. It is worth noting that the age of the animals used by Hendricson and colleagues, who showed an enhancement of CA1-LTD by ethanol, spanned a wide age range (P12-P20). In our study there was a significant decrease in CA1-LTD observed between P14 and P21 (Figure 7). Considering this significant age effect, it is reasonable to believe that pooling animals aged P12-P20 together may produce inauspicious results. The significant change in CA1-LTD with age is not a surprising result considering the vast cortical changes and neural development that occurs within that age group. It is interesting to note that the decrease of CA1-LTD at P21 corresponds very well with reported increases in the NR2A:NR2B NMDA receptor subpopulation ratio (Dumas 2005; Monyer et al. 1994; Zhong et al. 1995). In 2004, a controversial report on NMDA subunit contribution governing direction of synaptic plasticity was released (Liu et al. 2004). This group reported activation of NR2B containing population of NMDA (NR2B-NMDA) receptors led to LTD. In agreement with this, another group reported activation of extrasynaptic NR2B-NMDA receptors is responsible for LTD (Massey et al. 2004). However, since these studies, it has been shown that LTD is not completely dependent on NR2B-NMDA receptors (Morishita et

al. 2007). Our results, failing to block CA1-LTD with ifenprodil, an NR2B-NMDA receptor antagonist, also agree with Morishita's study, indicating that CA1-LTD is not dependent on NR2B-NMDA receptors (Figure 10). A revised hypothesis is that LTD is dependent on the ratio of NR2A:NR2B containing NMDA receptors. If true, this could explain why decreases in CA1-LTD occur after P14, that is, it correlates with a decrease of NR2B-NMDA receptors relative to NR2A containing ones. Consistent with this notion is the observation that 900 1 Hz pulses (the same LFS used in our study) produces NMDA receptor-dependent CA1-LTD (Dudek and Bear 1992). It must be noted, however, that speculation as to why CA1-LTD decreases with age is based on correlative data and we have not provided direct causal evidence.

In the dentate gyrus, we found the magnitude of LTD induced was smaller than in the CA1 and independent of age (Figure 6). Most reports of DG-LTD indicate that it is not NMDA receptor-dependent (Christie and Abraham 1992b; O'Boyle et al. 2004; Poschel and Manahan-Vaughan 2007; Trommer et al. 1996). It has been reported that DG-LTD induced by LFS (1 Hz at 900 pulses) is dependent on metabotropic glutamate receptors mGluRs (O'Mara et al. 1995; Poschel and Manahan-Vaughan 2005). Since this is the same protocol used in our preparation, it is reasonable to suspect that the DG-LTD induced in our animals was also mGluR-dependent. With this in mind, it is not surprising DG-LTD had different developmental characteristics compared to CA1-LTD, which is classically NMDA receptor-dependent (with LFS). Furthermore, mGluR expression does not change as dramatically during this developmental period as NMDA receptor expression (Condorelli et al. 1992; Simonyi et al. 1999). This would help to explain why DG-LTD is relatively constant across tested age groups (Figure 6). In contrast to our

study, there has been a report on age-dependent DG-LTD, where the amount of LTD induced decreased with age (Trommer et al. 1996). However, it must be noted that the LTD-induction protocol (5 Hz for 5 minutes) used in the aforementioned study was different than the LFS (1 Hz at 900 pulses) used in our study. Importantly, different induction protocols induce LTD with different expression mechanisms (Kemp et al. 2000; Poschel and Stanton 2007), which may explain why we did not observe an age-dependent effect even though one was previously reported. Although our results are consistent with the idea that the DG-LTD induced was mGluR-dependent, our evidence is correlative. In fact, heterosynaptic DG-LTD has been discovered to be NMDA receptor-dependent (Christie and Abraham 1992a) and further experimentation is required to ensure that our DG-LTD is mGluR-dependent. As well, further studies are required to conclusively determine whether the CA1-LTD induced in our study is NMDA receptor-dependent. These experiments could be performed by conducting the same control DG-LTD and CA1-LTD experiments in the presence of mGluR inhibitors or NMDA receptor inhibitors (e.g. (+)- α -methyl-4-carboxyphenylglycine (MCPG) or D-2-amino-phosphonopentanoate (APV), respectively).

Effects of Ethanol on Synaptic Plasticity

In direct contrast to Hendricson and colleagues (Hendricson et al. 2002), we discovered that ethanol inhibits CA1-LTD in P28 rats (Figure 4). This is in agreement with Izumi and colleague's report (Izumi et al. 2005). As stated, this discrepancy may be explained by the wide age range used by Hendricson et al. Even though in the youngest age group tested (P14) CA1-LTD is significantly attenuated but not completely ablated, we can still

be confident in this effect due to the stable and consistent LTD recordings observed in untreated slices (Figures 3 and 5). An interesting result was that there was no significant inhibition of CA1-LTD in the P21 age group (Figure 4). Although the younger age group showed significant attenuation and the older age group showed complete ablation, no significant effect was found in the middle age group. It is possible ethanol still inhibits CA1-LTD at this age group, but this effect was unresolvable due to significantly less control CA1-LTD at P21 animals compared to P14 animals. In other words, it's conceivable that CA1-LTD is still attenuated by ethanol at P21, however, due to significantly decreased CA1-LTD, we were unable to observe this attenuation. One way to resolve a difference at this age group (if a difference exists), is to saturate CA1-LTD in these ages by administering multiple LFS protocols. This would produce larger CA1-LTD, and it may be possible to resolve ethanol attenuation on saturated CA1-LTD. In the oldest animals (P28), CA1-LTD was completely blocked by ethanol, showing a much more dramatic inhibition of CA1-LTD than at the youngest age group (P14). By the oldest time point (P28), it is likely the animals had developed a mature chloride reversal potential. Meaning, at this age activation of GABA_A receptors lead to inhibitory postsynaptic potentials as opposed to excitatory postsynaptic potentials, which were likely found in the youngest animals. It is known that ethanol has inhibitory effects on NMDA receptors as well as potentiating effects on GABA_A receptors (Durand et al. 1981; Lovinger et al. 1989; Wan et al. 1996). It has also been discovered that activation of GABA_A receptors promote LTD synergistically with NMDA receptors when GABA_A current is depolarizing (Pavlov et al. 2004). Taken together, this may account for the complete inhibition of CA1-LTD in P28 animals compared to partial inhibition of CA1-

LTD in P14 animals. In P14 animals, where GABA_A receptors have been found to be excitatory (Rivera et al. 1999; Zhang et al. 1991), potentiating effects of ethanol on GABA_A would promote LTD. However, in P28 animals, when the Cl⁻ reversal potential is fully mature, ethanol potentiated GABA_A currents would lead to further inhibition of CA1-LTD. This along with inhibitory effects of ethanol on NMDA receptors would lead to more dramatic CA1-LTD inhibition in older animals than in younger animals. This hypothesis has not been confirmed. It remains to be seen whether our animals at P14 still had an immature reversal potential or whether KCC2 protein levels were significantly lower in P14 animals than P28 animals. Furthermore, direct actions of ethanol on both GABA_A receptors and NMDA receptors were not measured in our preparations. If true, however, it may be possible to resolve a difference between ethanol inhibition of CA1-LTD in P14 animals and P28 animals in the presence of a GABA_A receptor inhibitor (e.g. bicuculline-methiodide). We would suspect in P14 animals that ethanol inhibition of CA1-LTD would either be unaffected or enhanced in the presence of bicuculline-methiodide. However, in P28 animals a GABA_A receptor antagonist would be expected to partially occlude inhibition of CA1-LTD by ethanol.

In the dentate gyrus there was no significant effect of ethanol on LTD. This is consistent with the view that the DG-LTD induced was mGluR-dependent rather than NMDA receptor dependent. Ethanol has very limited effects on mGluRs and has been found to only affect mGluR₅ receptors (which are mainly presynaptic) but not any others (Minami et al. 1998). Since DG-LTD may be induced by mGluR₁ receptors (and even type II and III mGluRs), it seems ethanol inhibition on mGluR₅ is insufficient to inhibit DG-LTD (Poschel and Stanton 2007). Since ethanol has known inhibitory effects on

NMDA receptors (Lovinger et al. 1989), but very limited effects on mGluRs, it is reasonable that ethanol is inhibitory to CA1-LTD, which is generally NMDA receptor-dependent, but has little effect on DG-LTD, which is generally mGluR-dependent.

In examining the effects of ethanol on LTP, we were unable to reliably reproduce previous reports of ethanol inhibition on CA1-LTP (Blitzer et al. 1990; Durand and Carlen 1984; Izumi et al. 2005; Morrisett and Swartzwelder 1993; Mulkeen et al. 1987; Sinclair and Lo 1986). This was mainly due to our inability to reliably produce CA1-LTP in our animals. Upon HFS, slices were prone to seizure activity in the CA1 (Figure 12B). Since seizure activity originates from the CA3, axonal projections from this area were severed to reduce this epileptiform activity (Deuchars and Thomson 1996). Seizures were reduced, however, robust CA1-LTP was still lacking. In agreement with previous studies, we found acute ethanol exposure attenuates CA1-LTP (Figure 11A and C). Although we failed to convincingly show CA1-LTP inhibition by ethanol, numerous established studies suggest that CA1-LTP is indeed blocked by ethanol. Our failure to reliably induce robust CA1-LTP may reside in our induction protocol, which has mainly been used in the DG region in our lab.

We also found that inhibition of GABA_A receptors occlude the effect of ethanol on CA1-LTP, indicating ethanol inhibition of CA1-LTP is dependent on GABA_A receptor activation. Although our effect may be over-shadowed by the fact that we could not produce robust LTP, this is the same effect Izumi and colleagues reported (Izumi et al. 2005). Considering known potentiating effects of ethanol on GABA_A receptors, it is reasonable to believe that this would inhibit CA1-LTP, which is generally NMDA

receptor-dependent when using the same HFS protocol used in this study (Durand et al. 1981; Wan et al. 1996).

Ethanol was unable to significantly alter DG-LTP. However, it must be noted, to induce robust DG-LTP, inhibition by GABA_A was blocked. Therefore it is inconclusive whether a failure of ethanol to attenuate DG-LTP was due to regional differences between the CA1 and DG, or due to a GABA_A dependent mechanism. Although ethanol inhibition on CA1-LTP is at least partially dependent on GABA_A receptor activation (Izumi et al. 2005; Schummers and Browning 2001), we were unable to conclude whether this was the same in the DG. It may be possible to test this hypothesis in the DG, by inducing DG-LTP in the absence of a GABA_A antagonist. Reports of such DG-LTP have been made and characterized (Snyder et al. 2001). This type of DG-LTP (in the absence of a GABA_A antagonist), is NMDA receptor independent and correlated with neurogenesis in the DG but it is unclear whether ethanol inhibits it. It would not be surprising if DG-LTP in the absence of GABA_A receptor antagonists were immune to attenuation by ethanol since reports of local application of ethanol to the DG failed to inhibit DG-LTP *in vivo* (Steffensen et al. 1993). However it was found that systemic administration of ethanol does inhibit DG-LTP *in vivo* (Givens and McMahon 1995; Steffensen et al. 1993). In fact, reports have shown that ethanol inhibition on DG-LTP are due to effects on the lateral septum lateral hypothalamus, and ventral tegmental area, but not due to ethanol acting directly on the dentate gyrus (Criado et al. 1994; 1996; Wayner et al. 1997). If it held true that in *in vitro* slice preparations DG-LTP was not inhibited by direct application of ethanol, then it would seem there are regional differences between the effects of ethanol on DG-LTP and CA1-LTP. However, if it were not true, it would

be interesting whether ethanol inhibition of DG-LTP was GABA_A receptor-dependent as it is in CA1-LTP (Izumi et al. 2005; Schummers and Browning 2001).

Ethanol Effects

Ethanol is known to have numerous effects on the CNS many of which may be relevant in affecting synaptic plasticity. In our study, ethanol significantly attenuated baseline fEPSP responses, and this effect was reversible (Figure 8). Although AMPA receptors are far less sensitive to ethanol than NMDA receptors, ethanol still has partial inhibitory effects on AMPA receptors (Dildy-Mayfield and Harris 1992). AMPA receptors, not NMDA receptors, are the main contributors to the fEPSP, as exemplified by a complete ablation of the fEPSP with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline-2,3-dione (NBQX), a selective AMPA receptor antagonist but an unaffected fEPSP by APV (data not shown). Although, it must be noted that the ablation of the fEPSP by AMPA receptor inhibition is also due to AMPA receptor inhibition on presynaptic neurons as well as a direct inhibition of post-synaptic depolarization. Thus, the attenuation of the fEPSP by ethanol may have been mediated by inhibition of AMPA receptors. More likely however, are GABA_A receptors which are more sensitive to ethanol than AMPA receptors contributed to fEPSP attenuation. Attenuation of AMPA receptors may contribute to ethanol mediated decreases in synaptic plasticity (as observed in CA1-LTD and CA1-LTP), by diminishing the amount of depolarization of postsynaptic cells. This would decrease NMDA receptor activation leading to decreases in both CA1-LTD and CA1-LTP. Attenuation of AMPA receptors is largely ignored in reports of ethanol inhibition of synaptic plasticity since it is thought that pharmacologically relevant concentrations of ethanol do not affect AMPA receptors.

Ethanol did not significantly change paired pulse ratio implying the effects of ethanol were mainly postsynaptic, rather than presynaptic (Figure 9). A change in the paired pulse ratio would indicate changes in the amount of presynaptic glutamate released. Paired pulse facilitation, as observed in our experiments, is caused by a build up of Ca^{+2} in the presynaptic terminal accrued from the first pulse. Thus, upon a second pulse, more glutamate is released because more Ca^{+2} is available to signal release. Since the ratio of the first pulse to the second didn't change, there is no evidence that any presynaptic changes occurred. It was observed, however, that during ethanol wash, both pulses of the paired pulse had decreased in magnitude, although the ratio of between these two did not change. This is consistent with the fact that ethanol attenuates fEPSP (Figure 8), and contributes more evidence towards postsynaptic mechanisms of ethanol.

In an attempt to mimic ethanol inhibition of CA1-LTD, ifenprodil, a NR2B specific NMDA receptor antagonist was applied during CA1-LTD induction. In contrast to both Izumi et al. and Hendricson et al., we found that ifenprodil does not mimic the action of ethanol on CA1-LTD (Hendricson et al. 2002; Izumi et al. 2005). The inability to inhibit CA1-LTD with ifenprodil also contrasts the idea that CA1-LTD is solely NR2B-dependent (Liu et al. 2004). However, our study is in agreement with Morishita and colleagues (Morishita et al. 2007). Even though we have produced a negative result using ifenprodil, we are confident this is not due to ineffectiveness of the drug. From the same stock and concentration of ifenprodil, Eadie and colleagues, from our lab, were able to significantly alter isolated NMDA receptor currents in whole cell preparation (not yet published). These findings show that inhibition of NR2B containing NMDA receptors is insufficient to block CA1-LTD. Although NMDA receptor inhibition is thought to be the

main cause of ethanol inhibition of synaptic plasticity (Morrisett and Swartzwelder 1993), it is more likely that a number of factors are involved, including AMPA receptor inhibition and GABA_A receptor potentiation (Izumi et al. 2005; Schummers and Browning 2001).

Conclusions

Our findings show that ethanol has inhibitory effects on synaptic plasticity in the CA1 (CA1-LTD and CA1-LTP), yet did not significantly affect synaptic plasticity in the DG (DG-LTD and DG-LTP). Ethanol has a myriad of effects on the CNS, making it difficult to pinpoint the exact mechanisms contributing to the effects of ethanol on synaptic plasticity. Crucial in determining these mechanisms is understanding the underlying mechanism of LTD in both the CA1 and DG. Previous studies have shown that CA-LTD is NMDA receptor dependent in rats (Dudek and Bear 1992). Although DG-LTD induced by LFS is NMDA receptor dependent in mice (Vasuta et al. 2007), mounting evidence suggests that in rats DG-LTD is mGluR dependent (O'Mara et al. 1995; Poschel and Manahan-Vaughan 2007). It must be further investigated whether this holds true in our preparations. The results of our study along with previous studies shed light on the direction we should be looking in to determine the mechanism of action of ethanol on synaptic plasticity. It has been shown that GABA_A receptor activation is important in facilitating ethanol inhibition of CA1-LTP (Izumi et al. 2005; Schummers and Browning 2001). However, this has not been shown for CA1-LTD. Attempting to induce CA1-LTD in the presence of ethanol as well as a GABA_A receptor inhibitor would directly test this hypothesis. There has yet to be any studies showing inhibition of DG-LTP with direct application of ethanol on the DG. Blocking DG-LTP produced in the absence of GABA

inhibitors (like the DG-LTP produced by Snyder and colleagues in 2001) would show that DG-LTP is indeed susceptible to ethanol inhibition. As it stands, however, the synaptic plasticity in the DG is much more resilient to the effects of ethanol. Although our study reconciles the differences between two conflicting studies of ethanol inhibition on CA1-LTD (Hendricson et al. 2002; Izumi et al. 2005), there is still much to discover in the field of ethanol affects on synaptic plasticity.

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