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A Dissertation

entitled

Regulation of Neuronal L-type Voltage-Gated Calcium Channels by Flurazepam and Other Positive Allosteric GABA_A Receptor Modulators

by

Damien E. Earl

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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August 2011
Benzodiazepines (BZs) are clinically useful anxiolytics, sedatives, and anticonvulsants. Their mechanism of action is positive allosteric modulation of γ-aminobutyric acid type A (GABA_A) receptors, the main inhibitory neurotransmitter receptors in the mammalian central nervous system. Long-term administration of BZs and other positive allosteric GABA_A receptor modulators, neurosteroids, barbiturates, and ethanol can lead to physical dependence manifested by a characteristic withdrawal syndrome. A common mechanism proposed to contribute to this withdrawal syndrome is functional up-regulation of L-type voltage-gated calcium channels (L-VGCCs). Our lab models BZ dependence using a 1-week oral treatment of rats with flurazepam (FZP) followed by 1 or 2 days of withdrawal. This treatment paradigm resulted in a near doubling of voltage-gated Ca^{2+} currents in hippocampal CA1 neurons. Enhanced L-VGCC-mediated Ca^{2+} influx may activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which potentiated excitatory synaptic function in CA1 neurons correlating with expression of FZP withdrawal anxiety.

The current studies tested three hypotheses: 1) GABA_A receptor modulators directly inhibit recombinantly expressed L-VGCCs containing neuronal α_1 subunits, Ca_{v}1.2 or Ca_{v}1.3; 2) L-VGCC subunit expression is increased in the rat hippocampal CA1 region;
and 3) CaMKII enhances CA1 excitatory synaptic function via activation and autophosphorylation at Thr\(^{286}\) and/or enhanced localization to the postsynaptic density (PSD). The findings suggested that while the barbiturate pentobarbital and ethanol directly inhibit L-VGCCs at clinically relevant concentrations, the concentrations of BZs and neurosteroids required to inhibit recombinant L-VGCCs were likely too high to be clinically relevant. Interestingly, Ca\(_v\)1.2 channels were more sensitive to inhibition by pentobarbital and FZP and were less sensitive to inhibition by the L-VGCC benzothiazepine (BTZ) antagonist, diltiazem, than Ca\(_v\)1.3 channels. Selective inhibition could independently block Ca\(^{2+}\) signaling cascades mediated by Ca\(_v\)1.2 and Ca\(_v\)1.3 L-VGCCs. Mutation studies revealed that the pentobarbital L-VGCC binding site may overlap that of dihydropyridines, and despite structural similarities amongst BTZs and BZs, the BZ L-VGCC binding site is distinct from that of BTZs. No alteration in L-VGCC subunit expression was observed in PSD-enriched CA1 homogenates or immunostained hippocampal slices as a function of FZP withdrawal. Taken together, the data suggested that mechanisms other than direct inhibition of L-VGCCs and increased L-VGCC subunit expression mediate the enhanced Ca\(^{2+}\) influx observed following long-term FZP treatment. Post-translational modifications and/or enhanced trafficking of L-VGCCs to the membrane due to persistent BZ enhancement of GABA\(_A\) receptors are alternate possibilities. Additionally, after 2 days of FZP withdrawal, total CaMKII\(\alpha\) expression was decreased in CA1 PSDs with no alteration in the absolute amount of the autonomously active Thr\(^{286}\) autophosphorylated form of CaMKII. Alternate mechanisms of CaMKII activation by L-VGCC-mediated Ca\(^{2+}\) influx and for the loss of CaMKII from PSDs during FZP withdrawal are proposed.
ACKNOWLEDGEMENTS

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Krista Pettee performed one of the immunofluorescent trials and Dr. Boulineau assisted with confocal imaging. Dr. Das in collaboration with Dr. Alvarez prepared the tissues used in for CaMKII EM.
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TABLE OF CONTENTS

ABSTRACT ...................................................................................................................... iii

ACKNOWLEDGEMENTS .............................................................................................. v

TABLE OF CONTENTS ............................................................................................... vii

LIST OF TABLES .......................................................................................................... xii

LIST OF FIGURES ....................................................................................................... xiii

LIST OF ABBREVIATIONS ......................................................................................... xv

CHAPTER 1 - INTRODUCTION .................................................................................... 1

CHAPTER 2 - LITERATURE REVIEW ........................................................................... 7

2.1 Benzodiazepines and other positive allosteric GABA\(_A\) receptor modulators ........ 7

2.2 GABA\(_A\) receptor structure and function ................................................................. 8

2.3 Positive allosteric modulation of the GABA\(_A\) receptor .......................................... 11

2.4 Additional sites of action GABA\(_A\) receptor modulators ........................................ 15

2.5 Withdrawal from GABA\(_A\) receptor modulators .................................................... 18

2.6 The hippocampus and its role in drug dependence .................................................. 20

2.7 Hippocampal GABAergic network ........................................................................ 24

2.8 Molecular substrates of drug dependence: Role of GABA\(_A\) receptor dysfunction ................................................................. 25

2.9 Molecular substrates of drug dependence: Role of ionotropic glutamate receptors ........................................................................ 32
2.10 Molecular substrates of drug dependence: Role of L-type voltage gated calcium channels

2.11 Regulation of L-VGCCs

CHAPTER 3 - INHIBITION OF RECOMBINANT L-TYPE VOLTAGE-GATED CALCIUM CHANNELS BY POSITIVE ALLOSTERIC MODULATORS OF GABA-A RECEPTORS

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 Cell culture and transient transfection

3.3.2 Site-Directed Mutagenesis

3.3.3 Recording solutions

3.3.4 Whole-cell voltage-clamp electrophysiology

3.3.5 Drugs

3.3.6 Statistical analyses

3.4 Results

3.4.1 Functional Characterization of Recombinant L-VGCCs

3.4.2 GABA_\text{A}R Modulators Reversibly Inhibit Recombinant L-VGCCs

3.4.3 GABA_\text{A}R Receptor Modulators Enhance L-VGCC Current Decay

3.4.4 GABA_\text{A}R Modulators Induce a Negative Shift in L-VGCC Steady-State Inactivation

3.4.5 Inhibition of Cav1.2 Channels by Desalkylflurazepam is State- and Frequency-Dependent
3.4.6 Two Amino Acids Important for DHP Potency Reduce Pentobarbital Potency .................................................................59

3.4.7 A Single Amino Mutation that Affects Diltiazem Potency does not Affect Diazepam Potency ...............................................61

3.5 Discussion .........................................................................................62

3.5.1 GABAAR Modulator Pharmacology at L-VGCCs: Comparison to GABAARs ..............................................................62

3.5.2 GABAAR Modulator Manner of L-VGCC Inhibition: Comparison to L-VGCC Antagonists ..................................................62

3.5.3 Ca,1.2 and Ca,1.3 L-VGCC Differential Sensitivity to GABAAR Modulators ..........................................................64

3.5.4 GABAAR Modulator Site of Action .............................................65

3.5.5 Clinical Relevance of L-VGCC Inhibition by GABAAR Modulators and Implications for Physical Dependence ..................65

3.6 Footnotes ..........................................................................................69

3.7 Acknowledgements ..........................................................................70

3.8 References ......................................................................................71

3.9 Figures and Tables ..........................................................................77

CHAPTER 4 - CA²⁺/CALMODULIN-DEPENDENT PROTEIN KINASE II LOCALIZATION AND AUTOPHOSPHORYLATION WITHIN HIPPOCAMPAL CA1 EXCITATORY POSTSYNAPSES DURING FLURAZEPAM WITHDRAWAL ....................................................98

4.1 Abstract ..........................................................................................100
4.2 Introduction................................................................................................................................102
4.3 Materials and Methods..............................................................................................................106
  4.3.1 Long-term FZP treatment ..................................................................................................106
  4.3.2 Antibodies and specificity...............................................................................................106
    4.3.2.1 Antibodies used for immunoblot and immunofluorescent staining..........................106
    4.3.2.2 Antibodies used for post-embedding immunogold electron microscopic analysis......108
  4.3.3 CA1 minislice subcellular fractionation ..........................................................................109
  4.3.4 Western blot ...................................................................................................................110
  4.3.5 Hippocampal slice confocal immunofluorescence ..........................................................110
  4.3.6 Post-embedding immunogold electron microscopy .......................................................111
  4.3.7 Statistical analyses ........................................................................................................113
4.4 Results.....................................................................................................................................114
  4.4.1 Unaltered L-VGCC subunit expression in CA1 PSD-enriched fraction during FZP-withdrawal ..........................................................114
  4.4.2 CaMKII-α expression, but not Thr²⁸⁶ autophosphorylation is reduced in FZP-withdrawn CA1 asymmetric synapses .................................................116
4.5 Discussion................................................................................................................................119
  4.5.1 Molecular basis of FZP-induced enhancement of CA1 VGCC function .........................119
  4.5.2 Molecular basis of CaMKII activation during FZP withdrawal ...................................120
  4.5.3 Discrepancies with prior studies ....................................................................................122
LIST OF TABLES

3.1 Parameters for concentration-dependent inhibition of L-VGCCs by diltiazem and GABA\(_{\text{A}}\)R modulators

3.2 GABA\(_{\text{A}}\)R modulators enhance L-VGCC current decay

3.3 Negative shift in L-VGCC steady-state inactivation by diltiazem and GABA\(_{\text{A}}\)R modulators

4.1 CaMKII\(_{\alpha}\) immunogold synaptic labeling

4.2 pCaMKII immunogold synaptic labeling

4.3 CaMKII\(_{\alpha}\) and pCaMKII non-PSD immunogold synaptic labeling
LIST OF FIGURES

3.1 Chemical structures of the compounds used in the current studies (except for ethanol)

3.2 Functional expression of recombinant L-VGCCs in HEK293T cells

3.3 Concentration-dependent inhibition of Ca\textsubscript{v}1.3 Ba\textsuperscript{2+} currents by diazepam, and normalized, rundown-corrected time-courses of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-VGCC inhibition by diltiazem and GABA\textsubscript{A} receptor modulators

3.4 Concentration-response curves representing inhibition of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-VGCCs by diltiazem and GABA\textsubscript{A}R modulators

3.5 Diazepam enhances L-VGCC current decay

3.6 L-VGCC voltage-dependent steady-state inactivation curves in the presence and absence of GABA\textsubscript{A}R modulators

3.7 Ca\textsubscript{v}1.2 L-VGCCs are inhibited by desalkylflurazepam in a state- and frequency-dependent manner

3.8 The sensitivity of dihydropyridine-insensitive Ca\textsubscript{v}1.2 (DHPI) L-VGCCs to GABA\textsubscript{A}R modulators

3.9 A single amino acid mutation in Ca\textsubscript{v}1.2, I1150A, reduces diltiazem but not diazepam potency, and diazepam-mediated Ca\textsubscript{v}1.2 inhibition is not antagonized by diltiazem

4.1 Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 immunoblot analysis of subcellular fractions created from CA1 homogenates
4.2 Unaltered L-VGCC subunit expression in the PSD-enriched fraction from 1-day and 2-day FZP-withdrawn rats

4.3 Unaltered Ca\textsubscript{v}1.2 subunit expression detected by immunofluorescent staining of hippocampal slices from 2-day FZP-withdrawn rats

4.4 CaMKII and Thr286 autophosphorylated CaMKII expression in CA1 SR asymmetric synapses of control and 2-day FZP-withdrawn rats

4.5 Pre- and postsynaptic distribution of CaMKII and pCaMKII immunogold particles in CA1 asymmetric synapses of control and 2-day FZP-withdrawn rats

4.6 Distribution histograms of asymmetric synaptic profiles containing different numbers of CaMKII and pCaMKII immunogold particles within the PSD
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BDZ</td>
<td>benzodiazepine</td>
</tr>
<tr>
<td>BTZ</td>
<td>benzothiazepine</td>
</tr>
<tr>
<td>BZ</td>
<td>benzodiazepine</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_i)</td>
<td>intracellular (\text{Ca}^{2+}) concentration</td>
</tr>
<tr>
<td>CA1-4</td>
<td>\textit{cornu ammonis} fields 1-4</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinase II} )</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DHP</td>
<td>dihydropyridine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>E-LTP</td>
<td>early phase long-term potentiation</td>
</tr>
<tr>
<td>FZP</td>
<td>flurazepam</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>GCL</td>
<td>granule cell layer</td>
</tr>
<tr>
<td>(G_{\text{max}})</td>
<td>maximal conductance</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HVA</td>
<td>high voltage-activated</td>
</tr>
<tr>
<td>IC(_{50})</td>
<td>half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>ID</td>
<td>integrated density</td>
</tr>
<tr>
<td>KCC2</td>
<td>(\text{K}^+)-(\text{Cl}^-) cotransporter 2</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>L-LTP</td>
<td>late phase long-term potentiation</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>L-VGCC</td>
<td>L-type voltage-gated calcium channel</td>
</tr>
<tr>
<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
</tr>
<tr>
<td>mIPSC</td>
<td>miniature inhibitory postsynaptic current</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NKCC1</td>
<td>(\text{Na}^+-\text{K}^+-\text{Cl}^-) cotransporter 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAA</td>
<td>phenylalkylamine</td>
</tr>
<tr>
<td>PDZ</td>
<td>postsynaptic density-95/Disks large/zona occludens-1</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene) glycol</td>
</tr>
</tbody>
</table>
PKA  protein kinase A or cyclic adenosine monophosphate-dependent protein kinase
PKC  protein kinase C
PKG  protein kinase G or cyclic guanosine monophosphate-dependent protein kinase
PP2A  phosphatase 2A
PP2B  phosphatase 2B or calcineurin
PSD  postsynaptic density
SLM  \textit{stratum lacunosum-moleculare}
SP   \textit{stratum pyramidale}
SO   \textit{stratum oriens}
SR   \textit{stratum radiatum}
TBS  tris-buffered saline
TBST tris-buffered saline with 0.1\% Tween 20
TEA  tetraethylammonium
TSPO translocator protein 18 kDa
$V_{50}$ membrane potential of half-maximal activation or inactivation
$V_h$ holding potential
$V_{rev}$ reversal potential
$V_t$ test potential
WT  wild-type
CHAPTER 1

INTRODUCTION

Benzodiazepines (BZs) are clinically useful anxiolytics, sedative-hypnotics, and anticonvulsants attributable to positive allosteric modulation of γ-aminobutyric acid type A (GABA_A) receptors, the primary inhibitory neurotransmitter receptors in the mammalian central nervous system (CNS). However, the clinical usefulness of BZs is limited during long-term treatment by the development of tolerance to their sedative and anticonvulsant effects (File, 1985; Rosenberg & Chiu, 1985; Rosenberg et al., 1991) and physical dependence manifested by a characteristic withdrawal syndrome (Griffiths & Johnson, 2005). BZ withdrawal involves a number of symptoms including anxiety and insomnia (Griffiths & Johnson, 2005). Though with a lesser safety margin, similar anxiolytic, sedative-hypnotic, and anticonvulsant effects are by and large seen with other less selective positive allosteric GABA_A receptor modulators including neurosteroids, barbiturates, and ethanol. Further, although the withdrawal syndrome associated with each of these modulators includes symptoms similar to that of BZ withdrawal such as anxiety, withdrawal symptoms are often much more severe for barbiturates and ethanol (Hodding et al., 1980; Smith, 2002).

In animal models of the drug withdrawal phenomenon, anxiety and drug-induced seizure susceptibility are commonly measured outcomes (Emmett-Oglesby et al., 1990;
Chaix et al., 2007). Our lab studies tolerance and withdrawal phenomena in rats using a model in which the water-soluble BZ flurazepam (FZP) is administered orally for 1 week followed by drug withdrawal. This treatment paradigm consistently results in withdrawal anxiety after 1 day of FZP withdrawal (Van Sickle et al., 2004; Xiang & Tietz, 2007).

Due to the interaction of BZs with a binding site on GABA\textsubscript{A} receptors, many studies of BZ tolerance and withdrawal mechanisms have focused on the GABAergic system. These studies have shown that while dysfunction of GABAergic transmission may underlie tolerance to BZ effects (Tietz et al., 1986; Xie & Tietz, 1992; Zeng et al., 1995; Zeng & Tietz, 2000), dysfunction of both GABAergic and glutamatergic systems may be involved in the mechanisms of BZ physical dependence (Izzo et al., 2001; Allison & Pratt, 2003). Glutamate is the main excitatory neurotransmitter in the CNS and activates postsynaptic $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, which respond by depolarizing the postsynaptic membrane and mediating Ca\textsuperscript{2+} influx. FZP-induced withdrawal anxiety correlated with the progressive potentiation of AMPA receptor-mediated currents in hippocampal CA1 neurons (Van Sickle et al., 2004; Xiang & Tietz, 2007). AMPA receptor potentiation was observed after 1 and 2 days of withdrawal, but not immediately after FZP treatment (Van Sickle et al., 2004). A concomitant increase in GluA1, but not GluA2 subunit expression in the soma and proximal apical dendrites of CA1 neurons and postsynaptic incorporation of homomeric GluA1 AMPA receptors was observed during FZP withdrawal (Van Sickle et al., 2004; Song et al., 2007; Das et al., 2008; Shen et al., 2010).

In models of long-term potentiation (LTP) in the CA1 region, synaptic strength is enhanced in an activity-dependent manner requiring Ca\textsuperscript{2+} influx via NMDA receptors or
L-type voltage-gated calcium channels (L-VGCCs) dependent on the stimulation intensity and pathway (Grover & Teyler, 1990; Remondes & Schuman, 2003). Interestingly, high voltage-activated (HVA) Ca\(^{2+}\) channel currents were enhanced in CA1 neurons immediately after FZP treatment and continued through 2 days of withdrawal (Xiang et al., 2008). Moreover, systemic pre-injection on an L-VGCC antagonist, but not an NMDA receptor antagonist prevented withdrawal anxiety and AMPA receptor potentiation after 1 and 2 days withdrawal (Van Sickle et al., 2004; Xiang & Tietz, 2007; Xiang et al., 2008). Thus, Ca\(^{2+}\) influx through L-VGCCs appears to contribute to AMPA receptor potentiation and withdrawal anxiety following long-term FZP treatment. A role for up-regulated L-VGCC function has also been proposed to contribute to physical dependence on barbiturates (Rabbani et al., 1994; Rabbani & Little, 1999) and ethanol (Messing et al., 1986; Whittington et al., 1991; Katsura et al., 2006).

The mechanism of enhanced neuronal L-VGCC function following long-term treatment with various GABA\(_A\) receptor modulators is currently unknown. It may be related to the ability of BZs (Yamakage et al., 1999; Xiang et al., 2008) and other GABA\(_A\) receptor modulators, such as the neurosteroid, allopregnanolone (Hu et al., 2007), the barbiturate, pentobarbital (ffrench-Mullen et al., 1993), and ethanol (Messing et al., 1986) to directly inhibit L-VGCCs. Direct inhibition during FZP treatment would also attenuate downstream consequences of L-VGCC-mediated Ca\(^{2+}\) influx, explaining why AMPA receptor function is unaffected until withdrawal even though L-VGCC currents are enhanced during treatment. Although a direct interaction has been proposed, no studies have investigated the effects of GABA\(_A\) receptor modulators on channels composed of either of the two subtypes of neuronal L-VGCCs, Ca\(_V\)1.2 and Ca\(_V\)1.3. In
particular, expression in a heterologous system would isolate L-VGCC subtypes from GABA_A receptors and other VGCCs, such as N-, P/Q-, R- and T-type (Catterall et al., 2005). Moreover, Ca_{v}1.2 channels were found to be more sensitive than Ca_{v}1.3 channels to inhibition by the well-characterized organic L-VGCC antagonists, dihydropyridines (DHPs), benzothiazepines (BTZs), and phenylalkylamines (PAAs) (Koschak et al., 2001; Xu & Lipscombe, 2001; Tarabova et al., 2007), suggesting that GABA_A receptor modulators might also differentially inhibit L-VGCC subtypes. In particular, there are structural similarities amongst BZ and BTZ compounds. Therefore, the first aim of the current studies was to test the hypothesis that GABA_A receptor modulators inhibit recombinantly expressed Ca_{v}1.2 and/or Ca_{v}1.3-containing L-VGCCs at concentrations achieved in vivo during long-term drug exposure.

The mechanistic basis of enhanced voltage-dependent Ca^{2+} influx is also currently unknown. It could be due to increased expression of L-VGCC subunits, trafficking of L-VGCCs to the plasma membrane from intracellular compartments, or post-translational modifications, such as phosphorylation that enhance L-VGCC currents. Previous studies indicated an increased number of radiolabeled L-VGCC antagonist binding sites on neuronal membranes following long-term exposure to BZs (Katsura et al., 2007), barbiturates (Rabbani & Little, 1999), and ethanol (Messing et al., 1986; Whittington et al., 1991; Katsura et al., 2006), supporting increased plasma membrane expression of L-VGCCs. Moreover, one lab detected increased Ca_{v}1.2 and Ca_{v}1.3 subunit expression in cerebral cortical neurons following prolonged BZ or ethanol exposure in vitro and in vivo (Katsura et al., 2006; Katsura et al., 2007). Studies from our lab revealed a negative shift in the voltage-dependence of HVA Ca^{2+} currents following long-term FZP exposure.
(Xiang et al., 2008), suggesting that Ca\textsubscript{v}1.3 in particular might be up-regulated, since it activates at relatively negative membrane potentials compared to other HVA Ca\textsuperscript{2+} channels (Koschak et al., 2001; Xu & Lipscombe, 2001). Additionally, since Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-VGCCs are expressed near PSDs (Zhang et al., 2005; Day et al., 2006; Tippens et al., 2008; Zhang et al., 2008; Leitch et al., 2009), increased Ca\textsubscript{v}1.3 subunit expression near PSDs could mediate the AMPA receptor potentiation seen during FZP withdrawal. Accordingly, the second aim was to test the hypothesis that Ca\textsubscript{v}1.3 subunit expression is increased near hippocampal CA1 PSDs during FZP withdrawal. Based on evidence of increased Ca\textsubscript{v}1.2 expression in cerebral cortex following long-term BZ exposure (Katsura et al., 2007) and the fact that \( \alpha \), subunit phosphorylation can also enhance L-VGCC currents (Oliveria et al., 2007), the current studies additionally assessed Ca\textsubscript{v}1.2 subunit expression and phosphorylation state in the hippocampus during FZP withdrawal.

One of the downstream consequences of up-regulated L-VGCC function near PSDs may be enhanced Ca\textsuperscript{2+}-mediated activation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), a holoenzyme formed from up to 12 CaMKII\( \alpha \) and CaMKII\( \beta \) molecules (Bennett et al., 1983). In particular, after homomeric GluA1 AMPA receptors are inserted into CA1 PSDs during the first day of FZP withdrawal, increased AMPA receptor conductance was observed after 2 days of withdrawal attributable to phosphorylation of GluA1 at Ser\textsuperscript{831} by CaMKII (Shen et al., 2009; Shen et al., 2010). During NMDA receptor-mediated LTP, Ca\textsuperscript{2+} influx through NMDA receptors leads to autophosphorylation of CaMKII\( \alpha \) at Thr\textsuperscript{286} and CaMKII\( \beta \) at Thr\textsuperscript{287}. CaMKII autophosphorylation results in Ca\textsuperscript{2+}-independent, autonomous CaMKII activity, release of the CaMKII holoenzyme from the actin cytoskeleton, and translocation to binding
partners in the PSD where it mediates phosphorylation of GluA1 homomers (Fink & Meyer, 2002). However, during FZP withdrawal, although total CaMKIIα, but not CaMKIIβ expression was increased in CA1 homogenates, including a PSD-enriched subcellular fraction, there was no corresponding alteration in the level of Thr286/287 autophosphorylated CaMKII (Shen et al., 2010). This suggested that Ca^{2+} influx through L-VGCCs may lead to autonomous CaMKII activation and/or translocation to the PSD via a different upstream mechanism compared to Ca^{2+} influx through the NMDA receptor. It is also possible that similar CaMKII autophosphorylation and translocation does occur during FZP withdrawal, but that it was not detectable in PSD-enriched homogenates. Thus, the third aim tested the hypothesis that total and autophosphorylated CaMKIIα translocates to the PSD of asymmetric synapses located in CA1 neuron proximal apical dendrites during the second day of FZP withdrawal.
CHAPTER 2

LITERATURE REVIEW

2.1 Benzodiazepines and other positive allosteric GABA<sub>A</sub> receptor modulators

The first BZ, chlordiazepoxide, was discovered in the 1950’s by Leo Sternbach as a psychoactive compound with sedative-hypnotic, anticonvulsant, and muscle relaxant effects (Sternbach, 1972). Following clinical trials that verified its safety and efficacy as an anxiolytic and sedative-hypnotic drug, it was introduced into clinical practice in 1960. In the years that followed, numerous other BZs with varying pharmacokinetic and pharmacodynamic properties were introduced for the treatment of anxiety disorders, insomnia, muscle spasms, seizures, as well as alcohol dependence. Due to a combination of sedative and amnestic effects, BZs are also used as premedication for medical and surgical procedures (Dailey, 1990).

Similar behavioral effects are observed for neurosteroids, barbiturates, and ethanol. Like BZs, these drugs are positive allosteric modulators of GABA<sub>A</sub> receptors. Thus, they enhance the inhibitory action of GABA in the CNS, attributing to their clinical depressant effects. These agents are classified as “sedative-hypnotics” or “CNS depressants,” although numerous other drugs with mechanisms of action distinct from modulation of GABA<sub>A</sub> receptors are also grouped into this category. Prior to the introduction of BZs into clinical practice, barbiturates were the treatment of choice for anxiety disorders,
insomnia, and seizures. However, like ethanol, barbiturates have a high incidence of severe side effects, which may be related to their greater number of molecular targets at therapeutic concentrations. Due to the relative safety and efficacy of the more selective BZs, they have largely supplanted barbiturates in clinical practice.

2.2 GABA<sub>A</sub> receptor structure and function

GABA is the major inhibitory neurotransmitter in the mammalian CNS. It is synthesized from glutamate in GABAergic neurons by the enzyme glutamate decarboxylase. Release of GABA from nerve terminals activates both pre- and postsynaptic receptors. GABA is removed from the extracellular space by reuptake into both neurons and glial cells via high affinity transporters and is broken down by GABA-transaminase. GABA activates both ionotropic GABA<sub>A</sub> and metabotropic GABA<sub>B</sub> receptors. GABA<sub>B</sub> receptors are expressed both pre- and postsynaptically. They are G protein-coupled receptors that inhibit presynaptic N- and P/Q-type VGCCs (Bussieres & El Manira, 1999), enhance postsynaptic L-VGCCs (Bray & Mynlieff, 2009), or hyperpolarize the membrane via activation of K<sup>+</sup> channels (Innis et al., 1988). However, GABA<sub>B</sub> receptors are structurally distinct from GABA<sub>A</sub> receptors and not considered pharmacologic targets of GABA<sub>A</sub> receptor modulators (Chebib & Johnston, 1999).

GABA<sub>A</sub> receptors are members of a Cys-loop superfamily of ligand-gated ion channels, which also includes inhibitory glycine receptors, and excitatory nicotinic acetylcholine and serotonin (5-HT<sub>3</sub>) receptors. Each subunit of a Cys-loop receptor consists of a large extracellular N-terminal domain, which contains the conserved disulfide-linked cysteine residues separated by a 13-amino acid spacer, four α-helical transmembrane domains (M1-4), and a short extracellular C-terminal domain. The large
N-terminal region is an asymmetric domain made of β-sheets with minus and plus sides connected by the disulfide-linked Cys-loop. Each side is divided into three loops. Loops A-C form the plus side and loops D-F form the minus side. The minus side of one subunit always faces the plus side of an adjacent subunit. Ligands of Cys-loop receptors bind to a conserved region within loops A-F at the interface between two adjacent subunits (Sigel, 2002; Ernst et al., 2005; Miller & Smart, 2010). The structurally conserved M2 transmembrane helix of each subunit forms an anion or cation channel pore (Ernst et al., 2005).

GABA_A receptors are assembled from a combination of 19 subunits encoded by separate genes: α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3. ρ subunits form receptors sometimes referred to as GABA_C receptors based on their unique pharmacology, but are classified as a type of GABA_A receptor (Barnard et al., 1998; Olsen & Sieghart, 2008). The large majority of GABA_A receptors are formed from two copies of an α, two copies of a β, and one copy of either a γ2 or δ subunit, with (α1)_2(β2)_2γ2 representing the most common subunit composition in the brain (McKernan & Whiting, 1996). The primary differences between γ2 and δ subunit-containing GABA_A receptors are their subcellular localization, affinity for GABA, and decay kinetics. The γ2 subunit mediates synaptic expression, usually when combined with α1, α2, or α3 subunits (Olsen & Sieghart, 2008). Synaptic receptors have a lower affinity for GABA and fast desensitization (channel closure with ligand still bound), appropriate for phasic responses to the millimolar GABA concentrations that occur in the synaptic cleft during neurotransmission (Clements, 1996; Jones & Westbrook, 1996). The γ2 subunit is also expressed extrasynaptically, especially when combined with α4, α5, or α6 subunits (Olsen & Sieghart, 2008). These receptors
may have similar or higher affinity for GABA compared to synaptic GABA_A receptors, sometimes dependent on the β subunit expressed (Ebert et al., 1994; Saxena & Macdonald, 1996; Wafford et al., 1996). The δ subunit is expressed exclusively extrasynaptically and combines with mainly α4 and α6 subunits (Olsen & Sieghart, 2008). In addition, GABA_A receptors containing the δ subunit display an even higher affinity for GABA (5- to 25-fold) and slower desensitization kinetics compared to equivalent receptors containing a γ2 subunit (Saxena & Macdonald, 1994; Saxena & Macdonald, 1996; Brown et al., 2002; Wallner et al., 2003), which is appropriate for tonic responses to ambient micromolar concentrations of GABA (Lerma et al., 1986).

Most GABA_A receptors contain two binding sites for GABA, at the interface between the α and β subunits. Binding of both GABA molecules dramatically increases the open probability of the associated anion channel, which is permeable to both Cl⁻ and HCO₃⁻, among other anions. The relative permeability to these anions varies depending on the subunit composition ranging from 0.2 – 0.4 (HCO₃⁻/Cl⁻) (Bormann et al., 1987; Kaila, 1994). In most mature neurons, this results predominantly in Cl⁻ influx and hyperpolarization of the postsynaptic neuron, though a small HCO₃⁻ efflux accounts for a GABA_A receptor reversal potential more positive than that for Cl⁻ alone (Kaila & Voipio, 1987; Kaila et al., 1993). In some cases, such as in embryonic neurons and during certain pathologic conditions, a relatively large intracellular Cl⁻ concentration ([Cl⁻]₀) shifts the reversal potential in a depolarizing direction resulting in Cl⁻/HCO₃⁻ efflux and neuronal depolarization (Staley & Proctor, 1999; Blaesse et al., 2009). The [Cl⁻]₀ is maintained by cation-Cl⁻ cotransporters (CCC). During early postnatal development there is a physiologic switch in the GABA reversal potential from depolarizing to hyperpolarizing
due to decreased expression of the Cl\textsuperscript{−}-accumulating CCC, Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter 1 (NKCC1) and increased expression of the neuron-specific Cl\textsuperscript{−}-extruding CCC, K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter 2 (KCC2) (Plotkin et al., 1997; Rivera et al., 1999; Dzhala et al., 2005). Any alteration in the expression or activity of these transporters can result in GABA\textsubscript{A} receptor dysfunction via a shift in the Cl\textsuperscript{−} reversal potential (Staley et al., 1995; Dzhala et al., 2005).

2.3 Positive allosteric modulation of the GABA\textsubscript{A} receptor

Although the clinical effects of BZs were discovered in the 1950’s, it was not until the early 1980’s that a high affinity CNS BZ site was identified (Mohler & Okada, 1977) and a connection with the GABA receptor was established (Gavish & Snyder, 1981). The BZ binding site has since been characterized as homologous to the GABA binding site, but between the α and γ subunits (Sigel, 2002; Olsen & Sieghart, 2008). Upon binding, BZs enhance the affinity of the receptor for GABA, which results in an increase in the frequency of channel opening and mean channel open time (Study & Barker, 1981; Lavoie & Twyman, 1996). BZs are allosterically coupled to GABA, in that GABA binding also enhances the affinity of BZs (Klein & Harris, 1996). Studies using photoaffinity labeling identified a conserved histidine residue in α1 (His\textsuperscript{101}), α2 (His\textsuperscript{101}), α3 (His\textsuperscript{126}), and α5 (His\textsuperscript{105}) subunits that plays a critical role in BZ binding (Sigel, 2002). The analogous amino acid in α4 and α6 subunits is an arginine residue, making GABA\textsubscript{A} receptors formed from these subunits insensitive to BZ agonists (Mohler et al., 2004). Consistently, mutation of this histidine residue in α1/α2/α3/α5 subunits to an arginine renders the GABA\textsubscript{A} receptor insensitive to BZ agonists (Benson et al., 1998). Knock-in mice containing this mutation in α1 subunits showed reduced sedative, anticonvulsant,
and amnestic effects by diazepam, but are still sensitive to its anxiolytic and muscle relaxant effects (Rudolph et al., 1999; McKernan et al., 2000). α2-H101R knock-in mice lacked sensitivity to the anxiolytic and muscle relaxant effects by diazepam, but retained sensitivity to its sedative and anticonvulsant effects (Low et al., 2000; Crestani et al., 2001). α3-H126R knock-in mice showed a partial reduction in muscle relaxant effects of diazepam at high doses, but retained sensitivity to the anxiolytic, sedative, and anticonvulsant effects (Low et al., 2000; Crestani et al., 2001). α5-H105R knock-in mice displayed reduced muscle relaxant effects, but retained anxiolytic, sedative, and anticonvulsant effects of diazepam (Crestani et al., 2002). In addition, α5-H105R knock-in mice showed a deficit in α5-containing extrasynaptic GABA_A receptor expression in hippocampal dendrites and a corresponding enhancement in associative learning (Crestani et al., 2002), and α5 knock-out mice displayed enhancement in hippocampus-dependent spatial memory tasks (Collinson et al., 2002). Collectively, the findings strongly support a role for α1 subunits mediating the sedative and some of the anticonvulsant effects, α2 subunits in mediating the BZ anxiolytic effects, α2, α3, and α5 subunits mediating the muscle relaxant effects, and α1 and possibly α5 subunits mediating the amnestic effects of BZs.

Other GABA_A receptor modulators exert their actions at sites distinct from BZs, and these sites are allosterically linked to each other. For example, neurosteroids and barbiturates enhance BZ binding (Klein & Harris, 1996). Amino acid residues within the transmembrane domains of α and β subunits are important for GABA_A receptor sensitivity to neurosteroids (Mitchell et al., 2008), barbiturates (Olsen & Sieghart, 2008), and ethanol at higher concentrations (Wallner et al., 2006a). Moreover, δ-containing
GABA<sub>Α</sub> receptors displayed greater efficacy to neurosteroids and barbiturates (Brown et al., 2002) and greater potency to ethanol (Wallner et al., 2003) compared with γ2-containing receptors. The greater sensitivity of δ-containing receptors likely stems from the fact that these receptors display low GABA efficacy compared to γ2-containing receptors allowing more room for positive modulation of GABA efficacy (Olsen & Sieghart, 2008).

Despite the commonalities, these compounds appear to have distinct binding sites within GABA<sub>Α</sub> receptor subunits. Neurosteroids produce GABA-enhancing effects at lower, nanomolar concentrations and directly activate GABA<sub>Α</sub> receptors at higher, micromolar concentrations (Lambert et al., 1987; Hosie et al., 2006). The mechanism of the neurosteroid GABA-enhancing effect involves increased time spent in longer duration openings, without increasing single channel conductance or GABA affinity (Peters et al., 1988; Twyman & Macdonald, 1992). The neurosteroid binding pocket that mediates the GABA-enhancing action is likely located in M1 (Gln<sup>241</sup>) and M4 (Asn<sup>407</sup> and Tyr<sup>410</sup>) of α subunits. Direct activation of GABA<sub>Α</sub> receptors requires binding of a second neurosteroid molecule to a site within M1 (Thr<sup>236</sup>) of α subunits and M3 (Tyr<sup>283</sup>) of β subunits (Hosie et al., 2006). Interestingly, these studies were performed using γ-containing GABA<sub>Α</sub> receptors. The presence of the δ subunit enhanced neurosteroid efficacy, but not apparent affinity (Brown et al., 2002), which is likely related to enhanced allosteric modulation of GABA efficacy rather than neurosteroid binding.

Barbiturates also produce GABA-enhancing effects at lower, micromolar concentrations and directly activate GABA<sub>Α</sub> receptors at higher, sub-millimolar concentrations (Schulz & Macdonald, 1981). Also like neurosteroids, the GABA-
enhancing action involves increased time spent in longer duration openings, without increasing single channel conductance or GABA affinity (Peters et al., 1988; MacDonald et al., 1989; Steinbach & Akk, 2001; Feng et al., 2004). The barbiturate binding sites are likely located in the β subunit transmembrane domains, since mutation of Pro^{228} in M1 of β, but not α subunits reduces the GABA-enhancing effects of barbiturates, but not diazepam or the neurosteroid, alphaxalone (Greenfield et al., 2002). The direct activation of GABA_A receptors by barbiturates is dependent upon residues downstream from the middle of M2 of β subunits based on chimeric studies (Serafini et al., 2000). It should be noted that as with mutations affecting neurosteroid actions, it cannot be ruled out that these mutations affect the ability of barbiturates to allosterically modulate channel function, rather than affecting barbiturate binding. Binding studies are also not conclusive, as mutations can allosterically affect ligand binding affinity without being part of the binding site themselves (Greenfield et al., 2002). However, the α and β subunit transmembrane mutations affecting neurosteroid affinity are ideally positioned to accommodate hydrogen-bonding predicted to mediate neurosteroid binding (Hosie et al., 2006; Mitchell et al., 2008). In addition, the lack of evidence supporting a role for α subunits in mediating barbiturate affinity would seem to indicate a site of action solely within β subunits. Thus, the neurosteroids and barbiturates seem to act at distinct GABA_A receptor binding sites, consistent with results of earlier studies that found a lack of competitive interaction (Gee et al., 1988; Peters et al., 1988).

Similar to neurosteroids and barbiturates, ethanol increased mean GABA_A receptor open time via increased time spent in longer duration openings without a change in single channel conductance, but additionally increased the frequency of channel opening.
In addition to a low affinity ethanol binding site, likely within M2 and M3 of α and β subunits (Mihic et al., 1997; Ueno et al., 1999; Wallner et al., 2006a), a high affinity ethanol binding site may overlap that of the BZ inverse agonist, Ro15-4513. This was demonstrated by the fact that Ro15-4513 antagonized ethanol’s low-dose intoxicating and GABA-enhancing effects, and ethanol antagonizes [³H]-Ro15-4513 binding to recombinant α4β3δ, but not α4β3γ GABA_A receptors (Wallner et al., 2006b). Additionally, α6-R100Q mutation resulted in enhanced sensitivity of GABA_A receptors to ethanol and enhanced low-dose intoxicating effects of ethanol (Hanchar et al., 2005), suggesting that Arg^{100} of α4/6 subunits, in conjunction with adjacent residues in the δ subunit, may be important for high-affinity ethanol binding and/or allosteric effects. However, there are numerous labs that were unable to replicate the enhancement of δ-containing GABA_A receptors by low ethanol concentrations (Borghese & Harris, 2007; Harris et al., 2008), and the existence of a high-affinity ethanol site that explains threshold intoxication at low millimolar concentrations remains controversial.

### 2.4 Additional sites of action of GABA_A receptor modulators

Though many of the clinical effects of BZs can be explained by their action at GABA_A receptors, other sites of action are also implicated. At clinically relevant free plasma concentrations (nanomolar range), some BZs may also act at a peripheral BZ receptor (Szewczyk & Wojtczak, 2002), now known as translocator protein 18 kDa (TSPO). TSPO is located in the outer mitochondrial membrane and, among other things, is involved with steroid transport from the outer to inner mitochondrial membrane, the rate-limiting step in the synthesis of steroid hormones and neurosteroids (Rupprecht et al., 2010). In the CNS, TSPO is known to play a critical role in the formation of

(Tatebayashi et al., 1998). In addition to a low affinity ethanol binding site, likely within M2 and M3 of α and β subunits (Mihic et al., 1997; Ueno et al., 1999; Wallner et al., 2006a), a high affinity ethanol binding site may overlap that of the BZ inverse agonist, Ro15-4513. This was demonstrated by the fact that Ro15-4513 antagonized ethanol’s low-dose intoxicating and GABA-enhancing effects, and ethanol antagonizes [³H]-Ro15-4513 binding to recombinant α4β3δ, but not α4β3γ GABA_A receptors (Wallner et al., 2006b). Additionally, α6-R100Q mutation resulted in enhanced sensitivity of GABA_A receptors to ethanol and enhanced low-dose intoxicating effects of ethanol (Hanchar et al., 2005), suggesting that Arg^{100} of α4/6 subunits, in conjunction with adjacent residues in the δ subunit, may be important for high-affinity ethanol binding and/or allosteric effects. However, there are numerous labs that were unable to replicate the enhancement of δ-containing GABA_A receptors by low ethanol concentrations (Borghese & Harris, 2007; Harris et al., 2008), and the existence of a high-affinity ethanol site that explains threshold intoxication at low millimolar concentrations remains controversial.

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neurosteroids (Papadopoulos et al., 2006), and long-term diazepam treatment in rats resulted in decreased stress-induced production of the neurosteroid, allopregnanolone (Wilson & Frye, 1999), which may have been mediated by diazepam’s interaction with TSPO.

At higher, micromolar concentrations BZs inhibit VGCCs in both neuronal (Reuveny et al., 1993; Ishizawa et al., 1997; Xiang et al., 2008) and non-neuronal cells (Yamakage et al., 1999; Hara et al., 2001). Inhibition of L-VGCCs present in cardiac myocytes and arterial smooth muscle cells could be the cause of reduced ventricular force and hypotension observed during intravenous injection of BZs (Hara et al., 2001). L-VGCCs have several functions in neuronal cells, including regulation of firing frequency, intracellular signaling cascades affecting activity of other receptors and channels and transcriptional activity (Jones, 1998; Deisseroth et al., 2003). Therefore, inhibition of neuronal L-VGCCs by BZs could affect neuronal excitability and gene expression.

In addition to their action at the GABA_A receptor, neurosteroids, barbiturates, and ethanol also modulate activity of other receptors and channels at various concentrations. Like BZs, allopregnanolone does not appear to have many alternate molecular targets other than GABA_A receptors at physiologically relevant nanomolar brain concentrations. At higher, sub-micromolar concentrations neurosteroids have been shown to inhibit VGCCs (ffrench-Mullen et al., 1994; Hu et al., 2007), possibly via activation of a G protein-coupled signaling cascade leading to activation of protein kinase C (PKC) (ffrench-Mullen et al., 1994). Inhibition by neurosteroids could target presynaptic VGCCs and release of neurotransmitter, as well as postsynaptic L-VGCCs and corresponding effects on neuronal excitability and gene expression as noted above. The
activation of PKC may be an additional mechanism to modulate GABA_A receptors, which might even be inhibitory to receptor function (Brandon et al., 2000) and would affect numerous other ion channels as well. Thus, a greater number of non-selective effects would be expected, but further studies are needed to elucidate the role of G protein-coupled receptors in mediating neurosteroid actions.

Unlike BZs and neurosteroids, both barbiturates and ethanol have numerous other sites of action at clinically relevant brain concentrations. The barbiturates have been shown to inhibit excitatory glutamate receptors, including AMPA, NMDA, and kainate receptors (Hoffman & Tabakoff, 1993; Grasshoff et al., 2006), as well as pre- and postsynaptic VGCCs (Blaustein & Ector, 1975; Gross & Macdonald, 1988; ffrench-Mullen et al., 1993). Ethanol has been shown to affect activity of all Cys-loop receptors, including inhibition of excitatory nicotinic acetylcholine and 5-HT_3 receptors and enhancement of inhibitory glycine receptors (Crews et al., 1996; Aguayo et al., 2002; Harris et al., 2008). Various potassium channels and VGCCs are also inhibited by ethanol (Messing et al., 1986; Harris et al., 2008). Ethanol is also considered a potent inhibitor of NMDA receptors (Crews et al., 1996). A commonality in ethanol’s putative site of action is amphipathic α-helices containing residues like serine and threonine, where it likely displaces water molecules to affect protein function (Harris et al., 2008). Interestingly, the effects of ethanol on GABA_A receptors and some other channels depends on the activity of intracellular signaling cascades (Woodward, 1999; Aguayo et al., 2002), and a role of G protein-dependent signaling cascades in mediating ethanol’s actions has also been proposed (Harris et al., 2008), similar to neurosteroids.
2.5 Withdrawal from \textit{GABA}_A\ receptor modulators

Long-term exposure to \textit{GABA}_A\ receptor modulators can lead to the development of tolerance to their sedative and anticonvulsant effects, requiring higher doses to achieve the same level of effect (File, 1985). Higher doses of drug coupled with long-term exposure may also lead to physical dependence, manifested by withdrawal symptoms (Hodding et al., 1980; Woods & Winger, 1995; Kan et al., 2004). Although there are some similarities between the withdrawal syndromes of CNS depressants, there is substantial variability in the range of symptoms present and their severity, likely attributable to their different molecular targets. The BZ withdrawal syndrome includes anxiety and insomnia, as well as headaches, muscle aches, nausea, depression, increased sensitivity to light and sound, paresthesias, perceptual changes, depersonalization, and, rarely, seizures or psychosis (Lader, 1994; Griffiths & Johnson, 2005). Synthetic neurosteroids are currently used for surgical anesthesia, but not for chronic conditions; thus, withdrawal effects have not been observed following clinical treatment. However, withdrawal of endogenous neurosteroids occurs following prolonged physiologic elevation during the menstrual cycle or pregnancy. This physiologic withdrawal may result in perimenstrual catamenial epilepsy as well as the symptoms of premenstrual syndrome and postpartum depression (Smith, 2002; Reddy & Rogawski, 2009). These symptoms relate mainly to mood alterations and include depression, fatigue, anxiety, irritability, sleep disturbances, and decreased experience of pleasure (Amin et al., 2006), many which overlap BZ withdrawal symptoms.

Withdrawal symptoms are often far more severe for the non-selective CNS depressants, barbiturates and ethanol. Barbiturate withdrawal symptoms range in severity
from anxiety, insomnia, irritability, restlessness, depression, headaches, nausea, tremors, and diaphoresis to hallucinations, myoclonus, delirium, grand mal seizures, and severe fever (Hodding et al., 1980). The ethanol withdrawal syndrome is similar to that of barbiturates, including the rare appearance of the most severe condition, delirium tremens, which is a combination of a severely confused state, hallucinations, and symptoms of sympathetic overactivity such as diaphoresis, hypertension, and tachycardia (Hodding et al., 1980; McKeon et al., 2008).

The barbiturates and ethanol also have a greater abuse liability in terms of their reinforcing properties (Griffiths & Johnson, 2005). The mechanisms for this are not entirely clear, but ethanol’s reinforcing effects may relate to its ability to enhance activity of the mesolimbic dopaminergic reward pathway (Soderpalm et al., 2009). Patients taking BZs less seldom become addicted, and BZ abuse is generally limited to polydrug abusers (O’Brien C, 2005). Correspondingly, BZs have a limited abuse liability in terms of their reinforcing effects based on both preclinical data and studies in humans (Woods et al., 1992; Griffiths & Johnson, 2005), although some patients abuse BZs in a chronic quasi-therapeutic manner (i.e., self-administration) in order to relieve or avoid withdrawal symptoms (Griffiths & Johnson, 2005). The greater withdrawal severity and abuse liability of the barbiturates and ethanol likely pertains to their larger number of molecular targets compared to BZs.

Withdrawal phenomena observed in animal models includes measurable effects such as anxiety and susceptibility to pharmacologically induced seizures. The most commonly used drugs to measure seizure susceptibility are kainic acid, a glutamate receptor agonist; pentylenetetrazol, a GABA<sub>A</sub> receptor antagonist; and β-carboline compounds, methyl-
6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate and methyl-β-carboline-3-carboxylate, BZ inverse agonists (Chaix et al., 2007). Among the variety of tests used to measure anxiety, such as the open field test and the light-dark box, one of the most common and well-accepted measures is the open arm test, also known as the elevated plus maze. These behavioral tests take advantage of the natural exploratory tendencies of rodents. Animals with anxiety reliably spend less time exploring open and well-lit areas, presumably to avoid potential dangers. In the case of the elevated plus maze, anxious animals avoid the two open arms and spend more time in the two closed arms (Ramos, 2008). Anxiety is perhaps the most commonly measured outcome in animal models of withdrawal from CNS depressants and other drugs of abuse (Emmett-Oglesby et al., 1990), but increased seizure susceptibility is also frequently observed (Izzo et al., 2001; Smith, 2002).

2.6 The hippocampus and its role in drug dependence

The hippocampal formation is part of the limbic system, which functions to regulate learning, memory, and emotions. Anatomically, the limbic system consists of the limbic lobe, a phylogenetically primitive subdivision of the cerebral cortex, as well as other functionally and anatomically related structures. The limbic lobe consists of the parahippocampal gyrus (including entorhinal cortex), the cingulate gyrus, and the hippocampal formation. The additional structures of the limbic system include parts of the hypothalamus, the septal area, the nucleus accumbens, certain neocortical areas (e.g., orbitofrontal cortex) and the amygdala (Iversen et al., 2000). The hippocampal formation is subdivided into the hippocampus proper, the dentate gyrus, and the subiculum. The hippocampus proper is divided into four fields with distinct cytoarchitecture: CA1, CA2, CA3, and CA4 (abbreviated from *cornu ammonis*, or “Ammon’s horn”). The dentate
gyrus contains a layer of principle cells that forms a V-shaped pair of joined blades and a polymorphic hilar region located between the blades, which surrounds field CA4. These fields can be further subdivided into somatic, dendritic, and axonic layers. The alveus is the deepest layer, a white matter tract formed by the myelinated axons of the principle (pyramidal) cells in CA1 and subiculum. Next is stratum oriens (SO), formed by the basal dendrites of pyramidal cells in CA1-3, then stratum pyramidale (SP), the pyramidal cell bodies, then stratum radiatum (SR), the apical dendrites, and stratum lacunosum-moleculare (SLM), the distal extensions of the apical dendrites. Immediately adjacent to the SLM is the molecular layer (ML), containing the apical dendrites of the principle cells of the dentate gyrus, the granule cells. The SLM of the hippocampus proper is separated from the ML of the dentate gyrus by the hippocampal fissure. The granule cell layer (GCL) is formed by the somata of granule cells, and as mentioned above, constitutes a compact layer of cells forming two blades facing opposite directions (Amaral & Witter, 1989). Between the two blades is the hilar region, which is a layer containing various types of polymorphic GABAergic interneurons that innervate the nearby granule cells (Houser, 2007). This polymorphic region merges with the CA4 pyramidal cells, which also appear to innervate the nearby granule cells (Amaral & Witter, 1989).

The rat hippocampus is also divided into two domains along the longitudinal (septotemporal) axis, referred to as the dorsal and ventral hippocampus. These domains are different in both connectivity and putative function, but the intrinsic circuitry is maintained along the entire length of the hippocampus. In particular, the hippocampus can be viewed as a trisynaptic circuit, which represents unidirectional information flow.
The first path in this circuit and the main input to the hippocampal formation comes from the entorhinal cortex via the perforant pathway. These inputs synapse primarily onto the dendrites of granule cells (ML), but also onto the distal dendrites of CA3 and CA1 pyramidal neurons (SLM, some SO). Granule cell axons project to the dendrites of pyramidal neurons in CA3 (SR/stratum lucidum) via the mossy fiber pathway, and axons from CA3 (and CA2) pyramidal neurons project to the dendrites of pyramidal neurons in CA1 (SR/SO) via the Schaffer collateral pathway, completing the trisynaptic circuit. CA3 also projects to contralateral CA1 via the anterior commissure. Axons from CA1 pyramidal neurons project mainly to the subiculum, as well as pre- and postsubiculum (Amaral & Witter, 1989; Somogyi & Klausberger, 2005). The combined CA1 and subicular axons form the alveus and project to entorhinal cortex (among other cortical regions, discussed below), mammillary body, amygdala, hypothalamus, thalamus, lateral septal nucleus, bed nucleus of the stria terminalis, nucleus accumbens, and anterior olfactory nucleus (Swanson & Cowan, 1977). Although this unidirectional circuit seems to carry information in a plane transverse to the hippocampus, significant information transfer also occurs along the longitudinal axis (Amaral & Witter, 1989).

The hippocampus is best known for its role in memory formation, but also functions in anatomic circuits linked to the expression of anxiety (Degroot & Treit, 2004) and seizures (Griesemer & Mautes, 2007), both symptoms of withdrawal from GABA<sub>A</sub> receptor modulators. The circuits that mediate these various functions may be anatomically separated in the dorsal and ventral hippocampus. In particular, the dorsal hippocampus receives visuospatial input and projects to retrosplenial and anterior cingulate cortex (bidirectional connection), mammillary nuclei (via the fornix), anterior
thalamic nuclei, lateral septal nucleus, nucleus accumbens, and rostral caudate-putamen, which are collectively involved in spatial memory and environmental exploration, especially via connections to ventral tegmental area and the reticular part of substantia nigra. In contrast, the ventral hippocampus receives mainly olfactory, gustatory, and visceral input and projects to olfactory bulb, anterior olfactory nucleus, amygdala (bidirectional connection), medial prefrontal cortex (bidirectional connection), periventricular and medial zones of the hypothalamus, lateral septal nucleus, and bed nucleus of the stria terminalis, which are collectively involved in the regulation of emotions, motivated behavior, and affective state. A third intermediate region may be distinguished as receiving a more widespread mix of inputs and projecting to anterior olfactory nucleus, medial prefrontal cortex, amygdala (bidirectional connection), anterior hypothalamic nuclei, supramammillary nucleus, medial mammillary nucleus, and lateral septal nucleus (Cenquizca & Swanson, 2007; Fanselow & Dong, 2010). These projections are similar to the ventral hippocampus and may additionally function to translate spatial inputs into motivated behaviors (Fanselow & Dong, 2010).

In addition to the intermediate region mediating overlapping function, longitudinal information processing and widespread connections between hippocampal inputs and outputs (Fanselow & Dong, 2010) suggest that the function of dorsal hippocampus may not be restricted to encoding spatial information. In particular, some studies implicate a role of dorsal hippocampus in mediating anxiolytic or anxiogenic effects of various drugs (Bitran et al., 1999; File et al., 2000) and BZ-induced withdrawal anxiety directly correlates with hyperexcitability of dorsal hippocampal CA1 neurons (Xiang & Tietz, 2007). Thus, the hippocampus represents an important node in anatomic circuits linked to
the expression of anxiety, a common symptom observed following withdrawal from CNS depressants.

2.7 GABAergic interneurons and hippocampal network activity

Network activity of the hippocampus is complex and determined by both intrinsic glutamatergic and GABAergic signals and a variety of extrinsic inputs. Of particular relevance to the study of BZs and other GABA_A receptor modulators are the location of GABAergic interneurons and their synaptic connections within the hippocampus. Numerous classes of interneurons exist based on the location of their cell bodies and the arborization of their dendrites and axons. Within the CA1 field, GABAergic interneuron cell bodies are located mainly in the avleus, SO, SP, and SR-SLM border. Their dendritic trees and axonal projections vary greatly. Some project locally, while others project to different regions, such as the dentate gyrus, CA3, subiculum, or septum. A few types of interneurons specifically project to other interneurons. Many of these interneurons are also distinguished based on the expression pattern of various calcium-binding proteins and neuropeptides. Specifically, GABAergic interneurons express in differing combinations parvalbumin, calretinin, calbindin, cholecystokinin, somatostatin, neuropeptide Y, and vasoactive intestinal peptide (Somogyi & Klausberger, 2005). Each interneuron of a particular class appears to be activated in a similar manner and may even be connected by similar inputs, as well as gap junctions (Mann & Paulsen, 2007). Thus, the various classes of interneurons can be differentially activated depending on the source and firing frequency of the input, which will then define the output firing rate of CA1 pyramidal neurons or other neuronal projections (Somogyi & Klausberger, 2005; Mann & Paulsen, 2007).
GABA released from the axonal projections of interneurons generally targets synaptic γ2-containing GABAₐ receptors, but GABA spillover also activates extrasynaptic δ- or γ2-containing GABAₐ receptors, as well as GABAₜ receptors. Inhibitory GABAergic synapses can be morphologically distinguished from excitatory glutamatergic synapses. Specifically, inhibitory synapses have symmetric pre- and postsynaptic densities and are generally located on the axon, soma, or dendritic shaft, whereas excitatory synapses contain thicker postsynaptic densities and are mainly located on dendritic protrusions or spines. The greatest density of GABAergic synapses on CA1 pyramidal neurons occurs in the perisomatic region in the proximal SR and SO, followed by SLM, then distal SR and SO. The percent of inhibitory input in these regions is 98% in proximal SR, 48% in proximal SO, 15% in SLM, and 2-3% in both distal SR and SO. Synaptic inputs on the soma and axon initial segment are exclusively inhibitory. Thus, except for a relatively high inhibitory component in SLM, which would specifically modulate excitatory input from entorhinal cortex (and thalamus), synaptic inhibition occurs mainly perisomatically. This strong perisomatic inhibition serves as a veto function for the wide array of excitatory input, and synchronous GABAergic interneuron firing can therefore regulate the output frequency of CA1 neurons (Megias et al., 2001).

2.8 Molecular substrates of drug dependence: Role of GABAₐ receptor dysfunction

Dysfunction of GABAergic transmission has been studied as a putative molecular mechanism underlying drug tolerance and dependence. This can occur presynaptically via changes in the frequency or amount of GABA release, or postsynaptically via changes in receptor number, channel kinetics, channel conductance, or anionic gradients, as well as altered GABA or modulator sensitivity. Although a few studies have found changes in
the frequency or amount of GABA release following long-term treatment with GABA_A receptor modulators, especially following administration of the non-selective CNS depressants, e.g., barbiturates and ethanol (Hunt, 1983; Saunders & Ho, 1990; Yu & Ho, 1990), the most dramatic alterations occur postsynaptically. Thus, the following discussion will focus on the role of dysfunctional postsynaptic GABA_A receptors in mediating drug tolerance and dependence.

The mechanisms and expression of GABA_A receptor dysfunction vary depending on the dose and particular drug administered, as well as the route and duration of administration (Uusi-Oukari & Korpi, 2010). One of the first changes to appear and the most common finding during long-term exposure to various GABA_A receptor modulators in vitro (Hu & Ticku, 1994; Friedman et al., 1996) and in vivo (Gallager et al., 1984; Morrow et al., 1988; Allan et al., 1992) is allosteric uncoupling of modulator binding sites from the GABA binding site and from each other. Uncoupling following long-term treatment with various modulators or even GABA itself can be observed in binding studies as a reduced ability of GABA, neurosteroids, or barbiturates to enhance radiolabeled BZ binding (Friedman et al., 1996). Functionally, uncoupling is manifested as a decreased efficacy of allosteric modulators to enhance GABA-mediated Cl⁻ flux (Morrow et al., 1988; Allan et al., 1992; Hu & Ticku, 1994). Heterologous uncoupling also occurs in which long-term exposure to one modulator uncouples other modulator binding sites from the GABA or BZ binding sites (Buck & Harris, 1990; Hu & Ticku, 1994; Friedman et al., 1996). The mechanism for uncoupling may be due to receptor subunit changes, as the time course for both GABA_A receptor turnover and uncoupling occur with a t1/2 of about 1 – 3 days with maximal uncoupling after 7 – 10 days of drug
exposure (Borden et al., 1984; Hu & Ticku, 1994). However, uncoupling has also been observed in heterologous expression systems using recombinant GABA_A receptors, suggesting that post-translational modifications such as phosphorylation are also plausible mechanisms (Klein & Harris, 1996). Uncoupling likely contributes to the rapid tolerance that develops to particular drug effects, especially sedation. Heterologous uncoupling could also contribute to cross-tolerance that occurs between some GABA_A receptor modulators (Harris, 1990; Smith & Stoops, 2001).

Subsequent to an initial uncoupling event, long-term FZP treatment results in decreased miniature inhibitory postsynaptic current (mIPSC) amplitude, synaptic and unitary conductance, and the number of mIPSC events, including an absence of events in about one-third of CA1 pyramidal neurons at 2 days, but not 7 days of withdrawal. These changes were region specific as they were not observed in dentate granule cells (Poisbeau et al., 1997; Tietz et al., 1999b; Zeng & Tietz, 1999; Xiang & Tietz, 2008). Long-term barbiturate or ethanol treatment may also result in decreased GABA-mediated currents (Klein & Harris, 1996). A role for increased \([\text{Ca}^{2+}]_i\) in mediating the reduced GABA sensitivity during FZP withdrawal has been proposed (Zeng & Tietz, 1997; Xiang & Tietz, 2008) based on the known ability of \(\text{Ca}^{2+}\) to negatively regulate GABA_A receptor function (Stelzer et al., 1988; Houston et al., 2009). Recent findings have also shown that chronic blockade of neuronal activity or L-VGCC-mediated \(\text{Ca}^{2+}\) influx reduced synaptic GABA_A receptor expression by enhancing proteasome-dependent turnover and reducing synaptic insertion (Saliba et al., 2009). These findings suggest that basal L-VGCC-mediated \(\text{Ca}^{2+}\) influx is required to maintain synaptic GABA_A receptor expression. Therefore, reduced L-VGCC activity due to prolonged enhancement of GABA-mediated
inhibition by BZs may result in reduced synaptic GABA_A receptor expression during chronic treatment. Subsequently, enhanced Ca^{2+} influx partly due to impaired GABA-mediated inhibition could further disrupt GABA_A receptor function during BZ withdrawal.

In addition to reduced synaptic conductance, a slight depolarizing shift in the GABA_A receptor reversal potential from $-72$ mV in control to $-68$ mV in 2-day FZP-withdrawn CA1 neurons was observed, implicating a potential reduction in the Cl$^{-}$ driving force (Zeng & Tietz, 1997). When interfering Na$^{+}$ and K$^{+}$ ion fluxes were blocked, a GABA_A receptor-mediated depolarization was observed in FZP-withdrawn but not control CA1 neurons following high intensity stimulation, which typically occurred later than the early inhibitory postsynaptic potential (Zeng et al., 1995). The requirement for high intensity stimulation and delayed response suggests that large amounts of GABA release may have been needed, which could result in spillover and activation of extrasynaptic receptors. Interestingly, it was previously suggested that dendritic extrasynaptic receptors in CA1 pyramidal cells are depolarizing (Alger & Nicoll, 1982). However, whether extrasynaptic receptors are up-regulated in the hippocampus as a consequence of long-term FZP administration remains unknown (see below). It should be noted that a slight depolarizing response can still be inhibitory to membrane excitability due to the shunting effect of reduced membrane resistance when the channels are open (Alger & Nicoll, 1979).

Depolarizing shifts in the GABA reversal potential have also been observed following prolonged use of GABA_A receptors (Staley et al., 1995). This effect primarily occurs in the dendrites, where the large surface area to volume ratio allows for faster changes in ion concentration. In particular, repeated GABA_A receptor activation leads to
chronic Cl\(^-\) and HCO\(_3^-\) fluxes, neither of which is able to reach its respective reversal potential. Although intracellular and extracellular HCO\(_3^-\) levels are rapidly buffered by carbonic anhydrase enzymes, intracellular Cl\(^-\) accumulation can transiently overcome the maximal velocity of Cl\(^-\) transporters, like KCC2. This results in a reduced Cl\(^-\) driving force and transient GABA-mediated depolarization via HCO\(_3^-\) and possibly Cl\(^-\) efflux (Staley & Proctor, 1999). While this would explain a transient depolarizing response due to prolonged BZ-mediated GABA current enhancement, it does not explain the continued presence of a depolarized GABA reversal potential or the GABA-mediated depolarization observed after 2 days of FZP withdrawal. It is currently unknown whether dysfunctional Cl\(^-\) transport is responsible for the GABA-mediated depolarization, or whether this was a normal dendritic response unmasked by the decrease in IPSP amplitude (Zeng et al., 1995). It is unlikely due to increased expression of GABA\(_A\) receptors with higher relative permeability to HCO\(_3^-\), since the HCO\(_3^-\) conductance is selectively reduced during FZP withdrawal (Zeng & Tietz, 2000).

Changes in the expression of GABA\(_A\) receptor subunits could explain reduced IPSC amplitudes and decreased sensitivity to GABA and the allosteric modulators, but these changes are often inconsistent depending on the treatment model. One- and 4-week oral treatment with FZP (100 – 150 mg/kg/day) resulted in a down-regulation of BZ receptors across several brain regions measured by autoradiographic localization of flunitrazepam binding (Tietz et al., 1986). In contrast, once daily intraperitoneal (i.p.) injection of rats with diazepam (2.5 – 5 mg/kg) for 3 weeks did not affect BZ affinity or number of sites measured by radiolabeled flunitrazepam binding to cerebral cortical membranes (Gallager et al., 1984). Immediately after 1-week FZP treatment there was decreased α1
and β3 mRNA and protein in the hippocampus and cortex (Chen et al., 1999; Tietz et al., 1999a). These changes were largely reversed after 2 days of withdrawal (Tietz et al., 1999a; Tietz et al., 1999b). Thus, while down-regulation of α1/β3-containing GABA<sub>A</sub> receptors may explain tolerance to the sedative effects of FZP, dysregulation of GABA<sub>A</sub> receptor expression does not appear to adequately explain the manifestation of withdrawal anxiety or anticonvulsant tolerance up to 2 days after FZP withdrawal (Rosenberg et al., 1991; Van Sickle et al., 2004). Other studies using either i.p. injection or continuous infusion of rats with diazepam (5 – 20 mg/kg/day or 0.25 µg/g, ~0.9 µM, respectively) have also found decreased α1 mRNA or protein expression in hippocampus and cortex (Heninger et al., 1990; Wu et al., 1994; Impagnatiello et al., 1996). The decreased hippocampal α1 mRNA expression returned to baseline levels after 2 days of withdrawal (Wu et al., 1994). Thus, several but not all studies support decreased α1 subunit expression following long-term BZ treatment. However, there is no consistent finding that a particular subunit is up-regulated which might replace α1-containing GABA<sub>A</sub> receptors (Uusi-Oukari & Korpi, 2010). Although only a limited number of studies have analyzed BZ withdrawal or followed up on changes in mRNA expression by confirming changes at the protein level, overall the data seem to support changes in GABA<sub>A</sub> receptor subunit expression as a mechanism of BZ tolerance, but not withdrawal symptoms.

Unlike the effects observed for BZs, 2 to 3 days of continuous progesterone treatment in rats, the precursor to allopregnanolone, resulted in increased α4 subunit protein expression in the CA1 region. This correlated with decreased GABA-mediated current decay time and BZ sensitivity. The faster decay time of GABA currents in CA1 neurons
would reduce GABA-mediated inhibition, which may have contributed to the expression of anxiety that was also observed at these time points. These effects were dependent on allopregnanolone synthesis and were reversed after 5 to 6 days of treatment (Gulinello et al., 2001). However, all of these effects re-emerged after continued treatment for 3 weeks, followed by 8 to 24 hrs of withdrawal (Smith et al., 1998).

Measurement of subunit mRNA immediately after long-term treatment with pentobarbital revealed decreased $\alpha_1$ expression in the hippocampus (Tseng et al., 1994) and increased $\alpha_6$ and $\delta$ expression in the cerebellum (Ito et al., 1996; Lin & Wang, 1996). During withdrawal there was increased $\alpha_1$, $\beta_3$, and $\gamma_2$ expression in cerebral cortex (Tseng et al., 1993; Tseng et al., 1994) and increased $\alpha_1$ (Tseng et al., 1994) but decreased $\delta$ (Lin & Wang, 1996) expression in the cerebellum. Although changes in subunit protein expression were not confirmed, similar to BZs, GABA$_A$ receptor subunit regulation would appear to explain tolerance, but not the withdrawal phenomenon associated with barbiturate use.

Several alterations in GABA$_A$ receptor subunits have been observed following long-term ethanol treatment, many of which persist during withdrawal. In most brain regions decreased $\alpha_1$ subunit protein expression was observed. This was accompanied by increased $\alpha_4$, $\beta_{2/3}$, and $\gamma_1$ protein expression in the cerebral cortex (Devaud et al., 1997), decreased $\delta$ with increased $\alpha_4$ and $\gamma_2$ expression in the hippocampus (Cagetti et al., 2003), increased $\alpha_4$ and $\gamma_2$ in the basolateral amygdala (Diaz et al., 2011), and increased $\alpha_6$ expression in the cerebellum (Sanna et al., 2004a). The regulation of $\alpha_1$ subunit in the hippocampus required intermittent ethanol treatment, as it was not regulated by continuous treatment (Matthews et al., 1998). The subunit changes in the hippocampus
were also accompanied by a decrease in CA1 neuron mIPSC decay time and BZ sensitivity (Cagetti et al., 2003), similar to that of neurosteroid withdrawal. Interestingly, ethanol increased de novo synthesis of allopregnanolone in hippocampal slices (Sanna et al., 2004b), suggesting that some of ethanol’s long-term effects may be mediated by endogenous neurosteroids.

2.9 Molecular substrates of drug dependence: Role of ionotropic glutamate receptors

Dysfunction of GABA<sub>A</sub> receptors like that described above, as well as enhancement of ionotropic glutamate receptors, the main excitatory neurotransmitter receptors in the CNS, may contribute to withdrawal hyperexcitability (Izzo et al., 2001; Allison & Pratt, 2003; Van Sickle et al., 2004; Xiang & Tietz, 2007). Glutamate release from presynaptic terminals acts on several classes of pre- and postsynaptic receptors. Like GABA receptors, glutamate receptors can be divided into ionotropic and metabotropic categories. The ionotropic receptors include AMPA, kainate, NMDA, and ‘orphan’ receptors. As suggested by their names, the ionotropic receptors were initially classified pharmacologically by their agonists. Orphan receptors were classified as ionotropic receptors based on homology, but do not fit into a particular agonist class. It has subsequently been discovered that each class of ionotropic glutamate receptor is formed by a specific subset of genetically related subunits. These subunits assemble in the endoplasmic reticulum as a dimer of dimers to form functional homomeric or heteromeric tetramers. AMPA receptors are constructed from GluA1, GluA2, GluA3, or GluA4 subunits; kainate receptors from GluK1, GluK2, GluK3, GluK4, or GluK5 subunits; NMDA receptors from GluN1 (present in all NMDA receptors), GluN2A/B/C/D, or
GluN3A/B subunits; and orphan receptors from GluD1 or GluD2 (Collingridge et al., 2009). The metabotropic glutamate receptors are G protein-coupled receptors that are linked to different second-messenger signaling cascades. These receptors can be divided into 3 groups based on genetic and pharmacologic similarity: Group I receptors, mGluR1 and mGluR5, activate phospholipase C, and both Group II, mGluR2 and mGluR3, and Group III receptors, mGluR4, mGluR6, mGluR7, and mGluR8 inhibit adenylyl cyclase activity (Conn & Pin, 1997). Recent evidence suggests that orphan receptors may also paradoxically be G protein-coupled receptors rather than functional ion channels (Schmid & Hollmann, 2008).

The other ionotropic glutamate receptors can be distinguished as having different cationic permeability and kinetic properties depending on subunit composition. The conductive properties are determined by a specific Q/R/N site located in the pore-forming region of the subunit. For AMPA and kainate receptors, GluA2, GluK1, and GluK2 subunits undergo post-transcriptional editing of the genetically encoded glutamine to an arginine codon, which is developmentally regulated for kainate receptor subunits. Receptors composed entirely of unedited subunits containing a glutamine are permeable to Na⁺, K⁺, and Ca²⁺ and are sensitive to voltage-dependent block by intracellular polyamines. Receptors containing any edited subunits that have an arginine display reduced permeability to Ca²⁺ and channel conductance, and lack sensitivity to intracellular polyamines. NMDA receptor subunits contain an asparagine residue at the corresponding site and are permeable to Na⁺, K⁺, and Ca²⁺ and susceptible to voltage-dependent block by extracellular Mg²⁺ (Burnashev, 1998; Seeburg & Hartner, 2003).
The kinetics of excitatory postsynaptic currents (EPSCs) are determined by the particular subunit composition of the glutamate receptors assembled in vivo. AMPA receptors have a lower affinity for glutamate and fast activation and desensitization kinetics, mediating the fast component of EPSCs (Jonas & Spruston, 1994). In mature hippocampal neurons, the predominant population of AMPA receptors are composed of mainly Ca\(^{2+}\)-impermeable GluA1/GluA2 and GluA2/GluA3 heteromers and only about 8% are Ca\(^{2+}\)-permeable GluA1 homomers (Wenthold et al., 1996). Application of kainate to hippocampal neurons revealed a rapidly activating, non-desensitizing current that was not seen with AMPA application. It was later discovered using AMPA-selective antagonists that kainate activated AMPA receptors without desensitizing, and that kainate receptors themselves rapidly desensitize. Although kainate receptors mediate cation fluxes both pre- and postsynaptically, they do not appear to play a major role in shaping hippocampal EPSCs (Lerma et al., 1997). NMDA receptors exhibit higher affinity for glutamate and activate and desensitize slowly, contributing to the slow component of EPSCs (Jonas & Spruston, 1994). Although under normal conditions NMDA receptors represent the primary source of glutamate-mediated Ca\(^{2+}\) influx into dendritic spines, this can only occur if the Mg\(^{2+}\) block is removed by postsynaptic depolarization. Thus, NMDA receptors are Ca\(^{2+}\) signaling complexes that serve as coincident detectors of simultaneous pre- and postsynaptic activation.

NMDA receptors in CA1 pyramidal neurons are mainly composed of GluN1/GluN2A or GluN1/GluN2B subunit combinations (Monyer et al., 1994). GluN2B-containing NMDA receptors are expressed embryonically and postnatally, whereas GluN2A expression occurs during the first two weeks of postnatal development (Sheng et al.,
1994; Zhong et al., 1994). GluN2A- and GluN2B-containing NMDA receptors are expressed both synaptically and extrasynaptically in hippocampal neurons (Thomas et al., 2006). GluN2B-containing NMDA receptors are susceptible to lateral mobility into and out of PSDs (Tovar & Westbrook, 2002; Groc et al., 2004) and additionally serve as CaMKII binding partners (Strack et al., 2000; Robison et al., 2005) to regulate synaptic plasticity.

CaMKII serves an important role in both drug-induced and physiologic glutamatergic plasticity. CaMKII forms oligomeric holoenzymes containing up to 12 subunits (Bennett et al., 1983). The CaMKIIβ isoform mediates binding of CaMKII holoenzymes to the actin cytoskeleton within dendritic arbors (Shen et al., 1998). Upon elevation of intracellular Ca$^{2+}$ concentrations ($[\text{Ca}^{2+}]$), Ca$^{2+}$ binds to all four Ca$^{2+}$-binding sites on calmodulin (CaM), resulting in association with and activation of CaMKII via displacement of an autoinhibitory domain. If $[\text{Ca}^{2+}]$ remains elevated for sufficient time, CaMKII enzymes phosphorylate neighboring CaMKII molecules within the holoenzyme at Thr$^{286}$ on $\alpha$ or Thr$^{287}$ on $\beta$ isoforms. This autophosphorylation results in Ca$^{2+}$/CaM trapping and autonomous kinase activity without the need of elevated $[\text{Ca}^{2+}]$. Elevations in $[\text{Ca}^{2+}]$ also result in dissociation of CaMKII holoenzymes from the actin cytoskeleton, promoting binding with other proteins (Fink & Meyer, 2002).

Alterations in CaMKII activity and glutamate receptor function may contribute to the drug withdrawal phenomenon. While enhanced AMPA receptor function in the ventral tegmental area is associated with behavioral sensitization and addiction to drugs of abuse such as cocaine, amphetamine, morphine, ethanol, and nicotine (Wolf, 2003; Kauer & Malenka, 2007; Mameli et al., 2011), enhanced AMPA receptor function in other brain
regions, such as the cortex, hippocampus, amygdala, and thalamus may underlie BZ withdrawal symptoms (Izzo et al., 2001; Van Sickle & Tietz, 2002; Allison et al., 2005). Notably, potentiation of AMPA receptors in the dorsal hippocampal CA1 region significantly correlated with the severity of FZP withdrawal anxiety (Van Sickle et al., 2004; Song et al., 2007; Xiang & Tietz, 2007). Specifically, increased miniature EPSC (mEPSC) amplitude via enhanced AMPA receptor function was observed after 1 and 2 days of FZP withdrawal, with increased unitary conductance after 2 days of withdrawal (Van Sickle & Tietz, 2002; Van Sickle et al., 2004). The increased AMPA receptor current amplitude is initiated by the incorporation of GluA1 homomers during the first day of FZP withdrawal (Song et al., 2007; Das et al., 2008), followed by increased conductance via CaMKII-mediated phosphorylation of GluA1 during the second day of withdrawal (Shen et al., 2009; Shen et al., 2010). However, anxiety was not observed after 2 days of FZP withdrawal, likely attributable to a compensatory decrease in GluN1/GluN2B subunit expression and NMDA receptor function (Van Sickle et al., 2004; Shen et al., 2009; Das et al., 2010; Shen & Tietz, 2011). Moreover, prior systemic injection with MK-801 (an NMDA receptor antagonist) prevented the NMDA receptor down-regulation and unmasked anxiety on day 2 of withdrawal (Van Sickle et al., 2004).

In contrast to BZs, decreased kainate and increased NMDA receptor ligand binding was observed following long-term barbiturate treatment (Hoffman & Tabakoff, 1993). Similarly, decreased AMPA and increased NMDA receptor ligand binding with enhanced NMDA receptor function was observed following long-term ethanol administration or repeated ethanol withdrawal episodes (Hoffman & Tabakoff, 1993; Ulrichsen et al., 1996). In particular, GluN2A and GluN2B mRNA and/or protein expression were up-
regulated both *in vitro* and *in vivo* following long-term ethanol exposure (Nagy, 2004). The variability in glutamatergic regulation by GABA<sub>A</sub> receptor modulators may be due to the different molecular targets of these compounds.

Interestingly, FZP-induced regulation of AMPA receptors is similar to that described in the early phase of LTP (E-LTP) following Schaffer collateral stimulation. Induction of E-LTP requires high frequency (25 - 100 Hz) or theta-burst (5 Hz) stimulation of CA1 afferents resulting in Ca<sup>2+</sup> influx through postsynaptic NMDA receptors and subsequent activation of CaMKII and other kinases like PKC. The initial Ca<sup>2+</sup> influx and resultant change in kinase activity leads to insertion of GluA1 homomers into the PSD enhancing synaptic strength. Further activation of NMDA receptors and corresponding increases in [Ca<sup>2+</sup>]<sub>i</sub> results in CaMKII autophosphorylation and translocation to binding partners in the PSD, like the GluN2B subunit, where it mediates phosphorylation of GluA1 homomers on Ser<sup>831</sup> increasing synaptic conductance (Fink & Meyer, 2002; Boehm & Malinow, 2005). In contrast, late-phase LTP (L-LTP, >1 hr) induced by repeated high frequency (100 Hz) stimulation or theta-burst (5 Hz) stimulation requires gene expression and protein synthesis or dendritic protein translation, respectively (Malenka & Bear, 2004; Huang & Kandel, 2005). In particular, enhanced dendritic translation of CaMKII<sub>α</sub> and GluA1 and GluA2 AMPA receptor subunits may occur during L-LTP (Steward & Schuman, 2001; Miller et al., 2002; Ju et al., 2004). Induction L-LTP is also dependent on Ca<sup>2+</sup> influx through NMDA receptors (Huang & Kandel, 2005; Bengtson et al., 2010). In contrast to induction mechanisms of CA1 LTP, systemic injection of FZP-treated rats with MK-801 did not prevent the appearance of anxiety or up-regulation of AMPA.
receptor function after 1 day of withdrawal (Xiang & Tietz, 2007), suggesting that a source of Ca\(^{2+}\) other than NMDA receptors may mediate this function.

2.10 Molecular substrates of drug dependence: Role of L-type voltage gated calcium channels

VGCCs are Ca\(^{2+}\) permeable channels that open in response to membrane depolarization. They are composed of a large (\(\sim 190 - 250\) kDa) transmembrane pore-forming \(\alpha_1\) subunit (10 subtypes) and 2 - 3 auxiliary subunits. An intracellular \(\beta\) subunit (4 subtypes) acts as a chaperone and regulates gating properties. An extracellular \(\alpha_2\) subunit disulfide-linked to a transmembrane \(\delta\) subunit (4 \(\alpha_2\delta\) subtypes) regulates trafficking and voltage-dependent channel properties (Catterall, 2000). A transmembrane \(\gamma_1\) subunit was originally purified with skeletal muscle and cardiac Ca\(^{2+}\) channels, but not with neuronal VGCCs. Additional \(\gamma\) homologs (\(\gamma_2-8\)) are also not considered VGCC subunits, but may play a role in AMPA receptor trafficking (Dolphin, 2009). The \(\alpha_1\) subunit consists of intracellular N- and C-termini and four repeated domains connected by intracellular loops, each domain containing six transmembrane segments (S1 - S6), with S4 serving as the voltage-sensor (Catterall, 2000). The different classes of VGCCs are characterized by their \(\alpha_1\) pore-forming subunit, as the associated \(\beta\) and \(\alpha_2\delta\) subunits are common to all VGCCs. VGCCs are divided into five classes based on pharmacology, as well as kinetic and conductance properties: high voltage-activated (HVA) channels contain L- (Ca\(_{\alpha 1.1-1.4}\)), P/Q- (Ca\(_{\alpha 2.1}\)), N- (Ca\(_{\alpha 2.2}\)), or R-type (Ca\(_{\alpha 2.3}\)) subunits, and low voltage-activated channels contain T-type (Ca\(_{\alpha 3.1-3.3}\)) subunits. L-VGCCs are selectively inhibited by DHPs, BTZs, and PAAs. P/Q-VGCCs are inhibited by \(\omega\)-
agatoxin IVA, N-VGCCs by \( \omega \)-conotoxin-GVIA, and R-VGCCs by SNX-482. T-VGCCs are somewhat more sensitive to inhibition by mibefradil (Catterall et al., 2005).

The \( \alpha_1 \) subunits found in neuronal L-VGCCs are Ca\(_{1.2}\) and Ca\(_{1.3}\). Ca\(_{1.2}\) is four times more abundant than Ca\(_{1.3}\) in the rat cortex and hippocampus (Hell et al., 1993a) and is localized in neuronal soma, as well as proximal and distal dendrites near excitatory asymmetric synapses both in vitro (Obermair et al., 2004) and in vivo (Tippens et al., 2008; Leitch et al., 2009). Although recent evidence suggested expression of Ca\(_{1.3}\) at postsynaptic sites within CA1 neuron dendritic spines (Leitch et al., 2009), this study did not confirm antibody specificity for immunohistochemical labeling. However, other studies using a Ca\(_{1.3}\) antibody characterized as specific for immunohistochemistry using knockout tissues (Olson et al., 2005) confirmed Ca\(_{1.3}\) localization within dendritic spines in other brain regions, including the striatum (Day et al., 2006) and ventral horn (Zhang et al., 2008). In addition, studies in hippocampal cultures indicated that Ca\(_{1.3}\) binds to and co-localizes with clusters of the postsynaptic adaptor protein, Shank (Zhang et al., 2005). Thus, there is substantial evidence that both Ca\(_{1.2}\) and Ca\(_{1.3}\) L-VGCCs are situated near excitatory postsynapses where they can respond to neuronal activity and mediate Ca\(^{2+}\)-dependent synaptic plasticity and changes in gene expression (Dolmetsch et al., 2001; Striessnig et al., 2006; Barbado et al., 2009). Moreover, L-VGCCs are located in glial cells (Tippens et al., 2008), GABAergic interneurons (Vinet & Sik, 2006; Zhang et al., 2008), and in axons and nerve terminals (Tippens et al., 2008), suggesting that L-VGCCs can modulate neuronal plasticity via a variety of mechanisms.

In particular, both the early and late phases of LTP described above can also be induced by Ca\(^{2+}\) through L-VGCCs during high frequency (100 - 200 Hz) or relatively
long-lasting theta-burst stimulation (Grover & Teyler, 1990; Morgan & Teyler, 2001; Moosmang et al., 2005). Estimates of the contribution of L-VGCCs to these forms of LTP may be underestimated for two reasons. First, application of NMDA receptor antagonists often completely blocks LTP suggesting no role for other sources of Ca$$^{2+}$$. However, L-VGCCs may contribute to LTP but require the added NMDA receptor-mediated depolarization (Morgan & Teyler, 2001). Second, ineffectiveness of DHPs to block LTP may be due to how slowly they block L-VGCCs when activated by action potential stimuli even at high frequencies (Helton et al., 2005).

A role for L-VGCCs in contributing to withdrawal pathology from GABA$$\text{A}$$ receptor modulators has been proposed in numerous studies. Long-term treatment with BZs enhances Ca$$^{2+}$$ influx through L-VGCCs (Katsura et al., 2007; Xiang et al., 2008) and BZ withdrawal symptoms were attenuated when L-VGCC antagonists were administered during the BZ withdrawal phase (Dolin et al., 1990; Gupta et al., 1996; Xiang & Tietz, 2007). However, reports differ on the effectiveness of L-VGCC antagonists when they are co-administered with BZs, with at least one study showing a reduction in withdrawal symptoms (Hitchcott et al., 1992) and others showing no significant effects (Dolin et al., 1990; Mizoguchi et al., 1993). Concurrent L-VGCC antagonist administration during long-term barbiturate treatment, but not during the withdrawal phase protected against the withdrawal syndrome (Rabbani et al., 1994). Barbiturate withdrawal was associated with an increase in the number of DHP binding sites in cerebral cortex and increased DHP-sensitive K$$^+$$-stimulated Ca$$^{2+}$$ influx in hippocampal slices (Rabbani & Little, 1999). Concurrent administration of an L-VGCC antagonist with long-term ethanol treatment in mice attenuated an up-regulation of brain DHP binding sites, convulsions, and
hippocampal slice hyperexcitability seen during withdrawal (Whittington et al., 1991; Whittington & Little, 1991). Moreover, addition of an L-VGCC antagonist to perfusion medium also acutely attenuated hippocampal slice hyperexcitability during ethanol withdrawal (Whittington & Little, 1991). A role for L-VGCCs in mediating the neurosteroid withdrawal phenomenon has not yet been reported.

The combination of data support a role for increased L-VGCC-mediated Ca\(^{2+}\) influx in contributing to the GABA\(_A\) receptor modulator withdrawal syndrome. In the presence of a hyperexcitable state due to GABA\(_A\) receptor dysfunction, a dramatic increase in postsynaptic L-VGCC-mediated Ca\(^{2+}\) influx would ensue, contributing to withdrawal phenomena via localized Ca\(^{2+}\) signal transduction cascades. At least for BZs, L-VGCC-mediated Ca\(^{2+}\) signaling cascades may enhance AMPA receptor function (Xiang & Tietz, 2007), maintaining CA1 neuron hyperexcitability during withdrawal.

### 2.11 Regulation of L-VGCCs

Prolonged depolarization and activation of L-VGCCs leads to both voltage-dependent and Ca\(^{2+}\)-dependent inactivation, forms of negative feedback regulation. Unlike other Ca\(^{2+}\) channels, L-VGCCs inactivate slowly leading to long-lasting Ca\(^{2+}\) currents. Voltage- and Ca\(^{2+}\)-dependent inactivation are regulated by distinct sites within the \(\alpha_1\) subunit. In particular, Ca\(^{2+}\)-dependent inactivation is mediated by LA and IQ motifs located in the Ca\(_{v1.2}\) C-terminus, which bind CaM in different Ca\(^{2+}\)-bound states. The LA motif binds apo-CaM (Ca\(^{2+}\) free) at low [Ca\(^{2+}\)]\(_i\) (50 - 100 nM), as well as when [Ca\(^{2+}\)]\(_i\) is high (>1 µM). In contrast, the IQ motif binds CaM when Ca\(^{2+}\) is conducted through the channel. The transition from the LA to the IQ motif mediates Ca\(^{2+}\)-dependent inactivation, and may prime Ca\(^{2+}\)-bound CaM for downstream signal transduction
cascades, linking L-VGCC inactivation with Ca$^{2+}$ signaling (Morad & Soldatov, 2005). Voltage-dependent rearrangements in channel confirmation also affect inhibition of L-VGCCs by several drug classes, which generally show preferential interaction with open and inactive channel states (Hockerman et al., 1997).

In addition to inactivation, L-VGCC function can be modulated by changes in plasma membrane expression (Jarvis & Zamponi, 2007), proteolytic cleavage (Hell et al., 1996), or phosphorylation (Kepplinger et al., 2000). As mentioned above both the β and α$_{2}\delta$ subunits are involved in trafficking Ca$_{v}$.1.2 and Ca$_{v}$1.3 subunits to the membrane, as well as modulating activation and inactivation kinetics and open probability of the channels (Lacinova, 2005). Proteolytic cleavage of Ca$_{v}$.1.2 C-terminus by the Ca$^{2+}$-dependent protease, calpain, has been shown to increase Ca$^{2+}$ influx through the channel in hippocampal neurons (Hell et al., 1996). This cleavage also results in removal of S$^{1928}$, a critical phosphorylation site on Ca$_{v}$.1.2 (De Jongh et al., 1996; Yang et al., 2005; Hall et al., 2006; Oliveria et al., 2007; Yang et al., 2007). Some of the kinases and phosphatases involved in regulating L-VGCC function include PKA (Hell et al., 1993b; Gao et al., 1997; Hall et al., 2007), PKC (Yang et al., 2005; Baroudi et al., 2006), PKG (Yang et al., 2007), CaMKII (Lee et al., 2006; Grueter et al., 2008), PP2A (Davare et al., 2000; Hall et al., 2006), and PP2B (Oliveria et al., 2007), also known as calcineurin. Many of these kinases and phosphatases have been shown to act on Ca$_{v}$.1.2, but also regulate other α$_{1}$ (Liang & Tavalin, 2007) and β (Gao et al., 1997; Yang et al., 2007; Grueter et al., 2008) subunits. In particular, CaMKII is known to mediate Ca$^{2+}$-dependent facilitation of both Ca$_{v}$.1.2 (Lee et al., 2006) and Ca$_{v}$.1.3 (Calin-Jageman et al., 2007) channel function. G
protein-coupled receptors also regulate L-VGCCs, mainly via signaling cascades that alter activity of kinases or phosphatases (Olson et al., 2005; Hulme et al., 2006).

Ca\(^{2+}\) influx is rapidly buffered by numerous cytosolic Ca\(^{2+}\)-binding proteins, resulting in localized elevation of Ca\(^{2+}\) concentrations mainly within the microdomain created by channel clusters. Thus, \(\text{Ca}_v1.2\) and \(\text{Ca}_v1.3\) Ca\(^{2+}\) microdomains are capable of selectively activating distinct localized signaling cascades leading to different downstream targets. In particular, although both L-VGCCs contain class I PDZ (postsynaptic density-95/Discs large/zona occludens-1)-interaction motifs in their C-termini, the \(\text{Ca}_v1.2\) motif, VSNL, interacts with different scaffolding and adaptor proteins than the \(\text{Ca}_v1.3\) motif, ITTL (Calin-Jageman & Lee, 2008). In addition, because of the different voltage-dependent activation and inactivation properties of L-VGCC subtypes (Koschak et al., 2001; Xu & Lipscombe, 2001), they are selectively activated at different phases of the action potential and by different frequencies of neuronal activity (Zhang et al., 2006). Thus, \(\text{Ca}_v1.2\) and \(\text{Ca}_v1.3\) L-VGCCs can mediate distinct neuronal functions. In particular, \(\text{Ca}_v1.2\) channels are involved in memory formation related to their role in an NMDA-independent form of L-LTP (Moosmang et al., 2005), which may be stimulated solely by back-propagating action potentials (Dudek & Fields, 2002). \(\text{Ca}_v1.3\) channels on the other hand, stimulate Ca\(^{2+}\)-activated K\(^+\) channels enhancing the afterhyperpolarization and reducing further neuronal activity (Bowden et al., 2001), promote spontaneous firing activity in certain neurons (Putzier et al., 2009), and may promote age-related neuronal degeneration and cognitive dysfunction (Thibault & Landfield, 1996; Porter et al., 1997; Chan et al., 2010). Thus, differential regulation of \(\text{Ca}_v1.2\) and \(\text{Ca}_v1.3\) L-VGCCs could selectively target their distinct functions mediated by localized signaling cascades.
CHAPTER 3

INHIBITION OF RECOMBINANT L-TYPE VOLTAGE-GATED CALCIUM CHANNELS BY POSITIVE ALLOSTERIC MODULATORS OF GABA-A RECEPTORS

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Running Title: GABAAR modulators inhibit recombinant L-VGCCs

Abbreviations: ANOVA, analysis of variance; BDZ, benzodiazepine; BTZ, benzothiazepine; DMSO, dimethyl sulfoxide; DHP, dihydropyridine; L-VGCC, L-type voltage-gated calcium channel; PAA, phenylalkylamine; TEA, tetraethylammonium; WT, wild type; LB, Luria-Bertani; HEK, human embryonic kidney

Keywords: barbiturate; benzodiazepine; calcium channel mutation; electrophysiology; ethanol; neurosteroid

3.1 Abstract

Benzodiazepines (BDZs) depress neuronal excitability via positive allosteric modulation of inhibitory GABA\(\text{A}\) receptors (GABA\(\text{A}\)R). BDZs and other positive GABA\(\text{A}\)R modulators, including barbiturates, ethanol, and neurosteroids, can also inhibit L-type voltage-gated calcium channels (L-VGCCs), which could contribute to reduced neuronal excitability. Because neuronal L-VGCC function is up-regulated after long-term GABA\(\text{A}\)R modulator exposure, an interaction with L-VGCCs may also play a role in physical dependence. The current studies assessed the effects of BDZs (diazepam, flurazepam, and desalkylflurazepam), allopregnanolone, pentobarbital, and ethanol on whole-cell Ba\(^{2+}\) currents through recombinant neuronal Ca\(_v\)1.2 and Ca\(_v\)1.3 L-VGCCs expressed with \(\beta_3\) and \(\alpha_2\delta-1\) in HEK293T cells. Allopregnanolone was the most potent inhibitor (IC\(_{50}\), \(\sim\)10 \(\mu\)M), followed by BDZs (IC\(_{50}\), \(\sim\)50 \(\mu\)M), pentobarbital (IC\(_{50}\), 0.3–1 mM), and ethanol (IC\(_{50}\), \(\sim\)300 mM). Cav1.3 channels were less sensitive to pentobarbital inhibition than Cav1.2 channels, similar to dihydropyridine (DHP) L-VGCC antagonists. All GABA\(\text{A}\)R modulators induced a negative shift in the steady-state inactivation curve of Cav1.3 channels, but only BDZs and pentobarbital induced a negative shift in Cav1.2 channel inactivation. Mutation of the high-affinity DHP binding site (T1039Y and Q1043M) in Cav1.2 channels reduced pentobarbital potency. Despite the structural similarity between benzothiazepines and BDZs, mutation of an amino acid important for diltiazem potency (I1150A) did not affect diazepam potency. Although L-VGCC inhibition by BDZs occurred at concentrations that are possibly too high to be clinically relevant and is not likely to play a role in the up-regulation of L-VGCCs during long-
term treatment, pentobarbital and ethanol inhibited L-VGCCs at clinically relevant concentrations.
3.2 Introduction

Benzodiazepines (BDZs) are clinically useful anxiolytics, sedatives, and anticonvulsants. The clinical effects of BDZs are attributed to their ability to allosterically enhance the inhibitory action of γ-aminobutyric acid type A receptors (GABA\textsubscript{A}Rs). However, there is also evidence that BDZs directly inhibit L-type voltage-gated calcium channels (L-VGCCs) in muscle and nerve cells at low micromolar concentrations (Yamakage et al., 1999; Xiang et al., 2008). Other GABA\textsubscript{A}R modulators, such as the neurosteroid, allopregnanolone (3\textalpha-hydroxy-5\textalpha-pregnan-20-one), the barbiturate, pentobarbital, and ethanol can also inhibit neuronal L-VGCCs (Messing et al., 1986; ffrench-Mullen et al., 1993; Hu et al., 2007).

The interaction of GABA\textsubscript{A}R modulators with L-VGCCs could play a role in their acute effects to reduce neuronal excitability and may also contribute to the neuronal hyperexcitability seen following withdrawal from chronic drug exposure. Notably, chronic treatment with BDZs, barbiturates, and ethanol increases neuronal Ca\textsuperscript{2+} influx \textit{in vitro} and \textit{in vivo}, possibly through L-VGCCs (Messing et al., 1986; Rabbani & Little, 1999; Katsura et al., 2006; Katsura et al., 2007; Xiang et al., 2008). An upregulation of L-VGCC function during chronic treatment with the various GABA\textsubscript{A}R modulators is implicated in contributing to withdrawal hyperexcitability (Dolín et al., 1987; Rabbani & Little, 1999; Xiang & Tietz, 2007; Xiang et al., 2008).

Although it is known that GABA\textsubscript{A}R modulators inhibit L-VGCCs, there have been no studies to assess the selective effects of these drugs on the neuronal L-VGCC \(\alpha_1\) subtypes, Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3. Using the whole-cell voltage-clamp technique, the current studies investigated the effects of BDZs (diazepam, flurazepam, and desalkylflurazepam),
allopregnanolone, pentobarbital, and ethanol on recombinant L-VGCCs containing the neuronal L-type α₁ subunit, Caᵥ₁.2 or Caᵥ₁.3, along with β₃ and α₂δ-1 subunits. We found that positive allosteric GABAₐR modulators reversibly inhibited both Caᵥ₁.2- and Caᵥ₁.3-containing L-VGCCs in a state-dependent manner. The order of potency was allopregnanolone > BDZs > pentobarbital > ethanol, with BDZs displaying 2- to 6-fold less potency than the benzothiazepine (BTZ), diltiazem. Previous studies reported that Caᵥ₁.3 channels were less sensitive to inhibition by dihydropyridines (DHPs), phenylalkylamines (PAAs), and BTZs than Caᵥ₁.2 channels (Koschak et al., 2001; Xu & Lipscombe, 2001; Tarabova et al., 2007). The structural similarities among BDZs and BTZs (see Fig. 1) suggested the possibility that L-VGCCs might also display differential sensitivity to GABAₐR modulators. Compared to Caᵥ₁.2, recombinant Caᵥ₁.3 channels were 1.5-fold less sensitive to inhibition by flurazepam, 3-fold less sensitive to inhibition by pentobarbital, and 3-fold more sensitive to inhibition by diltiazem. Studies using Caᵥ₁.2 mutants revealed that pentobarbital potency was reduced 3.5-fold by mutation of the high-affinity DHP binding site (T1039Y and Q1043M). Despite the aforementioned structural similarities, diltiazem did not compete with diazepam inhibition, and mutation of an amino acid within the BTZ binding site (I1150A) had no effect on diazepam potency. Based on their concentration-dependent effects, inhibition of L-VGCCs by pentobarbital and ethanol may contribute to their acute clinical actions. A direct interaction of pentobarbital and ethanol with L-VGCCs may also contribute to the adaptive upregulation of L-VGCC function following chronic use of these non-selective CNS depressants.
3.3 Materials and Methods

3.3.1 Cell culture and transient transfection

SV40 large T-antigen stably transfected HEK293 (HEK293T) cells were utilized for transient expression of L-VGCCs. Cells were maintained at 37ºC and 5% CO₂ in Dulbecco’s Modified Eagle’s Medium with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown to ~80% confluency and transfected with α₁ (Ca,1.2 or Ca,1.3), β₃, α₂δ-1, and monomeric red fluorescent protein (mRFP) plasmids (1:1:1:0.5 µg, respectively) using Lipofectamine™ 2000 reagent (Invitrogen). One day after transfection, cells were trypsinized and replated on 35 mm culture dishes prior to electrophysiological recording 2 – 3 days post-transfection. Except for experiments with mutated Ca,1.2, all experiments utilized mouse neuronal Ca,1.2 (Helton et al., 2005), rat neuronal Ca,1.3 (+exon11, Δexon32, +exon42a, Xu & Lipscombe, 2001), rat neuronal β₃, and rat neuronal α₂δ-1 (Lin et al., 2004) subunit plasmids, which were generously provided by Dr. Diane Lipscombe (Brown Univ., Providence, RI). The mRFP plasmid was generously provided by Dr. Zi-Jian Xie (Univ. of Toledo College of Medicine, Toledo, OH). A DHP binding site (T1039Y and Q1043M) mutant Ca,1.2 (Hockerman et al., 2000) and its wild-type form from rat brain (Snutch et al., 1991) were generously provided by Dr. Gregory Hockerman (Purdue Univ., West Lafayette, IN).

3.3.2 Site-Directed Mutagenesis

A mutation previously shown to be important for diltiazem potency, I1150A (Hockerman et al., 2000), was inserted into rat brain wild-type Ca,1.2 using the
QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) and the following primers:

5’-GTGGAGATCTCCATCTTCTTCGCGATCTACATCATCATCATATGCCC-3’

5’-GGCAATGATGATGATGTAGATCGCAGAAGATGGAGATCTC-3’

(I1150A mutation in bold). The manufacturer’s protocol was followed with a few modifications. Briefly, 50 ng of Cav1.2 plasmid template was used in PCR with the following parameters: 1 cycle: 95°C, 30 s; 18 cycles: 95°C, 50 s, 60°C, 50 s, 68°C, 18 min (1.5 min/kb); 1 cycle: 68°C, 7 min. Following Dpn I treatment, the entire PCR product was ethanol precipitated, resuspended in 5 μL ddH2O, and transformed into XL10-Gold® ultracompetent cells. Transformed cells were plated on LB agar dishes containing 100 μg/mL ampicillin for >16 hrs at 37°C. Single colonies were grown for >16 hrs in LB broth containing 100 μg/mL ampicillin, then plasmid DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1). Mutation was confirmed by sequencing (Eurofins MWG Operon, Huntsville, AL). A clone positive for the mutation was grown in LB broth containing 100 μg/mL ampicillin, and the plasmid DNA was purified using QIAGEN Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA).

3.3.3 Recording solutions

The external solution (300 mOsm) contained 135 mM cholineCl, 1 mM MgCl2, 5 mM BaCl2, and 10 mM HEPES, adjusted to pH 7.4 with TEAOH. As indicated in the results, a few experiments used 2 mM Ca2+ as the charge carrier instead of 5 mM Ba2+. Glass recording pipettes were pulled from micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) to a resistance of 2-5 MΩ. To facilitate seal formation, recording pipette tips were filled with a lower osmolarity internal solution (270 mOsm)
lacking ATP. Pipettes were then back-filled with internal solution (285 mOsm) containing: 135 mM CsCl, 4 mM MgCl₂, 4 mM ATP, 10 mM HEPES, 10 mM EGTA, and 1 mM EDTA, adjusted to pH 7.2 with TEAOH.

3.3.4 Whole-cell voltage-clamp electrophysiology

Red-fluorescing cells were visualized on an Olympus IX51 fluorescent microscope. Whole-cell currents were recorded with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA). Data was sampled at 2 – 10 kHz, low pass-filtered at 1 kHz, and analyzed using p Clamp v9.2 (Molecular Devices). Following pipette and membrane capacitance compensation the series resistance was corrected by 80-90% with a 20-100 µs lag time. Cells with a series resistance greater than 20 MΩ were not used for analysis. After whole-cell break-in, Ba²⁺ currents were elicited in voltage-clamp mode by stepping from a holding potential (V_h) of -80 mV to test potentials (V_t) of 0 mV (Ca_v1.2) or -25 mV (Ca_v1.3) for 200 ms. Current rundown, plotted as total charge transfer elicited at 10 s intervals (or higher frequencies, as indicated), was fitted with a one- or two-phase exponential decay curve. Recordings using this protocol were not leak subtracted.

Drugs were applied by gravity flow and washed out rapidly by external solution applied with a separate pipette. Percent inhibition was calculated with the equation: % inhibition = (1 – [I_{actual}/I_{expected}]) × 100, where I_{actual} was the actual current remaining during steady-state inhibition by the drug, and I_{expected} was the expected current remaining at the same time-point based on the exponential decay fit of the current rundown. Concentration-response curves were fitted with a four-parameter logistic equation: % inhibition = % min + (% max – % min)/(1 + 10^{(log IC_{50} – log [drug]) × nH}), where % min represented percent inhibition by vehicle, % max represented maximal percent inhibition
by the drug, IC$_{50}$ was the drug concentration which yielded half-maximal inhibition, and $n_H$ was the Hill slope. % min was constrained to zero, since average inhibition by vehicle was not significantly different than zero. Current decay was measured as the ratio of current remaining after a 200 ms depolarization relative to peak current, which was defined as the r200 value, similar to previous studies (Cai et al., 1997).

For voltage-dependent activation and inactivation protocols, data obtained from each voltage step was leak-subtracted online by removing the sum of the current obtained from four consecutive waveforms of 1/4 amplitude and opposite polarity to the test potentials (a P/4 protocol). Voltage-dependence of activation was determined using either 5 mM Ba$^{2+}$ or 2 mM Ca$^{2+}$ as charge carriers. Channels were activated with 200 ms voltage steps from $V_h = -80$ mV to various test potentials, $V_t = -75$ mV to $+40$ mV (Cav1.3) or $V_t = -50$ mV to $+65$ mV (Cav1.2) in 5 mV steps. Peak current plotted against the test potential was fitted with the equation: $I = G_{max}(V_t - V_{rev})(1 + \exp[(V_{50} - V_t)/k])$, where $G_{max}$ was the maximal conductance of the cell, $V_{rev}$ was the reversal potential, $V_{50}$ was the potential for half-maximal activation, and $k$ was the slope factor of activation. As the test potential approached $V_{rev}$, currents deviated from the linear relationship predicted by this equation. Therefore, currents obtained at potentials more positive than 25 mV were excluded from fitting analysis.

The voltage-dependence of L-VGCC steady-state inactivation was determined by stepping to various conditioning pulse potentials for 1.5 s, preceded by a control pulse to -25 mV (Cav1.3) or 0 mV (Cav1.2) and followed by a test pulse to -25 mV (Cav1.3) or 0 mV (Cav1.2). The current elicited by the control pulse was used to control for current rundown. Inactivation curves were plotted as normalized peak currents (test peak/control
peak) versus the conditioning pulse potential and fit with the Boltzmann equation: 
$$I_n = I_{n\text{(min)}} + \frac{(I_{n\text{(max)}} - I_{n\text{(min)}})}{1 + \exp\left(\frac{V_{50} - V_t}{k}\right)}$$
where $I_n$ was the normalized peak current, $I_{n\text{(min)}}$ was minimal normalized current, $I_{n\text{(max)}}$ was maximal normalized current, $V_{50}$ was the potential for half-maximal inactivation, and $k$ was the slope factor of inactivation.

Drug effects on steady-inactivation were tested by directly incubating cells in external solution containing the drug for at least 5 min prior to running the inactivation protocol.

### 3.3.5 Drugs

The three BDZs tested were the prototype diazepam, the relatively water soluble flurazepam, and its major bioactive metabolite in rat and man, desalkylflurazepam (Lau et al., 1987). Flumazenil, a competitive BDZ antagonist, was also tested. Stocks of nimodipine, diazepam, desalkylflurazepam, flumazenil, and allopregnanolone were made in dimethylsulfoxide (DMSO). Stocks of (+)-cis-diltiazem, flurazepam (pH 5.8), and racemic pentobarbital were made in dH2O. Drug stocks were dissolved into external solution by vortexing. Drugs dissolved in DMSO were diluted at least 1:1000, except 300 mM diazepam and desalkylflurazepam stocks were diluted 1:300 to achieve the final concentration of 1 mM. DMSO diluted 1:1000 or 1:300 in external solution was used as a vehicle control for some experiments and resulted in negligible inhibition of currents (mean inhibition typically < 5% in each experiment). Absolute ethanol (empirical density of 0.75 g/mL) was dissolved directly into external solution. Flurazepam was generously provided by the NIDA Drug Supply Program. Flumazenil and desalkylflurazepam were obtained from Roche, Nutley, NJ. Allopregnanolone was obtained from Tocris Bioscience, Ellisville, MO. Ethanol was obtained from Pharmco-AAPER, Shelbyville, KY. All other drugs were obtained from Sigma-Aldrich, St. Louis, MO.
3.3.6 Statistical analyses

All curve fits were made using GraphPad Prism v5 (GraphPad Software, Inc., La Jolla, CA). Except for curve fit parameters, all data are reported as mean ± S.E.M. Curve fit parameters (i.e., IC\textsubscript{50}, Hill slope, V\textsubscript{50}, and slope factor \(k\)) are reported as the best fit value to the experimental data set with the associated 95% confidence interval from a Levenberg-Marquardt least squares fitting algorithm. Non-overlapping confidence intervals between values were accepted as significant differences (\(p < 0.05\)). A two-tailed Student’s \(t\)-test was used to compare percent inhibition by select drug concentrations between two experimental conditions. Threshold drug concentrations were defined as the lowest concentration to yield significant inhibition measured by repeated measures one-way ANOVA with post-hoc comparison by Dunnett’s test. Mean r200 values were analyzed by repeated measures one-way ANOVA with post-hoc comparison by Dunnett’s test or by a paired \(t\)-test. A p-value of 0.05 or less was considered significant.
3.4 Results

3.4.1 Functional Characterization of Recombinant L-VGCCs

As a confirmation that the transient transfection of HEK293T cells resulted in functional L-VGCCs, the voltage-dependence of activation was evaluated for both Ca\textsubscript{v}1.2- and Ca\textsubscript{v}1.3-containing L-VGCCs and was similar to previous reports for these channels (Koschak et al., 2001; Xu & Lipscombe, 2001). Fig. 2A shows Ca\textsubscript{v}1.3 and Ca\textsubscript{v}1.2 L-VGCC current-voltage responses, with peak 5 mM Ba\textsuperscript{2+} or 2 mM Ca\textsuperscript{2+} currents (pA) normalized to cell capacitance (pF). Notably, activation of Ca\textsubscript{v}1.3 channels occurred at more negative potentials than Ca\textsubscript{v}1.2 channels. Half-maximal inactivation also occurred at more negative potentials in Ca\textsubscript{v}1.3 compared to Ca\textsubscript{v}1.2 channels (see Fig. 6 and Table 3). As an additional test of channel integrity and appropriate pharmacologic response, channels were inhibited by the DHP, nimodipine in a concentration-dependent manner. As shown in Fig. 2B, Ca\textsubscript{v}1.3 channels were less sensitive to inhibition by nimodipine than Ca\textsubscript{v}1.2 channels, consistent with previous reports of recombinant L-VGCC inhibition by DHPs (Koschak et al., 2001; Xu & Lipscombe, 2001).

3.4.2 GABA\textsubscript{A}R Modulators Reversibly Inhibit Recombinant L-VGCCs

In control cells transfected with Ca\textsubscript{v}1.3 L-VGCCs (n = 4) in which no drug was applied, the average percent current remaining 2, 5, and 10 min from the start of recording was 71 ± 11%, 57 ± 13%, and 42 ± 9%, respectively. This extent of rundown was similar to that of Ca\textsubscript{v}1.2 L-VGCCs in this study and as reported previously (Kepplinger et al., 2000). There was no apparent correlation between the extent of rundown in different cells and drug concentration-response, suggesting that the mechanisms causing the rundown phenomenon were not a significant factor in modifying
L-VGCC inhibition by GABA_\(_A\)_R modulators. As seen in Fig. 3A, 5 mM Ba\(^{2+}\) currents through Ca\(_{\alpha 1.3}\) L-VGCCs were inhibited in a concentration-dependent manner by diazepam. Inhibition by all drugs tested showed at least partial current recovery upon washout. Figs. 3B and 3C illustrate that inhibition of Ca\(_{\alpha 1.2}\) and Ca\(_{\alpha 1.3}\) L-VGCCs, respectively, was rapid for most GABA_\(_A\)_R modulators tested. For BDZs, pentobarbital, and ethanol, a maximal response was typically obtained by the 3\(^{rd}\) activating pulse after drug application. Inhibition was slower for allopregnanolone and diltiazem, generally requiring at least 4 activation pulses to reach maximal inhibition. For allopregnanolone inhibition was slower in Ca\(_{\alpha 1.2}\) channels, and for diltiazem inhibition was slower in Ca\(_{\alpha 1.3}\) channels. Inhibition of Ca\(_{\alpha 1.3}\) channels by desalkylflurazepam was similar when 2 mM Ca\(^{2+}\) was used as the charge carrier (data not shown).

Figs. 4B and C show the concentration-response relationship of diltiazem and all GABA_\(_A\)_R modulators tested in Ca\(_{\alpha 1.2}\) and Ca\(_{\alpha 1.3}\) L-VGCCs, respectively. Threshold concentrations, IC\(_{50}\) values, Hill slopes, and numbers of cells tested are reported in Table 1. BDZs had relatively similar potencies, except Ca\(_{\alpha 1.3}\) channels were 1.5-fold less sensitive to flurazepam than Ca\(_{\alpha 1.2}\) channels (p < 0.05). The BDZs were about 2- to 6-fold less potent than diltiazem, with Ca\(_{\alpha 1.3}\) channels displaying 3-fold greater sensitivity to diltiazem than Ca\(_{\alpha 1.2}\) channels (p < 0.05). The BDZ receptor competitive antagonist, flumazenil had no effect to inhibit Ca\(_{\alpha 1.2}\) or Ca\(_{\alpha 1.3}\) channels at concentrations up to 300 µM (data not shown). Flumazenil (100 – 300 µM) was unable to antagonize diazepam inhibition of Ca\(_{\alpha 1.2}\) channels (Table 1). Interestingly, co-application with 100 µM flumazenil resulted in a 1.8-fold reduction in desalkylflurazepam potency in Ca\(_{\alpha 1.3}\) channels (Table 1). Although allopregnanolone was the most potent GABA_\(_A\)_R modulator
tested (about 6-fold more potent than BDZs), it incompletely inhibited L-VGCCs and was less efficacious at inhibiting Cav1.3 than Cav1.2 channels (29% versus 63% maximal response, respectively). Pentobarbital was about 7- to 21-fold less potent than BDZs. Cav1.3 channels were 3-fold less sensitive to pentobarbital than Cav1.2 channels (p < 0.05). Ethanol was the least potent drug tested; about 4,500-fold less potent than BDZs.

3.4.3 GABA<sub>A</sub>R Receptor Modulators Enhance L-VGCC Current Decay

Inhibition by various L-VGCC antagonists is known to depend on the state of the channel (Lee & Tsien, 1983; Bean, 1984; Uehara & Hume, 1985; Cai et al., 1997; Hockerman et al., 1997). In particular, antagonists can stabilize the inactive state or display enhanced binding during channel opening (Hockerman et al., 1997), resulting in increased current decay during prolonged depolarization. Fig. 5A illustrates that the decay of Cav1.3 Ba<sup>2+</sup> current is enhanced by diazepam. Fig. 5B shows that the ratio of residual current at 200 ms to peak current (r200) was reduced in a concentration-dependent manner by diazepam. Table 2 summarizes L-VGCC r200 values in the presence and absence of diltiazem and GABA<sub>A</sub>R modulators at approximately equipotent concentrations. A significant decrease in the r200 value of both L-VGCCs was observed for all GABA<sub>A</sub>R modulators, except for flurazepam at Cav1.3 channels and ethanol at Cav1.2 channels, due to variability and the low numbers of cells tested. By comparison, diltiazem at its IC<sub>50</sub> concentration resulted in substantially less enhancement of L-VGCC current decay.

3.4.4 GABA<sub>A</sub>R Modulators Induce a Negative Shift in L-VGCC Steady-State Inactivation
The voltage-dependence of L-VGCC steady-state inactivation was measured as described in the methods in the presence and absence of drug. As illustrated in Fig. 6A, there was a significant shift in the voltage-dependence of Ca,1.2 L-VGCC steady-state inactivation towards more negative potentials in the presence of approximately equipotent concentrations of diazepam (60 μM) and pentobarbital (300 μM), but not allopregnanolone (30 μM) or ethanol (300 mM). The potentials of half-maximal inactivation (V_{50}) in the presence and absence of diltiazem and GABA<sub>A</sub>R modulators are reported in Table 3. Notably, there was a concentration dependent effect of diazepam to shift Ca,1.2 inactivation towards more negative potentials, which was significant at 60 and 100 μM. 100 μM desalkylflurazepam caused a significant shift in the Ca,1.2 inactivation curve, but was smaller in magnitude than the shift by 100 μM diazepam. A similar result was obtained with 30 μM diltiazem. Consistent with data obtained from 0.1 Hz activation, flumazenil (300 μM) did not shift Ca,1.2 inactivation and was unable to antagonize the shift by 100 μM diazepam.

Fig. 6B shows a drug-induced shift of Ca,1.3 steady-state inactivation towards more negative potentials in the presence of approximately equipotent concentrations of diazepam (60 μM), allopregnanolone (30 μM), pentobarbital (1 mM), and ethanol (300 mM), although ethanol was weakly effective relative to the other drugs. As shown in Table 3, 100 μM desalkylflurazepam caused a significant shift in the Ca,1.3 inactivation curve, but was of smaller magnitude than the shift by 100 μM diazepam. Diltiazem at its IC<sub>50</sub> concentration of 10 μM was completely ineffective to shift the Ca,1.3 inactivation curve.
3.4.5 Inhibition of Cav1.2 Channels by Desalkylflurazepam is State- and Frequency-Dependent

To test the hypothesis that BDZ potency is enhanced by channel inactivation, the inhibitory action of desalkylflurazepam was measured on channels activated from a more depolarized holding potential resulting in a greater extent of channels in the inactive state. As illustrated in Fig. 7A, desalkylflurazepam inhibited Cav1.2 channels 2-fold more potently when channels were activated from a more depolarized holding potential, with a significantly greater degree of inhibition occurring at 30 and 100 µM desalkylflurazepam. For Cav1.3 channels, which inactivate at more negative membrane potentials, a holding potential of -60 mV was used. As illustrated in Fig. 7B, desalkylflurazepam was similarly potent when Cav1.3 channels were activated from a more depolarized holding potential, and the degree of inhibition was not significantly different at any concentration at or above threshold.

To test whether BDZ potency might be similarly enhanced by the frequency of channel activation, desalkylflurazepam potency was tested on L-VGCCs activated at 1 Hz. As illustrated in Fig. 7C, desalkylflurazepam inhibited Cav1.2 L-VGCCs 2-fold more potently when channels were activated at 1 Hz compared to 0.1 Hz, with a significantly greater degree of inhibition occurring at 10, 30, and 100 µM desalkylflurazepam. As illustrated in Fig. 7D, inhibition of Cav1.3 L-VGCCs by desalkylflurazepam was not significantly different when channels were activated at 1 Hz compared to 0.1 Hz and showed no significant difference in inhibition at any concentration at or above threshold.

3.4.6 Two Amino Acids Important for DHP Potency Reduce Pentobarbital Potency
While the various classes of L-VGCC antagonists have unique binding sites, they also have overlapping amino acids that contribute to binding. Thus, it was of interest to see if single- and double-amino acid mutations already characterized to affect binding of specific L-VGCC antagonists, might also affect binding of GABA\(_A\)R modulators. Previous studies have found two amino acids in domain IIIS5 of Ca\(_{v1.2}\), T1039 and Q1043, which are critical for DHP affinity (Mitterdorfer et al., 1996; Hockerman et al., 2000). Mutation of these two amino acids thus creates a DHP insensitive Ca\(_{v1.2}\) (DHPI). As can be seen in Fig. 8A, half maximal inhibition of wild-type (WT) Ca\(_{v1.2}\) L-VGCCs occurred at about 0.1 \(\mu\)M nimodipine and maximal block at 1 \(\mu\)M (data represent inhibition at 0 mV test potential shown in Fig. 2B). In two cells transfected with DHPI L-VGCCs, application of 1 \(\mu\)M nimodipine resulted in negligible inhibition (5 ± 14%). Application of 10 \(\mu\)M nimodipine resulted in substantial inhibition (58 ± 3%), but was about 100-fold less potent than in WT, confirming that DHPI channels are resistant to DHPs.

As shown in Fig. 8B, DHPI L-VGCCs were significantly less sensitive to inhibition by pentobarbital compared to WT. Although this suggests that T1039 and Q1043 may play a role in pentobarbital binding to Ca\(_{v1.2}\), the 3.5-fold reduction in potency is quite small compared to the estimated 100-fold reduction in nimodipine potency. As shown in Figs. 8C and E, inhibition of Ca\(_{v1.2}\) channels by diazepam and ethanol, respectively was unaffected by mutation of the DHP binding site. Fig. 8D illustrates that DHPI channels were slightly more sensitive to allopregnanolone than WT Ca\(_{v1.2}\) channels, but only 10 \(\mu\)M allopregnanolone resulted in significantly more inhibition.
It should be noted that the WT Ca\textsubscript{v}1.2 used for comparison of nimodipine, allopregnanolone, and ethanol inhibition was a mouse brain clone, whereas the DHPI mutant was created from a rat brain Ca\textsubscript{v}1.2 clone. Thus, the small difference in inhibition by allopregnanolone might be due to species differences between the two channels. Additional controls in rat WT Ca\textsubscript{v}1.2 were obtained for diazepam, pentobarbital, and diltiazem (see below).

3.4.7 A Single Amino Mutation that Affects Diltiazem Potency does not Affect Diazepam Potency

Based on the structural similarity between BDZs and BTZs, it was hypothesized that they might share a similar binding site at L-VGCCs. A single amino acid mutation, I1150A, was previously shown to affect diltiazem potency, but not other classes of L-VGCC antagonists (Hockerman et al., 2000). As shown in Fig. 9A, mutation of I1150 in rat Ca\textsubscript{v}1.2 resulted in a significant 2-fold decrease in diltiazem potency, with a significantly lower degree of inhibition by 10, 30, and 300 μM diltiazem. However, as shown in Fig. 9B, diazepam potency was not significantly affected by the mutation, with no difference in the degree of inhibition by any concentration tested. In order to confirm that diazepam likely does not share a binding site with diltiazem, inhibition of WT Ca\textsubscript{v}1.2 channels by diazepam was tested in the presence of an IC\textsubscript{50} concentration of diltiazem (30 μM). As seen in Fig. 9C, the presence of diltiazem did not significantly affect inhibition at any diazepam concentration tested.
3.5 Discussion

3.5.1 GABA<sub>A</sub>R Modulator Pharmacology at L-VGCCs: Comparison to GABA<sub>A</sub>Rs

Prior studies investigated inhibition of native L-VGCCs by GABA<sub>A</sub>R modulators either in non-neuronal or neuronal cells that also contain GABA<sub>A</sub>Rs and multiple types of calcium channels, making it difficult to ascertain the selective effects of these drugs on the neuronal L-VGCC subtypes, Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. The current studies analyzed the inhibitory action of GABA<sub>A</sub>R modulators on recombinant neuronal Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 L-VGCCs assembled with β<sub>3</sub> and α<sub>2</sub>δ subunits. Inhibition by modulators was not due to an indirect action at the GABA<sub>A</sub>R, since HEK293T cells do not express functional GABA<sub>A</sub>Rs (Davies et al., 2000).

The BDZs were about 3 orders of magnitude less potent (Thomas et al., 1997), allopregnanolone about 1.5 orders of magnitude less potent (Maitra & Reynolds, 1998), pentobarbital about 1 order of magnitude less potent (Thomas et al., 1997), and ethanol slightly less than 1 order of magnitude less potent (Wallner et al., 2003) at L-VGCCs than at GABA<sub>A</sub>Rs. Though diazepam was several-fold more potent at GABA<sub>A</sub>Rs than flurazepam (Thomas et al., 1997), they displayed similar potency at L-VGCCs. The competitive BDZ antagonist, flumazenil did not inhibit L-VGCCs, yet it antagonized BDZ inhibition of Ca<sub>v</sub>1.3, but not Ca<sub>v</sub>1.2 channels. This suggests that the Ca<sub>v</sub>1.3 α<sub>1</sub> subunit may have a flumazenil binding site analogous to GABA<sub>A</sub>Rs.

3.5.2 GABA<sub>A</sub>R Modulator Manner of L-VGCC Inhibition: Comparison to L-VGCC Antagonists

Maximal inhibition by L-VGCC antagonists is dependent upon channel use, with PAAs displaying high levels of use-dependence, followed by BTZs, then DHPs, which
display little use-dependence (Lee & Tsien, 1983; Uehara & Hume, 1985). Although the current studies were not designed to assess use-dependence, maximal inhibition by BDZs, pentobarbital, and ethanol consistently required fewer activating pulses compared to inhibition by allopregnanolone and diltiazem (Fig. 3B and C). While these time-course differences may be due to differential kinetics of drug binding and/or drug action, it could also suggest that the former compounds may not be as dependent on channel opening to bind and can better access their L-VGCC binding sites when channels are in a resting confirmation. Alternatively, antagonists that slow recovery from inactivation may result in an accumulation of channels in the inactive state during repeated activation and have greater effect at higher frequencies of activation (Uehara & Hume, 1985). Although inhibition of Ca\textsubscript{v}1.2 channels by desalkylflurazepam was slightly enhanced in a frequency-dependent manner (Fig. 7), it is possible that these modulators have a limited ability to slow recovery from inactivation relative to BTZs and PAAs (Uehara & Hume, 1985).

GABA\textsubscript{A}R modulators enhance L-VGCC current decay, similar to other L-VGCC antagonists (Lee & Tsien, 1983; Cai et al., 1997). This could represent open channel block and/or stabilization of the inactive state. In support of the latter possibility, desalkylflurazepam potency was increased at Ca\textsubscript{v}1.2 channels by recording paradigms that enhance channel inactivation (Fig. 7). Additionally, GABA\textsubscript{A}R modulators induced a significant negative shift in the Ca\textsubscript{v}1.3 steady-state inactivation curve, and BDZs and pentobarbital also induced a significant negative shift in the Ca\textsubscript{v}1.2 steady-state inactivation curves (Fig. 6 and Table 3). Altogether, the data provide evidence for state-
dependent block by GABA<sub>K</sub> modulators, preferentially interacting with inactive and possibly open states.

3.5.3 Ca<sub>1.2</sub> and Ca<sub>1.3</sub> L-VGCC Differential Sensitivity to GABA<sub>K</sub> Modulators

Recombinant Ca<sub>1.3</sub> channels have been shown to be less sensitive to inhibition by DHPs than Ca<sub>1.2</sub> channels (Koschak et al., 2001; Xu & Lipscombe, 2001). Native Ca<sub>1.3</sub> channels in auditory hair cells were also found to be less sensitive to inhibition by BTZs and PAAs, though this effect may be cell-type specific (Tarabova et al., 2007). Indeed, recombinant Ca<sub>1.3</sub> channels were 3-fold more sensitive to diltiazem inhibition than Ca<sub>1.2</sub> channels. For the GABA<sub>K</sub> modulators, Ca<sub>1.3</sub> channels were 1.5-fold less sensitive to flurazepam and 3-fold less sensitive to pentobarbital than Ca<sub>1.2</sub> channels. Diazepam, desalkylflurazepam, allopregnanolone, and ethanol displayed no significant differential potency at L-VGCCs. However, since desalkylflurazepam inhibition of Ca<sub>1.2</sub>, but not Ca<sub>1.3</sub> channels was increased by protocols that enhance inactivation (Fig. 7), it is possible that Ca<sub>1.2</sub> L-VGCCs may display greater sensitivity to the latter compounds in a state-dependent manner as proposed previously for DHPs (Koschak et al., 2001). Whether this differential state-dependent effect may be related to the greater extent of inactivation observed for Ca<sub>1.2</sub> than Ca<sub>1.3</sub> channels (Table 2) or a more specific BDZ interaction with the channels is currently unknown. The differential potency of these drugs at Ca<sub>1.2</sub> and Ca<sub>1.3</sub> channels may allow them to selectively modify distinct L-VGCC subunit-mediated neuronal functions. In particular, reduced Ca<sub>1.2</sub> channel function impairs hippocampal spatial memory, whereas reduced Ca<sub>1.3</sub> channel function affects neuronal firing patterns and may be protective in reducing age-
related neuronal degeneration, as occurs in Parkinson’s disease (Striessnig & Koschak, 2008).

3.5.4 GABA<sub>A</sub>R Modulator Site of Action

Organic L-VGCC antagonists have unique binding sites on the Ca<sub>v</sub>1.2 subunit that share a number of overlapping amino acids (Hockerman et al., 1997). Thus, it was of interest to see if amino acid mutations that are known to affect binding of specific L-VGCC antagonists could similarly reduce inhibition by GABA<sub>A</sub>R modulators. Two Ca<sub>v</sub>1.2 mutants were used in the current studies: a double amino acid mutation, T1039Y and Q1043M, that reduces DHP potency (Mitterdorfer et al., 1996; Hockerman et al., 2000), and a single mutation, I1150A, that reduces diltiazem potency (Hockerman et al., 2000). Neither mutation affected diazepam potency, suggesting that BDZs may have an L-VGCC binding site distinct from DHPs and BTZs. Additionally, diltiazem did not competitively interfere with diazepam inhibition of Ca<sub>v</sub>1.2 channels and these compounds were somewhat dissimilar in their manner of block in terms of state- and use-dependence. Thus, despite the structural similarity between BDZs and BTZs and their relatively similar potencies, the evidence suggests that these drugs act via distinct sites. Interestingly, pentobarbital potency was significantly reduced 3.5-fold by the DHP mutant, suggesting that the pentobarbital binding site on Ca<sub>v</sub>1.2 might overlap that of DHPs.

3.5.5 Clinical Relevance of L-VGCC Inhibition by GABA<sub>A</sub>R Modulators and Implications for Physical Dependence

Calcium influx through postsynaptic L-VGCCs is linked to neuronal activity and gene transcription, and L-VGCC inhibition has anxiolytic, antidepressant, and
anticonvulsant actions (Striessnig et al., 2006). Thus, inhibition of neuronal L-VGCCs could play a role in the acute clinical actions of GABAAR modulators. Further, chronic inhibition of L-VGCCs may lead to an adaptive upregulation of L-VGCC function during prolonged GABAAR modulator treatment and contribute to physical dependence (Dolin et al., 1987; Rabbani & Little, 1999; Xiang & Tietz, 2007; Xiang et al., 2008).

Free plasma and cerebrospinal fluid concentrations of BDZs, barbiturates, and ethanol exist in a ~1:1 ratio (Richards, 1972; Hallstrom et al., 1980; Harris et al., 2008). Taking into account extensive binding (>97%) of diazepam and its active metabolite, desmethyldiazepam to plasma proteins (Hallstrom et al., 1980; Divoll & Greenblatt, 1981), free plasma concentrations of about 20 – 60 nM are necessary for acute relief of convulsant activity (Rey et al., 1999) and about 50 – 100 nM to produce sedation (Lundgren, 1987). Due to rapid tolerance to the sedative effects of diazepam, higher concentrations up to 200 nM are achieved during chronic treatment for psychiatric therapy (Rutherford et al., 1978; Hallstrom et al., 1980; Greenblatt et al., 1981). Since the threshold concentration of BDZs to significantly inhibit recombinant L-VGCCs was about 10 μM, inhibition of neuronal L-VGCCs is unlikely to contribute to BDZ clinical actions. However, studies in hippocampal cultures revealed that 1 μM flurazepam significantly inhibited VGCC current in a use-dependent manner (Xiang et al., 2008), suggesting that greater potency can be observed in neuronal systems. The threshold concentration of allopregnanolone to inhibit L-VGCCs was 3 μM, which is much greater than the 5 nM concentration estimated to be produced from progesterone in the brain (Uzunova et al., 1998). The threshold concentration of pentobarbital to inhibit L-VGCCs was 100 μM, within the 200 μM range required to induce anesthesia (Richards, 1972).
Unlike pentobarbital, phenobarbital has anticonvulsant actions with minimal sedative effects at 20 to 90 μM (Schulz & Macdonald, 1981). It would be of interest to see if phenobarbital inhibits L-VGCCs at concentrations required for anticonvulsant activity, or if L-VGCC inhibition is more likely to pertain to the anesthetic effects of barbiturates (ffrench-Mullen et al., 1993). The threshold concentration of ethanol to inhibit L-VGCCs was 30 mM, within range of intoxicating ethanol concentrations, 10 to 100 mM (Harris et al., 2008). Thus, inhibition of L-VGCCs may attribute to ethanol’s intoxicating effects, including mood alteration and memory impairment.

The enhancement of neuronal L-VGCC-mediated Ca\(^{2+}\) influx observed following chronic BDZ exposure is unlikely to be due to a direct effect at L-VGCCs, since this upregulation occurs with BDZ concentrations as low as 0.3 μM \textit{in vitro} (Katsura et al., 2007) and \sim 1 μM measured in rat brain homogenates during chronic BDZ treatment \textit{in vivo} (Xiang et al., 2008). Thus, persistent allosteric enhancement of GABA\(_A\)Rs seems the most plausible mechanism associated with L-VGCC regulation during chronic BDZ administration, since L-VGCC upregulation in cortical cultures can be prevented by nanomolar concentrations of the BDZ competitive antagonist, flumazenil (Katsura et al., 2007). In addition, a bicarbonate-dependent GABA\(_A\)R-mediated depolarization that arises during BDZ withdrawal (Zeng & Tietz, 2000) may contribute to the hyperexcitable state (Van Sickle et al., 2004), which could exacerbate the downstream Ca\(^{2+}\)-mediated effects of L-VGCC upregulation. As with other GABA\(_A\)R modulators, withdrawal from chronic exposure to allopregnanolone is associated with a withdrawal syndrome characterized by anxiety and increased seizure susceptibility (Smith, 2002). However, a role for L-VGCC upregulation in this phenomenon has not been established. Inhibition of L-VGCCs may
contribute to the upregulation of L-VGCC function following chronic exposure to barbiturates (Rabbani & Little, 1999), as well as ethanol (Messing et al., 1986; Katsura et al., 2006). Collectively, these findings may provide a molecular basis for the adaptive changes that contribute to drug withdrawal symptoms and physical dependence on GABA_4R modulators.
3.6 Footnotes

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3.8 References


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3.9 Figures and Tables

Figure 3.1

![Chemical structures]

- Diazepam
- Flurazepam
- Desalkyflurazepam
- Pentobarbital
- Allopregnanolone
- Diltiazem
- Nimodipine
Figure 3.1

Chemical structures of the compounds used in the current studies (with the exception of ethanol). Note the similarity in the core structure of the BTZ, diltiazem to the BDZs. Structures were created using CS ChemDraw software, version 5.0 (CambridgeSoft Corporation, Cambridge, MA).
Figure 3.2

Functional expression of recombinant L-VGCCs in HEK293T cells. A, the average current density (picoamperes/picofarads, pA/pF) of recombinant Ca_{v}1.3 (squares) and Ca_{v}1.2 (circles) L-VGCCs expressed with β_3 and α_2δ-1 subunits in HEK293T cells is shown plotted against the membrane test potential using either 5 mM Ba^{2+} (open symbols) or 2 mM Ca^{2+} (filled symbols) as the charge carrier. Using Ba^{2+} as the charge carrier, potentials of half-maximal activation (V_{50}) were -41.3 ± 2.1 mV (n = 15) and -12.9 ± 3.8 mV (n = 6) for Ca_{v}1.3 and Ca_{v}1.2 channels, respectively. Using Ca^{2+} as the charge carrier, activation V_{50} values were -35.3 ± 4.3 mV (n = 6) and -12.5 ± 5.1 mV (n = 8) for Ca_{v}1.3 and Ca_{v}1.2 channels, respectively. B, average peak Ba^{2+} currents through Ca_{v}1.3 channels in the absence (open squares, n = 3) and presence of 1 μM (half-filled squares, n = 3) or 10 μM nimodipine (filled squares, n = 3) and Ca_{v}1.2 channels in the absence (open circles, n = 2) and presence of 0.1 μM (half-filled circles, n = 2) or 1 μM nimodipine (filled circles, n = 2).
Figure 3.3
Figure 3.3

A, concentration-dependent inhibition of Ca$_{v}$.1.3 Ba$^{2+}$ currents by diazepam. Ca$_{v}$.1.3 L-VGCCs were activated by 200 ms test pulses to $-25$ mV every 10 s from a holding potential of $-80$ mV. The total charge transfer is normalized to its initial value and plotted as a function of time post break-in. Diazepam inhibited Ca$_{v}$.1.3 currents in a concentration-dependent manner and was reversible upon washout. VEH represents 1:1000 DMSO. Exemplar current traces in the presence and absence of diazepam are shown above. Normalized, rundown-corrected time-courses of Ca$_{v}$.1.2 (B) and Ca$_{v}$.1.3 (C) L-VGCC inhibition by diltiazem (filled circles, 30 and 10 µM for Ca$_{v}$.1.2 and Ca$_{v}$.1.3, respectively), flurazepam (open triangles, 30 µM), pentobarbital (half-filled diamonds, 0.3 and 1 mM for Ca$_{v}$.1.2 and Ca$_{v}$.1.3, respectively), ethanol (half-filled circles, 300 mM), and allopregnanolone (half-filled squares, 10 µM). Time point zero represents current just prior to drug application. Because the time-courses were taken from the middle of a concentration-response, current at time point zero had not in all cases completely recovered from the prior concentration of drug applied. Thus, inhibition that occurs during the first pulse after drug application (10 sec) does not accurately represent closed-channel block. Numbers of cells tested are the same as shown in Table 1.
Figure 3.4

Concentration-response curves representing inhibition of Ca\textsubscript{v1.2} (A) and Ca\textsubscript{v1.3} (B) L-VGCCs by GABA\textsubscript{A}R modulators. Diltiazem (filled circles) is shown for comparison. The rank order of potency amongst GABA\textsubscript{A}R modulators was allopregnanolone (half-filled squares) > BDZs > pentobarbital (half-filled diamonds) > ethanol (half-filled circles). The BDZs tested, diazepam (open circles), flurazepam (open triangles), and desalkylflurazepam (open squares), were about 2- to 6-fold less potent than diltiazem. Ca\textsubscript{v1.2} and Ca\textsubscript{v1.3} L-VGCCs had similar sensitivities to inhibition by diazepam, desalkylflurazepam, allopregnanolone, and ethanol. Ca\textsubscript{v1.3} channels were 1.5-fold less sensitive to flurazepam, 3-fold less sensitive to pentobarbital, and 3-fold more sensitive to diltiazem than Ca\textsubscript{v1.2} channels. VEH represents inhibition by vehicle. IC\textsubscript{50} values, Hill slopes, and numbers of cells tested are reported in Table 1.
**Figure 3.5**

Diazepam enhances L-VGCC current decay. A, current traces from the same cell shown in Fig. 3A are normalized to peak current. Diazepam enhanced Ca\textsubscript{v1.3} Ba\textsuperscript{2+} current decay during prolonged depolarization in a concentration-dependent manner. B, the ratio of residual current at 200 ms normalized to peak current (r200 value) is shown for various diazepam concentrations. 30 and 100 μM diazepam significantly reduced the r200 value compared to vehicle (n = 3). This effect was reversed upon washout. *, p < 0.05.
Figure 3.6

L-VGCC voltage-dependent steady-state inactivation curves in the presence and absence of GABA<sub>A</sub>R modulators. Normalized peak currents are plotted against the membrane potential of a 1.5 s conditioning pulse. A, the Ca<sub>v</sub>1.2 steady-state inactivation curve was significantly shifted towards more negative potentials by approximately equipotent concentrations of diazepam (open circles, 60 μM) and pentobarbital (half-filled diamonds, 300 μM), but not by allopregnanolone (half-filled squares, 30 μM) or ethanol (half-filled circles, 300 mM). The V<sub>50</sub> values (potential of half-maximal inactivation) in the presence and absence (filled circles) of each drug are plotted in the inset. Error bars were omitted for clarity in both A and B. *, p < 0.05. B, the Ca<sub>v</sub>1.3 steady-state inactivation curve was significantly shifted towards more negative potentials by approximately equipotent concentrations of diazepam (60 μM), allopregnanolone (30 μM), pentobarbital (1 mM), and ethanol (300 mM). The inactivation V<sub>50</sub> values are plotted in the inset. *, p < 0.05 relative to control V<sub>50</sub>. Inactivation V<sub>50</sub> values, slope factors, and numbers of cells tested are reported in Table 3.
Figure 3.7

A

B

C

D

% inhibition

IC_{50} (μM)

VEH

log [desalkyflurazepam] (M)

Ca_{1.2}

Ca_{1.3}

% inhibition

IC_{50} (μM)

VEH

log [desalkyflurazepam] (M)

Ca_{1.2}

Ca_{1.3}

% inhibition

IC_{50} (μM)

VEH

log [desalkyflurazepam] (M)

Ca_{1.2}

Ca_{1.3}

% inhibition

IC_{50} (μM)

VEH

log [desalkyflurazepam] (M)

Ca_{1.2}

Ca_{1.3}

% inhibition

IC_{50} (μM)

VEH

log [desalkyflurazepam] (M)

Ca_{1.2}

Ca_{1.3}
Figure 3.7

Cav1.2 L-VGCCs are inhibited by desalkylflurazepam in a state- (A and B) and frequency-dependent (C and D) manner. A, desalkylflurazepam (30 and 100 μM) inhibited significantly more current when Cav1.2 channels were activated from -45 mV (open circles) compared to activation from -80 mV (filled circles). The inset illustrates a corresponding significant 2-fold decrease in the desalkylflurazepam IC50 value at Vh = -45 mV (28 μM, 23 – 34 μM, n = 4) compared to Vh = -80 mV (55 μM, 45 – 68 μM, n = 3). VEH represents inhibition by vehicle. *, p < 0.05, **, p < 0.01. B, inhibition by desalkylflurazepam was not significantly different when Cav1.3 channels were activated from -60 mV (open circles) compared to -80 mV (filled circles). The inset illustrates the similarity in desalkylflurazepam IC50 values at Vh = -60 mV (32 μM, 13 – 80 μM, n = 4) and Vh = -80 mV (37 μM, 29 – 48 μM, n = 6). C, desalkylflurazepam (10, 30, and 100 μM) inhibited significantly more current when Cav1.2 channels were activated at 1 Hz (open circles) compared to activation at 0.1 Hz (filled circles). The inset illustrates a corresponding significant 2-fold decrease in the desalkylflurazepam IC50 value at 1 Hz (28 μM, 21 – 37 μM, n = 4) compared to 0.1 Hz activation (55 μM, 45 – 68 μM, n = 3). *, p < 0.05. D, inhibition by desalkylflurazepam was not significantly different when Cav1.3 L-VGCCs were activated at 1 Hz (open circles) compared to 0.1 Hz (filled circles). The inset illustrates the similarity in desalkylflurazepam IC50 values at 1 Hz (35 μM, 17 – 70 μM, n = 3) and 0.1 Hz activation (37 μM, 29 – 48 μM, n = 6).
Figure 3.8
Figure 3.8

DHPI contains mutations of two amino acids important for high-affinity DHP binding, T1039Y and Q1043M. The sensitivity of DHPI L-VGCCs to GABA_AR modulators was tested. A, DHPI channels were about 100-fold less sensitive to inhibition by nimodipine compared to WT Ca_{1.2} channels. B, DHPI channels were significantly less sensitive to inhibition by pentobarbital compared to WT Ca_{1.2} channels. The inset illustrates a significant 3.5-fold increase the pentobarbital IC_{50} value in DHPI (758 µM, 579 – 992 µM, \( n = 4 \)) compared to WT Ca_{1.2} channels (215 µM, 167 – 276 µM, \( n = 4 \)). VEH represents inhibition by vehicle. *, \( p < 0.05 \), **, \( p < 0.01 \), ***, \( p < 0.001 \). C, no difference in inhibition by diazepam was observed between WT and DHPI channels. Diazepam IC_{50} values for WT (41 µM, 31 – 55 µM, \( n = 4 \)) and DHPI channels (48 µM, 32 – 73 µM, \( n = 3 \)) are shown in the inset. D, DHPI channels were slightly more sensitive to inhibition by allopregnanolone than WT Ca_{1.2} channels, with 10 µM allopregnanolone inhibiting significantly more current through DHPI channels. As shown in the inset, there was no significant difference in allopregnanolone potency to inhibit in DHPI (3 µM, 2 – 5 µM, \( n = 3 \)) compared to WT channels (7 µM, 3 – 16 µM, \( n = 5 \)). E, no difference in inhibition by ethanol was observed between WT and DHPI channels. Ethanol IC_{50} values for WT (183 mM, 46 – 723 mM, \( n = 3 \)) and DHPI channels (157 mM, 117 – 216 mM, \( n = 3 \)) are shown in the inset.
Figure 3.9
Figure 3.9

A single amino acid mutation in Ca v1.2, I1150A reduces diltiazem, but not diazepam potency. A, as reportedly previously (Hockerman et al., 2000), the I1150A mutation reduced diltiazem potency, with significantly less inhibition by 10, 30 and 300 µM diltiazem. The inset illustrates a significant 2-fold increase the diltiazem IC 50 value in I1150A (53 µM, 40 – 70 µM, n = 6) compared to WT channels (26 µM, 21 – 32 µM, n = 4). VEH represents inhibition by vehicle. *, p < 0.05, **, p < 0.01. B, the I1150A mutation had no effect on diazepam potency. Diazepam IC 50 values for WT (41 µM, 31 – 55 µM, n = 4) and I1150A channels (50 µM, 32 – 77 µM, n = 4) are shown in the inset. C, as a confirmation that diazepam likely does not share a binding site with diltiazem, inhibition of WT Ca v1.2 channels by diazepam was assessed in the presence of an IC 50 concentration of diltiazem (30 µM). There was no significant difference in inhibition by diazepam at any concentration tested when compared to inhibition in the absence of diltiazem. The inset illustrates that there was no significant difference in diazepam potency in the presence (38 µM, 21 – 68 µM, n = 3) compared to the absence of diltiazem (49 µM, 38 – 64 µM, n = 4).
Table 3.1 Parameters for concentration-dependent inhibition of L-VGCCs by diltiazem and GABA\(_A\)R modulators

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca(_{\text{v}1.2}) Threshold</th>
<th>IC(_{50}^{a}) (\mu M)</th>
<th>IC(_{50}) 95% CI</th>
<th>Hill slope</th>
<th>(n)</th>
<th>Ca(_{\text{v}1.3}) Threshold</th>
<th>IC(_{50}^{a}) (\mu M)</th>
<th>IC(_{50}) 95% CI</th>
<th>Hill slope</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diltiazem</td>
<td>1</td>
<td>26*</td>
<td>21 – 32</td>
<td>1.0 ± 0.2</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>6 – 12</td>
<td>1.1 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>diazepam</td>
<td>30</td>
<td>49</td>
<td>38 – 64</td>
<td>1.5 ± 0.4</td>
<td>4</td>
<td>10</td>
<td>53</td>
<td>20 – 138</td>
<td>1.3 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>flurazepam</td>
<td>10</td>
<td>48*</td>
<td>40 – 57</td>
<td>1.2 ± 0.2</td>
<td>4</td>
<td>10</td>
<td>72</td>
<td>59 – 88</td>
<td>1.2 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>desalkylflurazepam</td>
<td>10</td>
<td>55</td>
<td>45 – 68</td>
<td>1.3 ± 0.3</td>
<td>3</td>
<td>10</td>
<td>37</td>
<td>29 – 48</td>
<td>1.1 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>diazepam +100 (\mu M) flumazenil</td>
<td>N.D.</td>
<td>66</td>
<td>56 – 77</td>
<td>1.5 ± 0.3</td>
<td>2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>diazepam +300 (\mu M) flumazenil</td>
<td>10</td>
<td>56</td>
<td>39 – 79</td>
<td>1.1 ± 0.4</td>
<td>5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>desalkylflurazepam +100 (\mu M) flumazenil</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>10</td>
<td>65(^{\dagger})</td>
<td>51 – 82</td>
<td>1.3 ± 0.3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>allopregnanolone</td>
<td>3</td>
<td>7</td>
<td>3 – 16</td>
<td>0.8 ± 0.4</td>
<td>5</td>
<td>10</td>
<td>11</td>
<td>4 – 30</td>
<td>1.0 ± 0.6</td>
<td>3</td>
</tr>
<tr>
<td>pentobarbital</td>
<td>100</td>
<td>336*</td>
<td>266 – 424</td>
<td>1.0 ± 0.2</td>
<td>3</td>
<td>100</td>
<td>1053</td>
<td>860 – 1291</td>
<td>1.5 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>ethanol</td>
<td>30000</td>
<td>182523</td>
<td>46091 – 722802</td>
<td>1.4 ± 0.9</td>
<td>3</td>
<td>30000</td>
<td>276452</td>
<td>35612 – 2146000</td>
<td>1.0 ± 0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^{a}\) IC\(_{50}\) values can be considered absolute for all drugs except allopregnanolone, which has a relative IC\(_{50}\) since maximal L-VGCC inhibition by allopregnanolone was less than 100%.

N.D., not determined

*, p < 0.05, compared to respective Ca\(_{\text{v}1.3}\) IC\(_{50}\) value.

\(^{\dagger}\), p < 0.05, compared to Ca\(_{\text{v}1.3}\) desalkylflurazepam IC\(_{50}\) value in the absence of flumazenil.
### Table 3.2 GABA<sub>A</sub>R modulators enhance L-VGCC current decay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca&lt;sub&gt;1.2&lt;/sub&gt;</th>
<th>Ca&lt;sub&gt;1.3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON r200&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Drug r200</td>
</tr>
<tr>
<td>30/10 μM diltiazem&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.03</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>30 μM diazepam</td>
<td>0.49 ± 0.09</td>
<td>0.26 ± 0.08**</td>
</tr>
<tr>
<td>30 μM flurazepam</td>
<td>0.35 ± 0.09</td>
<td>0.25 ± 0.07*</td>
</tr>
<tr>
<td>30 μM desalkylflurazepam</td>
<td>0.36 ± 0.08</td>
<td>0.26 ± 0.07*</td>
</tr>
<tr>
<td>30 μM allopregnanolone</td>
<td>0.50 ± 0.08</td>
<td>0.24 ± 0.06***</td>
</tr>
<tr>
<td>0.3/1 mM pentobarbital&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44 ± 0.10</td>
<td>0.19 ± 0.06*</td>
</tr>
<tr>
<td>300 mM ethanol</td>
<td>0.29 ± 0.12</td>
<td>0.12 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> 30 and 10 μM diltiazem were analyzed for Ca<sub>1.2</sub> and Ca<sub>1.3</sub>, respectively.

<sup>b</sup> 0.3 and 1 mM pentobarbital were analyzed for Ca<sub>1.2</sub> and Ca<sub>1.3</sub>, respectively.

<sup>c</sup> r200 is the ratio of current remaining after a 200 ms depolarization relative to the peak current. Control r200 values were obtained in the same cells in the absence of drug.

<sup>d</sup> Average percent drug-induced decrease in the r200 value.

*, p < 0.05, **, p < 0.01, ***, p < 0.001, Drug versus CON r200, paired t-test.
Table 3.3 Negative shift in L-VGCC steady-state inactivation by GABA<sub>A</sub>R modulators

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>Ca&lt;sub&gt;1.2&lt;/sub&gt;</th>
<th>Ca&lt;sub&gt;1.3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;50&lt;/sub&gt;</td>
<td>k</td>
</tr>
<tr>
<td>control</td>
<td>-25.7 ± 1.7</td>
<td>7.6 ± 1.6</td>
</tr>
<tr>
<td>30/10 µM diltiazem&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-29.9 ± 1.9*</td>
<td>7.7 ± 1.8</td>
</tr>
<tr>
<td>30 µM diazepam</td>
<td>-28.0 ± 1.6</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td>60 µM diazepam</td>
<td>-33.5 ± 1.1*</td>
<td>7.4 ± 1.0</td>
</tr>
<tr>
<td>100 µM diazepam</td>
<td>-36.9 ± 1.3*</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>100 µM desalkylflurazepam</td>
<td>-30.9 ± 2.0*</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>300 µM flumazenil</td>
<td>-27.2 ± 2.9</td>
<td>9.6 ± 3.0</td>
</tr>
<tr>
<td>100 µM diazepam + 300 µM flumazenil</td>
<td>-37.3 ± 1.2*</td>
<td>6.6 ± 1.1</td>
</tr>
<tr>
<td>30 µM allopregnanolone</td>
<td>-25.1 ± 4.2</td>
<td>12.0 ± 4.6</td>
</tr>
<tr>
<td>0.3/1 mM pentobarbital&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-31.2 ± 1.5*</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>300 mM ethanol</td>
<td>-25.2 ± 1.5</td>
<td>6.7 ± 1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> 30 and 10 µM diltiazem were used for Ca<sub>1.2</sub> and Ca<sub>1.3</sub>, respectively.

<sup>b</sup> 0.3 and 1 mM pentobarbital were used for Ca<sub>1.2</sub> and Ca<sub>1.3</sub>, respectively.

N.D., not determined.

* = p < 0.05 compared to respective control inactivation V<sub>50</sub> in the absence of drug
CHAPTER 4

CA2+/CALMODULIN-DEPENDENT PROTEIN KINASE II LOCALIZATION AND AUTOPHOSPHORYLATION WITHIN HIPPOCAMPAL CA1 EXCITATORY POSTSYNAPSES DURING FLURAZEPAM WITHDRAWAL

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98
**Abbreviations:** AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BZ, benzodiazepine; CaM, calmodulin; CaMKII, Ca\(^{2+}\)/calmodulin-dependent protein kinase II; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FZP, flurazepam; GABA\(_A\)R, γ-aminobutyric acid type A receptor; L-VGCC, L-type voltage-gated Ca\(^{2+}\) channel; NMDAR, N-methyl-D-aspartate receptor; PEG, poly(ethylene) glycol; PSD, postsynaptic density; TBST, Tris-buffered saline with 0.1% Tween 20
4.1 Abstract

Benzodiazepines (BZs) are clinically useful anxiolytics, sedatives, and anticonvulsants attributable to their positive allosteric modulation of GABA\textsubscript{A} receptors. Long-term use can lead to development of physical dependence manifested by withdrawal symptoms. One week oral administration of rats with the benzodiazepine flurazepam (FZP) results in withdrawal anxiety, correlated with hippocampal CA1 neuron hyperexcitability mediated by enhancement of GluA1-containing AMPARs. A CaMKII-mediated increase in AMPAR conductance after 2 days of FZP withdrawal may result from potentiation of L-type voltage-gated Ca\textsuperscript{2+} channel (L-VGCC) currents that precedes the GluA1-AMPAR enhancement and continues through 2 days of withdrawal. The mechanistic basis of L-VGCC potentiation and CaMKII activation remains unknown. The current studies assessed L-VGCC Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunit expression by immunoblot and Ca\textsubscript{v}1.2 immunohistochemistry. Total and autophosphorylated (Thr\textsuperscript{286}) CaMKII expression and distribution were examined in CA1 asymmetric synapses by postembedding immunogold electron microscopy. The findings suggested that up-regulated L-VGCC function was not due to increased subunit expression, rather may result from trafficking from intracellular stores or post-translational modifications such as phosphorylation. Interestingly, postsynaptic CaMKII\textsubscript{a} expression was decreased with no change in autophosphorylated CaMKII expression. Decreased CaMKII expression may be due to the corresponding removal of GluN1/GluN2B-containing N-methyl-D-aspartate receptors from CA1 synapses after 2 days of FZP withdrawal, suggesting CaMKII binding to GluN2B during FZP withdrawal. An enhanced CaMKII-GluN2B interaction may reflect autonomous
CaMKII activation stimulated by L-VGCC-mediated Ca\(^{2+}\) influx in this model of BZ withdrawal-induced glutamatergic plasticity.
4.2 Introduction

Benzodiazepines (BZs) are clinically useful anxiolytics, sedative-hypnotics, and anticonvulsants attributable to their enhancement of Cl− flux through γ-aminobutyric acid type A receptors (GABAARs), the main inhibitory neurotransmitter receptors in the mammalian central nervous system. However, long-term treatment can result in the development of tolerance to BZ effects and physical dependence manifested by withdrawal symptoms such as anxiety and insomnia (Griffiths & Johnson, 2005). Current evidence suggests that while tolerance may result from impaired GABAergic function, withdrawal symptoms such as anxiety and in some cases increased seizure susceptibility may relate to neuronal hyperexcitability mediated by enhanced function of the glutamatergic system (Izzo et al., 2001; Van Sickle et al., 2004; Song et al., 2007; Xiang & Tietz, 2007). Specifically, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs) are the excitatory ionotropic receptors that respond to glutamate neurotransmission, and AMPAR potentiation in particular may mediate withdrawal hyperexcitability. For example, long-term treatment of rats with the BZ diazepam lead to withdrawal anxiety and increased seizure susceptibility after 96 hrs of withdrawal, which correlated with increased AMPAR GluA1 subunit mRNA and protein in the cortex and hippocampus, with no alteration in AMPAR GluA2 or NMDAR GluN1 subunits (Izzo et al., 2001).

In flurazepam (FZP)-treated rats, withdrawal anxiety significantly correlated with potentiation of AMPAR currents in hippocampal CA1 pyramidal neurons (Van Sickle et al., 2004; Xiang & Tietz, 2007). The initial increase in AMPAR current amplitude resulted from incorporation of GluA1 homomeric receptors into the postsynaptic density.
(PSD) during the first day of withdrawal (Song et al., 2007; Das et al., 2008; Shen et al., 2010). Increased AMPAR conductance after 2 days of withdrawal was attributable to Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated phosphorylation of GluA1 subunits at Ser\textsuperscript{831} (Shen et al., 2009; Shen et al., 2010).

Although FZP withdrawal-induced glutamatergic plasticity is similar to that of long-term potentiation (LTP), several differences are notable. First, following AMPAR potentiation, a compensatory removal of GluN1 and GluN2B NMDAR subunits and reduced NMDAR current amplitude was observed 2 days after FZP withdrawal. Down-regulation of GluN2B-containing NMDARs normalized excitatory total charge transfer in CA1 neurons and precluded expression of anxiety (Van Sickle et al., 2004; Shen et al., 2009; Das et al., 2010; Shen & Tietz, 2011). Anxiety was unmasked after 2 days of withdrawal by preventing the NMDAR down-regulation via systemic pre-injection of an NMDAR antagonist (Van Sickle et al., 2004). Second, while LTP is induced by Ca\textsuperscript{2+} influx through NMDARs during high frequency stimulation (Malenka & Nicoll, 1999), a near doubling of high-voltage-activated (HVA) Ca\textsuperscript{2+} currents was observed in CA1 neurons of FZP-treated rats, preceding the AMPAR enhancement and continuing through 2 days of withdrawal (Xiang et al., 2008). Moreover, blockade of L-type voltage-gated calcium channels (L-VGCCs), but not NMDARs reversed the withdrawal anxiety and AMPAR potentiation at 1 and 2 days of FZP withdrawal (Van Sickle et al., 2004; Xiang & Tietz, 2007; Xiang et al., 2008). Taken together, the data suggest that Ca\textsuperscript{2+} influx through L-VGCCs contributes to the increased number and function of synaptic homomeric GluA1 AMPARs during FZP withdrawal via a CaMKII-mediated mechanism.
Although we reported that CaMKII mediates the phosphorylation of GluA1 homomers during the second day of FZP withdrawal, the mechanism of CaMKII activation remains uncertain. During LTP, persistent Ca\(^{2+}\) influx through NMDARs leads to activation and autophosphorylation of CaMKII at Thr\(^{286}\) resulting in partially autonomous (Ca\(^{2+}\)-independent) activity (Coultrap et al., 2010). During FZP withdrawal, total CaMKII\(\alpha\), but not \(\beta\) expression was increased in PSD-enriched CA1 homogenates from FZP-withdrawn rats without a concomitant increase in autophosphorylated Thr\(^{286}\)-CaMKII (pCaMKII) (Shen et al., 2010), suggesting that alternate mechanisms of CaMKII autonomous activation and/or PSD localization may occur, perhaps through binding to membrane-incorporated GluN2B subunits (Bayer et al., 2001; Bayer et al., 2006). It is interesting to speculate that CaMKII activation and/or translocation may be differentially mediated by the near doubling of Ca\(^{2+}\) influx through L-VGCCs during FZP withdrawal. One mechanism by which this might occur is through increased expression of the L-VGCC \(\alpha_1\) pore-forming subunits, Ca\(_v\)1.2 and Ca\(_v\)1.3, near CA1 PSDs of FZP-withdrawn rats. A hyperpolarizing shift in the L-VGCC activation curve (Xiang et al., 2008) suggested that Ca\(_v\)1.3, which activates at relatively negative membrane potentials (Xu & Lipscombe, 2001; Earl & Tietz, 2011), might be selectively enhanced in CA1 neurons as a consequence of long-term FZP treatment.

The current studies first assessed Ca\(_v\)1.2 and Ca\(_v\)1.3 L-VGCC subunit expression in FZP-withdrawn and control tissues by immunoblot of PSD-enriched CA1 homogenates and confocal immunofluorescent analysis of Ca\(_v\)1.2-labeled hippocampal slices. Next, post-embedding immunogold labeling was used to assess CaMKII\(\alpha\) and autophosphorylated (Thr\(^{286}\)/Thr\(^{287}\) \(\alpha/\beta\)-CaMKII expression and localization within
asymmetric synapses located in *stratum radiatum* (SR) of CA1 neurons from 2-day FZP-withdrawn and matched control rats.
4.3 Materials and Methods.

4.3.1 Long-term FZP treatment

All procedures involving the use of animals were performed in compliance with The University of Toledo College of Medicine Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health guidelines. One-week oral treatment of rats with the relatively water-soluble benzodiazepine, FZP was as described previously (Van Sickle et al., 2004). Briefly, male Sprague-Dawley rats (P22-25, Harlan, Indianapolis, IN) were acclimated 2 - 4 days to 0.02% saccharin water. Rats were then offered saccharin water containing FZP (pH 5.8) as the sole drinking source. The FZP concentration was periodically adjusted based on the body weight and volume consumed to yield doses of 100 mg/kg/day for 3 days, then 150 mg/kg/day for 4 days. The final average daily FZP dose was always greater than 100 mg/kg, generally 125 - 130 mg/kg, with a goal of achieving a minimum average daily dose of 120 mg/kg (Das et al., 2010). Related to the half-life (< 12 hrs) of FZP and its major bioactive metabolites in rats (Lau et al., 1987), this treatment paradigm results in BZ levels of about 1.2 µM measured in rat brain homogenates by radioreceptor assay (Xie & Tietz, 1992). After 1-week FZP treatment, rats were offered saccharin water for 1 or 2 days. FZP withdrawal consistently results in anxiety-like behavior after 1 day of withdrawal (Van Sickle et al., 2004; Xiang & Tietz, 2007), which can be masked or expressed on day 2 of withdrawal as a function of NMDAR down-regulation (Van Sickle et al., 2004; Shen & Tietz, 2011). Matched control rats were offered saccharin water for the same experimental period.

4.3.2 Antibodies and specificity

4.3.2.1 Antibodies used for immunoblot and immunofluorescent staining
A polyclonal anti-Cav1.3 antibody (2.49 mg/mL stock) which recognizes the N-terminal sequence, MQHQRQQQEDHANEANYARGTRKC was generously provided by Dr. Amy Lee (University of Iowa, Iowa City, Iowa). This antibody was previously characterized as recognizing two bands consistent in size with Cav1.3 (>170 kDa and >250 kDa) in hippocampal lysates from wild-type, but not Cav1.3 knockout mice. Immunoblots of rat brain lysates revealed a 250 kDa band (Jenkins et al., 2010). A polyclonal anti-Cav1.2 antibody (0.85 mg/mL stock) which recognizes an epitope located in the intracellular loop between domains II and III, TTKINMDLQPSENEDKS was from Alomone Labs (Jerusalem, Israel, Cat. # ACC-003). Antibodies generated against this epitope were previously characterized as recognizing a 250 kDa band consistent with Cav1.2, which was absent from cortical and hippocampal lysates of Cav1.2 conditional knockout mice (Tippens et al., 2008). Immunoblot of rat hippocampal lysates revealed a smeared band with a molecular weight >170 kDa (Tippens et al., 2008). Control experiments used in the current studies included omitting the primary antibody and pre-incubation with the antigenic peptide, both of which abolished bands on immunoblot (see Fig. 1B) and immunofluorescent labeling (see Fig. 3B). A monoclonal anti-Cav1.2 antibody (clone N263/31) generated using a peptide fragment containing amino acids 808-874 (intracellular loop between domains I and II) was from NeuroMab (UC Davis, Davis, CA). In the current studies, this antibody recognized full-length and truncated Cav1.2 size-forms with identical apparent molecular weights as those recognized by the Alomone antibody (data not shown). Polyclonal anti-pS\textsuperscript{1928}-Cav1.2 (0.34 mg/mL stock) was generously provided by Dr. William Catterall (University of Washington, Seattle, WA). This antibody was previously characterized as specifically recognizing Cav1.2
phosphorylated at Ser\textsuperscript{1928} (Hulme et al., 2006). Polyclonal anti-Ca\textsubscript{v}\textbeta\textsubscript{3} antibody (0.3 mg/mL stock) was from Alomone Labs (Cat. # ACC-008) and recognized a prominent 57 kDa band by immunoblot of PSD-enriched protein consistent with the Ca\textsubscript{v}\textbeta\textsubscript{3} subunit (Ludwig et al., 1997; Catterall, 2000), but also recognized fainter 60 and 69 kDa bands that likely represent Ca\textsubscript{v}\textbeta\textsubscript{3} subunit splice variants (Hullin et al., 1992; Ludwig et al., 1997), the latter of which was enriched in the cytosolic and Triton-soluble membrane subcellular fractions (data not shown, see subcellular fractionation below).

4.3.2.2 Antibodies used for post-embedding immunogold electron microscopic analysis

A monoclonal anti-CaMKII\textalpha antibody (clone 6G9-2, 1 mg/mL stock) generated using purified CaMKII\textalpha protein (Kennedy et al., 1983a; Kennedy et al., 1983b) was from Chemicon-Millipore (Billerica, MA, Cat. # MAB8699). This antibody recognized a specific band at \textasciitilde 50 kDa, whereas no bands were observed in hippocampal lysates from knockout mice (Silva et al., 1992). Additional controls for post-embedding immunogold labeling in the current studies included omitting the primary antibody, which yielded 0.01 15-nm particles/bouton, 0.10 particles/spine, and 0.01 particles/PSD in 69 asymmetric synaptic profiles (compare to CaMKII\textalpha immunogold labeling in these same compartments in Table 1), and replacing the monoclonal anti-CaMKII\textalpha with the polyclonal anti-pCaMKII antibody, which yielded no particles pre- or postsynaptically in 60 asymmetric synaptic profiles. A polyclonal anti-pCaMKII antibody which recognizes the epitope, MHRQET(PO\textsubscript{4})VDCLKKKFN was from Promega (Madison, WI, Cat. # V1111). This antibody recognizes phosphorylated Thr\textsuperscript{286/287} likely on all CaMKII isoforms (Lorenz et al., 2002; Larsson & Broman, 2006). Labeling using this antibody
was substantially reduced when used in immunocytochemistry of visual cortex from mice with CaMKIIα Thr<sup>286</sup> mutated to alanine (Taha et al., 2002). Pre-embedding immunogold pCaMKII labeling using this antibody revealed increased postsynaptic labeling in hippocampal cultures exposed to NMDA (Dosemeci et al., 2002). Additional controls for post-embedding immunogold labeling in the current studies included omitting the primary antibody which yielded 0.30 10-nm particles/bouton, 0.24 particles/spine, and 0.06 particles/PSD in 63 asymmetric synaptic profiles (compare to pCaMKII immunogold labeling in these same compartments in Table 2), and replacing the polyclonal anti-pCaMKII with monoclonal anti-CaMKII, which yielded no particles pre- or postsynaptically in 60 asymmetric synaptic profiles.

4.3.3 CA1 minislice subcellular fractionation

Subcellular fractionation to obtain a PSD-enriched membrane fraction was performed as described previously (Song et al., 2007; Shen et al., 2010). Briefly, following decapitation the CA1 region of the dorsal hippocampus was rapidly dissected on ice and stored at -80°C for future use (2-3 CA1 minislices/group) or homogenized immediately, then stored at -80°C. Homogenates contained phosphatase and protease inhibitors (P8340, Sigma, St. Louis, MO). After removing nuclei and debris, membranes were separated from cytosol with a 10,000 g spin. Subcellular fractions used in the current studies included a cytosolic fraction (S2), a 0.5% Triton X-100-soluble membrane fraction (S3), and a Triton X-100-insoluble membrane fraction (P3). The P3 fraction was previously shown to be ~40% enriched in PSDs (Song et al., 2007). Moreover, changes in AMPAR and NMDAR subunits detected in the P3 fraction (Song et al., 2007; Shen et al., 2010; Shen & Tietz, 2011) were also detected at CA1 synapses using immunogold techniques.
(Das et al., 2008; Das et al., 2010). Protein content was determined using either the Bradford or bicinchoninic acid (BCA) protein assays using bovine serum albumin as a standard. Proteins were mixed with Laemmli sample buffer and heated to 70ºC for 10 min prior to blotting.

4.3.4 Western blot

Protein samples were electrophoretically separated in 4-12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA) along with a Novex Sharp pre-stained protein standard (Invitrogen) in SDS-MOPS running buffer (Invitrogen), then wet transferred to polyvinylidene fluoride membrane (Immobilon-FL, Millipore) in NuPAGE methanol-free transfer buffer (Invitrogen) at 90 V for 4 hr on ice. Transferred protein bands were detected by 5 min incubation with 0.5% Ponceau S (w/v) in 1% acetic acid (v/v). As indicated in the results, in some cases Ponceau S-stained bands were scanned and used as loading controls in lieu of actin. Blots were blocked with 5% non-fat milk in Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl, 2.3 mM CaCl₂), probed with primary antibodies as indicated in the results, then secondary antibodies conjugated to infrared probes (700 or 800 nm, LiCOR, Lincoln, NE). Reacted blots were scanned using the Odyssey Infrared Imaging System (LiCOR) at an intensity that yielded band densities in a linear range. Integrated band densities (ID) were quantified using Image J software (NIH, Bethesda, MD), and signals were normalized to β-actin ID as a loading control. Data from each blot were expressed as a percent of the control average.

4.3.5 Hippocampal slice confocal immunofluorescence

Hippocampal tissue preparation, immunofluorescent staining, and confocal analysis were performed as described previously (Song et al., 2007). Briefly, 1 cm hippocampal
blocks were post-fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 1 hr at room temperature, then fixed overnight at 4°C. Fixed brains were incubated in 15% sucrose overnight at 4°C. Coronal dorsal hippocampal slices (50 μm) were quenched in 0.5 M NH₄Cl, blocked 1 hr in 10% normal goat serum (NGS, from here forward solutions contained 1% fish gelatin and 0.1% Tween-20), incubated overnight at 4°C in primary antibody (anti-Caᵥ1.2, 4.25 µg/mL, Alomone Labs), blocked 3 x 10 min in 5% NGS, then incubated 2 hr in secondary antibody (goat anti-rabbit IgG conjugated to Alexa488, 1:500). Confocal images of each slice were captured at 5 μm intervals on a Leica TCS SP5 system (Leica Microsystems Inc., Bannockburn, IL). Leica Application Suite Advanced Fluorescence software was used to create two-dimensional projections for each slice using only the maximal intensity pixels from each slice’s collection of confocal images. Regions of interest were drawn in several hippocampal regions (as noted in the results) and the mean gray value measured using Image J software. Each region’s mean gray value was averaged from 2 - 5 slices for each rat. A total of 4 separate immunofluorescent experiments were performed with 1 - 2 rats per treatment group in each experiment (n = 6 rats/group). Data from each experiment were expressed a percent of the mean control gray value in order to reduce interexperimental variability resulting from differences in confocal laser intensity, gain, and offset.

4.3.6 Post-embedding immunogold electron microscopy

Transcardial fixation, cryosubstitution, and post-embedding immunogold labeling procedures were as described previously, and the ultrathin cut sections used in the current studies were cut from the same tissues as those used in a previous study of GluN1 and GluN2A/B subunits at CA1 neuron asymmetric postsynapses (Das et al., 2010). Briefly,
isoflurane anesthetized rats were transcardially perfused with an oxygenated vascular rinse followed by 4% paraformaldehyde and 0.5% glutaraldehyde. Hippocampal slices (200 or 500 μm) were slam-frozen (-190°C, Leica EM CPC, Bannockburn, IL), cryosubstituted, and flat-embedded in lowicryl resin. Ultrathin CA1 sections (80 nm) were collected on nickel grids, equilibrated in Tris-buffered saline with 0.1% Triton X-100 (TBST, pH 7.6), quenched in 1% NaBH₄ and 50 mM glycine, blocked in 10% normal goat serum (NGS), then incubated in primary antibodies (1:20 anti-CaMKIα and/or 1:10 anti-pCaMKII with 1% NGS) 2 hrs at room temperature, then overnight at 4°C. Tissues were switched to pH 8.2 TBST, incubated in 0.5% poly(ethylene) glycol (PEG), then in secondary antibody containing 0.5% PEG (1:25 goat anti-rabbit IgG conjugated to 10-nm gold and/or 1:25 goat anti-mouse IgG conjugated to 15-nm gold, BBInternational, UK) for 1.5 hr. Sections were counterstained with 5% uranyl acetate and Reynold’s lead citrate. The CA1 proximal SR region of reacted tissues was scanned using a Phillips CM10 PW6020 transmission electron microscope to randomly identify profiles of asymmetric synapses. Images were captured at 52,000X magnification. The negatives were developed and scanned, then immunogold labeling was measured using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD) as previously described (Das et al., 2010). Pre- and postsynaptic immunogold particles were binned into distances perpendicular to the cleft surface of the PSD out to 300 nm. Immunogold particles were also assigned to PSD, active zone, perisynaptic, membrane, or intracellular compartments: particles within 20 nm of the PSD were counted as PSD labeling as described previously (Das et al., 2008); particles within 20 nm of the presynaptic membrane, but not within 20 nm of the PSD membrane were considered within the active
zone; particles within 20 nm of the membrane were considered membrane-bound; and postsynaptic membrane-bound particles within 100 nm lateral to the PSD were considered perisynaptic (Das et al., 2008). All procedures and measurements were performed with the experimenter blinded to the experimental group.

4.3.7 Statistical analyses

Data from immunoblot and immunofluorescent analyses were normalized to mean control values within each experiment so that data could be pooled from multiple experiments for statistical analysis. Immunoblot and immunofluorescent means were compared between control and FZP-withdrawn groups by Student’s unpaired $t$-test. Presynaptically and postsynaptically binned immunogold particles were analyzed by Two-way Repeated Measures ANOVA with post-hoc comparisons between control and FZP-withdrawn groups within each bin using Bonferroni’s Multiple Comparison test. Percent labeling and the mean number of immunogold particles were compared between control and FZP-withdrawn groups by Student’s $t$-test. Relative frequencies of the numbers of immunogold particles per PSD between control FZP-withdrawn groups (0 to 5) were compared by Mann-Whitney $U$-test. Data were statistically analyzed and graphs generated using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Values are reported as mean ± SEM and were considered significantly different if the p-value was less than or equal to 0.05.
4.4 Results

4.4.1 Unaltered L-VGCC subunit expression in CA1 PSD-enriched fraction during FZP-withdrawal

To investigate the mechanistic basis of the voltage-gated Ca\(^{2+}\) current enhancement in CA1 neurons during FZP withdrawal (Xiang et al., 2008), L-VGCC subunit expression was assessed by immunoblot of CA1 homogenates from rats withdrawn 1 or 2 days from 1-week FZP treatment. Figs. 1A and B show the staining pattern of Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}1.3}\) immunoblots, respectively in different subcellular fractions. Importantly, signals with a molecular weight consistent with that of Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}1.3}\) were primarily seen in the PSD-enriched, Triton-insoluble membrane fraction. Long and short size-forms of both Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}1.3}\) were observed with apparent molecular weights of 220 and 180 kDa. The different Ca\(_{\text{v}1.2}\) size-forms arise from calpain-mediated cleavage of the C-terminus resulting in full-length and truncated peptides (Hell et al., 1996). For Ca\(_{\text{v}1.3}\), these size forms likely result from neuronal splice variation, which yields full-length and C-terminal truncated peptides (Safa et al., 2001; Xu & Lipscombe, 2001; Singh et al., 2008), similar to Ca\(_{\text{v}1.2}\). In the Triton-soluble membrane fraction, a weakly staining diffuse band with an approximate molecular weight between 180 and 220 kDa was observed for both Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}1.3}\) subunits. The cytosolic fraction revealed no signal in the range of 180 – 220 kDa. Other signals above (for Ca\(_{\text{v}1.3}\)) and below the 180 – 200 kDa range were observed. Based on previous characterizations using knockout tissue, the low molecular weight signals may not be calcium channel \(\alpha_1\) subunits, including the prominent 120 kDa band observed in Ca\(_{\text{v}1.2}\) immunoblots that was removed by peptide pre-incubation (Fig. 1B).
Fig. 2A illustrates that there was no change in Ca\textsubscript{v}1.2 or Ca\textsubscript{v}1.3 subunit expression in the PSD-enriched fraction after 1 day of FZP withdrawal. Although increased expression of the auxiliary Ca\textsubscript{v}\textbeta\textsubscript{3} subunit could enhance trafficking of L-VGCCs to the plasma membrane from the endoplasmic reticulum (Bichet et al., 2000), no alteration in Ca\textsubscript{v}\textbeta\textsubscript{3} subunit expression was detected (Fig. 2A). Interestingly, each Ca\textsubscript{v}1.2 size form was expressed as a doublet of bands separated by about 10 kDa. These doublets could represent splice variants (Tang et al., 2004), each of which undergoes the same proteolytic cleavage by calpain. Separate measurements of the integrated densities of each doublet band also revealed no change in the expression of Ca\textsubscript{v}1.2 (data not shown). Calpain-mediated cleavage of the Ca\textsubscript{v}1.2 C-terminus increases Ca\textsuperscript{2+} currents through the channel (Klockner et al., 1995), which could be a factor in L-VGCC subunit regulation during FZP withdrawal. However, there was also no difference in the ratio of the short to long size-form by measuring integrated density of the doublet bands together (CON ratio: 1.16 ± 0.10, FZP ratio: 1.30 ± 0.08, p = 0.30, n = 4/group) or separately (top band: CON ratio: 0.96 ± 0.08, FZP ratio: 1.07 ± 0.11, p = 0.42; bottom band: CON ratio: 1.86 ± 0.22, FZP ratio: 1.88 ± 0.10, p = 0.96, n = 4/group). Fig. 2B illustrates that Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3, and Ca\textsubscript{v}\textbeta\textsubscript{3} subunit expression was similarly unaltered after 2 days of FZP withdrawal. The Ca\textsubscript{v}1.2 doublet bands were not easily distinguishable on this immunoblot, and were not separately analyzed. The Ca\textsubscript{v}1.2 short to long size-form ratio was also unaltered after 2 days of FZP withdrawal (CON ratio: 0.60 ± 0.09, n = 3; FZP ratio: 0.57 ± 0.03, n = 4; p = 0.81).

Phosphorylation of Ca\textsubscript{v}1.2 at Ser\textsuperscript{1928} enhances Ca\textsuperscript{2+} current density (Oliveria et al., 2007). Using an antibody specific for pS\textsuperscript{1928}-Ca\textsubscript{v}1.2, immunoblot analysis of CA1 PSD-
enriched proteins revealed no change in the Ca\textsubscript{v}1.2 phosphorylation state when normalized to Ponceau S-stained protein bands (CON: 1.00 ± 0.06, n = 8, FZP: 1.09 ± 0.16, n = 6, p = 0.56, pooled data from two blots) or when normalized to total Ca\textsubscript{v}1.2 expression measured using the NeuroMab monoclonal antibody (CON: 1.00 ± 0.06, n = 8, FZP: 1.36 ± 0.16, n = 6, p = 0.08 with Welch’s correction, pooled data from two blots). The anti-pS\textsuperscript{1928}-Ca\textsubscript{v}1.2 antibody recognized doublet bands with apparent molecular weights of about 220-240 kDa, similar to full-length Ca\textsubscript{v}1.2. This is consistent with the fact that the C-terminal truncated short-form of Ca\textsubscript{v}1.2 does not contain Ser\textsuperscript{1928}.

Consistent with immunoblot data, experiments in which fixed hippocampal slices were immunostained with the Alomone anti-Ca\textsubscript{v}1.2 antibody also revealed no change in expression after 2 days of FZP withdrawal (Fig. 3A and C). Control experiments in which the primary antibody was omitted or pre-incubated with the Ca\textsubscript{v}1.2-specific antigenic peptide abolished the labeling (Fig. 3B). Immunohistochemical experiments could not be conducted for Ca\textsubscript{v}1.3, since the antibody used for immunoblot is not specific for hippocampal immunohistochemical staining (Amy Lee, unpublished observations).

4.4.2 CaMKII\textsubscript{\alpha} expression, but not Thr\textsuperscript{286} autophosphorylation is reduced in FZP-withdrawn CA1 asymmetric synapses

Prior evidence suggested that Ca\textsuperscript{2+} influx through L-VGCCs might activate CaMKII, which then phosphorylates GluA1 subunits at Ser\textsuperscript{831} increasing AMPAR conductance after 2 days of FZP withdrawal (Xiang & Tietz, 2007; Xiang et al., 2008; Shen et al., 2009; Shen et al., 2010). To further evaluate this hypothesis, ultrathin cut sections of the CA1 region were co-labeled with anti-CaMKII\textsubscript{\alpha} and anti-autophosphorylated Thr\textsuperscript{286/287} CaMKII\textsubscript{\alpha}/\beta (anti-pCaMKII) antibodies recognized using secondary antibodies.
conjugated to 15- or 10-nm gold, respectively. Dual labeling was assessed in asymmetric synapses in the proximal dendritic SR region, where decreased GluN1 and GluN2B subunit postsynaptic expression was previously detected in these same tissues (Das et al., 2010). Increased postsynaptic expression of homomeric GluA1 AMPA receptors was also detected in this region after 2 days of FZP withdrawal (Das et al., 2008). Similar levels of labeling were observed in tissues reacted with CaMKII or pCaMKII antibodies alone (data not shown) rather than as a cocktail, suggesting that the antibodies did not sterically interfere with each other during dual reaction.

Fig. 4 illustrates dual labeling observed in asymmetric synapses from control (A,B) and FZP-withdrawn (C,D) rats. Immunogold particles were counted within 300 nm pre- and postsynaptic to the cleft surface of the PSD. There was no significant change in the percentage of boutons, spines, or PSDs labeled with CaMKII or pCaMKII antibodies (Fig. 4E-G, Tables 1 and 2, respectively). Similarly, no significant change in the mean number of CaMKII or pCaMKII immunogold particles was observed in boutons, spines, or PSDs (Fig. 4H-J, Tables 1 and 2, respectively). Interestingly, there was a trend towards an increased ratio of pCaMKII/CaMKII expression in PSDs (Fig. 4J). A significant decrease in the number of CaMKII immunogold particles was observed in spines and PSDs, but not boutons when only positively labeled (≥1 immunogold particle) boutons, spines, or PSDs were considered (Table 1). No alteration in pCaMKII labeling was observed in any of these synaptic compartments (Table 2). A significant decrease in the percent labeling and mean number of CaMKII immunogold particles was also observed in FZP-withdrawn active zones, but no alteration was observed in pre- or postsynaptic membrane or intracellular CaMKII or pCaMKII immunogold labeling (Table 3).
Fig. 5 illustrates CaMKII and pCaMKII expression within binned distances pre- and postsynaptic to the PSD membrane for control and FZP-withdrawn rats. Analysis of binned CaMKII expression revealed a significant interaction between experimental group and binned distance with a significant decrease 60 nm postsynaptic to the PSD membrane (see Fig. 5A). There was no significant interaction between experimental group and binned distance for presynaptic CaMKII expression or pCaMKII either pre- or postsynaptically. Decreased CaMKII expression within the 60 nm postsynaptic bin without a change in pCaMKII expression indicates an alteration in PSD labeling. Moreover, as illustrated in Fig. 6, comparing the relative frequency of numbers of 15- (CaMKII) and 10-nm (pCaMKII) particles within PSDs (0 to 5 immunogold particles) between control and FZP-withdrawn groups revealed a significant decrease in the frequency of PSDs labeled with two 15-nm particles in FZP-withdrawn tissues. Taken together the data support decreased CaMKIIα, but not absolute pCaMKII expression in PSDs within the CA1 region of 2-day FZP-withdrawn rats.
4.5. Discussion

4.5.1 Molecular basis of FZP-induced enhancement of CA1 VGCC function

The current results suggest that a mechanism other than increased expression of L-VGCC subunits mediates the increased HVA Ca\(^{2+}\) current density in CA1 neurons of FZP-treated and withdrawn rats (Xiang et al., 2008). It is possible that L-VGCC surface expression is enhanced via trafficking from intracellular compartments, which went undetected by the methods used in the current studies. This could be assessed in surface protein crosslinking experiments. Although it was reported that the number of postsynaptic profiles with plasma membrane-bound Ca\(_{v}1.2\) immunogold labeling was relatively low (Tippens et al., 2008), it may also be possible to detect increased surface expression of Ca\(_{v}1.2\) or Ca\(_{v}1.3\) L-VGCCs using immunogold EM methods. The specificity of anti-Ca\(_{v}1.2\) and Ca\(_{v}1.3\) antibodies for pre- or post-embedding immunogold labeling will need to be confirmed using knockout tissues to determine if the low level of surface labeling represents actual Ca\(_{v}1.2\) and Ca\(_{v}1.3\)-containing surface-expressed L-VGCCs near CA1 PSDs.

Another possible L-VGCC regulatory mechanism involves phosphorylation at sites that enhance channel currents and may also result in channel activation at more negative membrane potentials (Gao et al., 2006). Although the phosphorylation state of Ca\(_{v}1.2\) Ser\(^{1928}\) was unchanged during FZP withdrawal, several other phosphorylation sites regulate channel gating. In particular, Ca\(^{2+}\)-dependent facilitation of both Ca\(_{v}1.2\) and Ca\(_{v}1.3\) is mediated by CaMKII (Hudmon et al., 2005; Lee et al., 2006; Jenkins et al., 2010). This facilitation occurs during increased channel activity (Hudmon et al., 2005), though BZs would likely decrease channel activity via enhancement of GABA\(_{A}\)R-
mediated neuronal inhibition. Nevertheless, a depolarizing shift in the GABA<sub>A</sub>R reversal potential and a GABA<sub>A</sub>R-mediated depolarization was observed during FZP-withdrawal (Zeng et al., 1995; Zeng & Tietz, 1997), which could contribute to CA1 neuron hyperexcitability and activate L-VGCCs during FZP withdrawal. The precise mechanism of the observed GABA-mediated depolarization and whether it exists during drug treatment to enhance L-VGCC activity remains unknown.

4.5.2 Molecular basis of CaMKII activation during FZP withdrawal

L-VGCC-mediated Ca<sup>2+</sup> influx may related to CaMKII activation and the observed phosphorylation of GluA1 homomers and AMPAR potentiation during FZP withdrawal (Shen et al., 2010). CaMKII forms oligomeric holoenzymes containing up to 12 subunits (Bennett et al., 1983). The β-CaMKII isoform mediates binding of CaMKII holoenzymes to the actin cytoskeleton within dendritic arbors (Shen et al., 1998). Upon elevation of intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]), Ca<sup>2+</sup> binds to all four Ca<sup>2+</sup>-binding sites on calmodulin (CaM), resulting in association with and activation of CaMKII via displacement of an autoinhibitory domain. Elevations in [Ca<sup>2+</sup>], also result in dissociation of CaMKII holoenzymes from the actin cytoskeleton. If [Ca<sup>2+</sup>], remains elevated for a sufficient length of time, CaMKII enzymes phosphorylate neighboring CaMKII molecules within the holoenzyme at Thr<sup>286</sup> on α and Thr<sup>287</sup> on β isoforms. This autophosphorylation results in Ca<sup>2+</sup>/CaM trapping and autonomous kinase activity without the need for continued elevation of [Ca<sup>2+</sup>], (reviewed in Fink & Meyer, 2002).

The mechanism of drug withdrawal-induced CaMKII activation prior to the phosphorylation of GluA1 homomeric AMPARs observed after 2 days of FZP withdrawal is unclear. The trend towards an increased ratio of pCaMKII/CaMKII
immunogold PSD labeling suggests that a greater proportion of CaMKII molecules within the PSD are autophosphorylated at Thr^{286} in FZP-withdrawn rats. However, since the absolute amount of pCaMKII was unaltered, this suggests that an additional mechanism of CaMKII activation may occur during FZP withdrawal. A possible hint to an alternate activation mechanism was the loss of CaMKIIα from CA1 asymmetric synapses, which may be related to the corresponding loss of GluN1/GluN2B NMDA receptors observed in a prior EM study using tissue sections from the same experimental groups (Das et al., 2010). Thus, there may be an increased interaction between CaMKII and GluN2B prior to the second day of FZP withdrawal. Direct binding of CaMKII to GluN2B resulted in autonomous CaMKII activation independent of autophosphorylation (Bayer et al., 2001). A loss of GluN2B-containing NMDARs after 2 days of withdrawal would serve to homeostatically reset the CA1 glutamatergic system mediating recovery from FZP withdrawal-induced anxiety (Shen & Tietz, 2011). Since a correlation in the loss of both CaMKII and GluN2B from PSDs does not in itself indicate an enhanced interaction between these molecules, future experiments will address this hypothesis directly using co-immunoprecipitation studies.

The amino acid sequence surrounding the Thr^{286/287} autophosphorylation site is nearly identical in each CaMKII isoform and phospho-specific antibodies will recognize this epitope on all isoforms. In particular, β-CaMKII is the second highest expressed isoform in the brain (Bennett et al., 1983; Erondu & Kennedy, 1985). Autophosphorylation of β-CaMKII results in increased affinity for Ca^{2+}/CaM, leading to dissociation from the actin cytoskeleton promoting association of CaMKII holoenzymes with other proteins. Although, there was no change in β-CaMKII expression observed in CA1 PSD-enriched
homogenates from 2-day FZP-withdrawn rats (Shen et al., 2010), autophosphorylation of β-CaMKII cannot be ruled out.

4.5.3 Discrepancies with prior studies

The decrease in postsynaptic expression of total CaMKIIα was surprising given that prior studies detected increased CaMKIIα expression in the PSD-enriched fraction after 2 days of FZP withdrawal (Shen et al., 2010). One possibility to explain this discrepancy is that the Triton-insoluble PSD-enriched fraction contains other subcellular compartments in which CaMKII expression may be increased. For example, CaMKII is expressed in a heavy microsomal cytoskeletal compartment insoluble in 0.5% Triton X-100 (Dosemeci et al., 2000). It is possible that immunoblot analysis revealed increased CaMKII expression due to translocation to this cytoskeletal compartment, rather than to PSDs. One caveat is that the heavy microsomal cytoskeleton was pelleted from the cytosolic, rather than the membrane fraction. Yet, CaMKII expression may also be enriched in other non-PSD, Triton-insoluble compartments (Song et al., 2007) after 2 days of FZP withdrawal.

Another discrepancy is that CaMKII inhibitors were able to reverse the enhanced AMPAR currents and conductance in CA1 neurons recorded in hippocampal slices from 2-day FZP-withdrawn rats (Shen et al., 2009; Shen et al., 2010). If CaMKII is already removed from dendritic spines at this time-point, it seems unlikely that CaMKII inhibitors would be effective. This may be explained by the fact that the level of pCaMKII within the PSD remained unchanged in the current studies. Thus, although total CaMKII is being removed from postsynaptic sites along with the GluN2B subunit, the level of autophosphorylated CaMKII remains constant to phosphorylate the significantly greater
number of homomeric GluA1 AMPARs in 2-day FZP-withdrawn rats (Song et al., 2007; Das et al., 2008). Phosphatase activity may also be reduced in FZP-withdrawn tissues, so that the same level of kinase activity is unopposed by a corresponding amount of phosphatase activity, as may occur during LTP in CA1 synapses (Blitzer et al., 1998). This possibility remains to be tested in this model of drug withdrawal-induced plasticity.
4.6 Footnotes

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Parts of this work were previously presented at the following meetings: Earl DE and Tietz EI. (2010). Increased Ca\textsubscript{v}1.3, but not Ca\textsubscript{v}1.2 L-VGCC subunit expression in the rat hippocampus during flurazepam withdrawal, in *2010 Abstract Viewer*, 2010 Nov 13–17; San Diego, CA. Society for Neuroscience, Washington, DC.
4.7 Acknowledgements

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4.8 References


type voltage-gated calcium channel, Cav1.2 alpha1 subunit. *J Biol Chem*, 279(43), 44335-44343.


4.9 Figures and Tables

Figure 4.1
Figure 4.1

\( \text{Ca}_{\text{v}}1.2 \) and \( \text{Ca}_{\text{v}}1.3 \) immunoblot analysis of subcellular fractions created from CA1 homogenates. (A) Immunoblot with the polyclonal anti-\( \text{Ca}_{\text{v}}1.2 \) antibody \((4.25 \mu \text{g/mL})\) revealed a differential staining pattern in various subcellular fractions \((25 \text{ – } 30 \mu \text{g})\). Notably, a long \((220 \text{ kDa})\) and short \((180 \text{ kDa})\) size-form was observed in the PSD-enriched, Triton-insoluble membrane fraction (P3), consistent with previous reports for \( \text{Ca}_{\text{v}}1.2 \) (Hell et al., 1996). A smeared band was observed in the Triton-soluble membrane fraction (S3), which also contained an enriched \(~170 \text{ kDa}\) band. A 120 kDa band enriched in the cytosolic fraction (S2) was not seen following antigenic peptide pre-incubation. (B) Immunoblot with anti-\( \text{Ca}_{\text{v}}1.3 \) antibody \((12.45 \mu \text{g/mL}, \text{Jenkins et al., 2010})\) also revealed different staining patterns in the various fractions. Notably, two size-forms were detected in the PSD-enriched fraction with apparent molecular weights of 220 and 180 kDa. A weakly staining smear of protein surrounding an \(~200 \text{ kDa}\) band was observed in the Triton-soluble membrane fraction, and signals with unclear specificity outside the 180 - 220 kDa range were detected in the cytosolic fraction.
Figure 4.2

No change in L-VGCC subunit expression was detected in the PSD-enriched fraction after either 1 day (A) or 2 days (B) of FZP withdrawal. Blots were probed with the polyclonal anti-Cav1.2 (4.25 µg/mL, 25 - 30 µg protein/lane), anti-Cav1.3 (7.11 µg/mL, 50 µg protein/lane), or anti-Cavβ3 antibodies (1.5 µg/mL, 50 µg protein/lane). For the day 2 withdrawal point, data for Cav1.2 was averaged from 3 control (CON) and 4 FZP samples, and data for Cavβ3 represents an average of 7 CON and 8 FZP samples pooled from two blots. All other graphs represent data from a single blot in which 4 CON and 4 FZP samples were loaded. All statistical comparisons resulted in p-values > 0.05.
Figure 4.3

No change in Ca\textsubscript{v1.2} subunit expression was detected by immunofluorescent staining of hippocampal slices from control (CON) and 2-day FZP-withdrawn rats. (A) Immunostaining with the polyclonal anti-Ca\textsubscript{v1.2} antibody (4.25 µg/mL) revealed comparable fluorescent intensities between CON and FZP-withdrawn hippocampi in every subregion, including the CA1 regions, stratum oriens (SO), s. pyramidale (SP), s. radiatum (SR), and s. lacunosum-moleculare (SLM), and the dentate gyrus regions, molecular layer (ML) and granule cell layer (GCL). (B) Omission of the primary antibody abolished the immunofluorescent signal. Pre-incubation of anti-Ca\textsubscript{v1.2} with its antigenic peptide resulted in only sparse non-specific signal. (C) Averaged data from 4 separate experiments indicated no statistically significant difference in the Ca\textsubscript{v1.2} immunofluorescent intensity between CON and FZP-withdrawn tissues in any subregion (p > 0.05 for each region, n = 6 rats/group).
Figure 4.4
Figure 4.4

Images of asymmetric synapses in CA1 region of control (CON, A,B) and FZP-withdrawn (C,D) rats co-labeled with anti-CaMKIIα (1:20, recognized with 15-nm gold, arrows) and anti-pCaMKII antibodies (1:10, recognized with 10-nm gold, arrowheads). PSD-associated (black arrows), as well as membrane-bound and intracellular (white arrows) CaMKII immunogold particles were observed. Compared to CaMKII, pCaMKII presynaptic expression (white arrowheads) was higher relative to postsynaptic expression (black arrowheads, see also Fig. 5). (E-G) There was no significant change in the percent of boutons (E), spines (F), or PSDs (G) labeled with either CaMKII or pCaMKII (p > 0.05 in each case, Student’s t-test). (H-J) There was no significant change in the mean number of either CaMKII or pCaMKII immunogold particles located within boutons (H), spines (I), or PSDs (J, measured per PSD length in µm, p > 0.05 in each case, Student’s t-test). There was a trend towards decreased CaMKII labeling in spines (61% of control, p = 0.20, I), as well as a trend towards decreased CaMKII immunogold particles/PSD length (38% of control, p = 0.12, G and J, respectively). Coupled with unaltered pCaMKII labeling, this resulted in a trend towards an increased pCaMKII/CaMKII ratio in PSDs of 2-day FZP-withdrawn rats (J, right panel, p = 0.12).
Figure 4.5

A

CaMKII

Particles/bin length (µm)

Presynaptic (nm) Postsynaptic (nm)

-300 -200 -100 0 100 200 300

CON FZP

B

pCaMKII

Particles/bin length (µm)

Presynaptic (nm) Postsynaptic (nm)

-300 -200 -100 0 100 200 300
Figure 4.5

Pre- and postsynaptic distribution of CaMKII and pCaMKII immunogold particles in CA1 asymmetric synapses of control and FZP-withdrawn rats. (A) Analysis of CaMKII postsynaptic labeling revealed a significant interaction between experimental group and binned distance (p < 0.05, F(6,48) = 3.13, Two-way Repeated Measures ANOVA) with significantly decreased CaMKII labeling 60 nm postsynaptic to the PSD membrane in FZP-withdrawn relative to control synapses (38% of control, p < 0.01, Bonferroni’s posttest). In contrast, no significant interaction between treatment and binned distance was observed for presynaptic CaMKII (p > 0.05, Two-way Repeated Measures ANOVA). (B) There was no significant interaction between experimental group and binned distance for pre- or postsynaptic pCaMKII labeling (p > 0.05, Two-way Repeated Measures ANOVA).
Figure 4.6

A

CaMKII

Relative frequency

0.0 0.2 0.4 0.6 0.8 1.0

Number of 15 nm particles

CON
FZP

B

pCaMKII

Relative frequency

0.0 0.2 0.4 0.6 0.8 1.0

Number of 10 nm particles
Figure 4.6

Distribution histograms of postsynaptic profiles containing different numbers of CaMKII (A) and pCaMKII (B) immunogold particles within the PSD. Histograms represent the average relative frequencies of synapses lacking (0 particles) CaMKII or pCaMKII immunogold particles or containing 1 to 5 immunogold particles. There was a significant decrease in the fraction of FZP-withdrawn synapses containing two CaMKII immunogold particles (p < 0.05, Mann-Whitney U-test), whereas no significant difference was detected in the distribution of pCaMKII immunogold particles (p > 0.05, Mann-Whitney U-test).
Table 4.1

CaMKIIα Immunogold Synaptic Labeling

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*, p < 0.05, Student’s t-test
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<td>0.85</td>
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Table 4.3

CaMKIIα and pCaMKII Immunogold Non-PSD Synaptic Labeling

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<tr>
<td>pCaMKII</td>
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<td>FZP</td>
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*, p < 0.05, Student’s t-test. Values represent mean ± SEM.
CHAPTER 5

CONCLUSIONS AND SUMMARY

5.1 Conclusions

The following novel findings were generated during the dissertation work outlined in Chapters 3 and 4:

1. GABAA receptor modulators inhibited recombinant neuronal Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-VGCCs in a concentration-dependent manner, yet only pentobarbital and ethanol, but not BZs and allopregnanolone inhibited L-VGCCs at concentrations achieved \textit{in vivo}.

2. L-VGCC inhibition by GABAA receptor modulators was state-dependent, with preference for inactivate and possibly open channel states.

3. Pentobarbital and flurazepam were more potent inhibitors of Ca\textsubscript{v}1.2 than Ca\textsubscript{v}1.3 channels, and desalkylflurazepam was also more potent at Ca\textsubscript{v}1.2 channels in a state-dependent manner.

4. Diltiazem was a more potent inhibitor of Ca\textsubscript{v}1.3 channels and inhibited Ca\textsubscript{v}1.3 with greater apparent use-dependence than Ca\textsubscript{v}1.2 channels.

5. In addition to a dissimilar manner of L-VGCC inhibition between diltiazem and BZs, mutation of a Ca\textsubscript{v}1.2 amino acid important for diltiazem potency did not alter diazepam
potency, and diltiazem did not antagonize diazepam-mediated inhibition of Ca\textsubscript{v}1.2 channels, suggesting that despite their structural similarity, BZs and BTZs act at distinct L-VGCC binding sites.

6. Mutation of the DHP binding site in Ca\textsubscript{v}1.2 channels reduced pentobarbital potency, suggesting that the barbiturate L-VGCC binding site may overlap that of DHPs.

7. Flumazenil, a competitive BZ antagonist at the GABA\textsubscript{A} receptor, did not inhibit L-VGCCs, yet antagonized BZ-mediated inhibition of Ca\textsubscript{v}1.3 but not Ca\textsubscript{v}1.2 channels, suggesting that a flumazenil binding site might exist on Ca\textsubscript{v}1.3 channels analogous to the GABA\textsubscript{A} receptor.

8. Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3, Ca\textsubscript{v}\beta\textsubscript{3}, and pSer\textsuperscript{1928}-Ca\textsubscript{v}1.2 expression was not altered in PSD-enriched CA1 homogenates from 1- and 2-day FZP-withdrawn rats, suggesting that an alternate mechanism of up-regulated L-VGCC function occurs following long-term FZP treatment.

9. CaMKII\textalpha, but not Thr\textsuperscript{286/287} autophosphorylated CaMKII expression was decreased in PSDs of CA1 SR asymmetric synapses in 2-day FZP-withdrawn rats.
5.2 Summary and inferences

Long-term administration of positive GABA<sub>A</sub> receptor modulators can lead to tolerance, especially to their sedative and anticonvulsant effects (File, 1985; Rosenberg & Chiu, 1985; Rosenberg et al., 1991), and with higher doses also increases the risk of developing physical dependence manifested by a characteristic withdrawal syndrome that is more severe for barbiturates and ethanol relative to BZs (Hodding et al., 1980; Griffiths & Weerts, 1997). Differences in the manifestation and severity of withdrawal from the various GABA<sub>A</sub> modulators likely pertain to their differential effects at other molecular targets. However, one common molecular mechanism proposed to contribute to withdrawal hyperexcitability induced by long-term exposure to BZs (Katsura et al., 2007; Xiang et al., 2008), barbiturates (Rabbani & Little, 1999), and ethanol (Messing et al., 1986; Katsura et al., 2006) is enhanced Ca<sup>2+</sup> influx through L-VGCCs. One aim of the current studies was to assess whether various GABA<sub>A</sub> receptor modulators directly inhibit L-VGCCs, both as a putative mechanism to mediate the enhanced L-VGCC function and a mechanism to prevent downstream affects of L-VGCCs until drug withdrawal.

Findings related to the possibility that GABA<sub>A</sub> receptor modulators directly inhibit recombinantly expressed Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 L-VGCCs were published in the Journal of Pharmacology and Experimental Therapeutics manuscript in Chapter 3 (Earl & Tietz, 2011). These findings supported a differential role of direct L-VGCC inhibition by various GABA<sub>A</sub> receptor modulators in contributing to the neuronal adaptations that take place during long-term drug exposure. While pentobarbital and ethanol directly inhibit L-VGCCs at clinically relevant concentrations, the concentrations of BZs and
allopregnanolone required to inhibit L-VGCCs are likely too high to be clinically relevant. These findings are consistent with the relative selectivity of these drugs for the GABA_A receptor, with ethanol having the greatest number of alternate molecular targets at clinically relevant concentrations, followed by barbiturates, then neurosteroids and BZs. Barbiturate and ethanol actions at alternate molecular targets, including L-VGCCs, may also contribute to the toxic effects of these drugs, such as memory, cognitive, and psychomotor impairment and potential for overdose toxicity, whereas BZs exhibit a relatively low toxicity profile (Griffiths & Johnson, 2005). Nevertheless, acute drug effects measured using recombinantly expressed L-VGCCs in HEK cells may not represent that which occurs during long-term treatment at native channels expressed in cultured neurons or *in vivo*. Notably, a significant concentration and use-dependent inhibition of VGCCs was measured by preincubation of cultured hippocampal neurons with flurazepam at concentrations as low as 1 µM (Xiang et al., 2008). However, when working with neuronal preparations effects on non-L-VGCC subtypes cannot be ruled out. The contribution of different VGCCs to BZ effects could be addressed in future experiments by pharmacologically and/or electrophysiologically isolating L-VGCC currents.

Interestingly, Ca_v 1.2 channels were more sensitive to inhibition by pentobarbital and FZP and were less sensitive to inhibition by the L-VGCC benzo-thiazepine antagonist, diltiazem, than Ca_v 1.3 channels. Selective inhibition could independently block the different Ca^{2+} signaling cascades mediated by Ca_v 1.2 and Ca_v 1.3 L-VGCCs. In particular, Ca_v 1.2 channels play a role in a protein synthesis-dependent form of L-LTP involved in memory formation (Moosmang et al., 2005), whereas Ca_v 1.3 channels impact neuronal
excitability and firing rates (Bowden et al., 2001; Putzier et al., 2009) and may promote age-related neuronal degeneration and cognitive dysfunction, as occurs in Alzheimer’s and Parkinson’s diseases (Thibault & Landfield, 1996; Porter et al., 1997; Chan et al., 2010). Thus, not only does selective inhibition provide a useful pharmacologic tool in dissecting the differential functions of L-VGCC subtypes, but may also be valuable for treating age-related neuronal degeneration, without impairing Ca\textsubscript{v}1.2-dependent memory. However, other side-effects related to Ca\textsubscript{v}1.3 inhibition may be an issue for therapeutic treatment. For example, Ca\textsubscript{v}1.3 knockout mice are congenitally deaf and display arrhythmias due to the important role of Ca\textsubscript{v}1.3 channels in the function of cochlear hair cells and sinoatrial node cells, respectively (Platzer et al., 2000).

\textit{GABA\textsubscript{A} receptor modulators inhibited L-VGCCs in a state-dependent manner, which appeared to involve a more selective interaction with the inactive and/or open state of channels. This state-dependent interaction lead to more potent inhibition of Ca\textsubscript{v}1.2 channels by desalkylflurazepam relative to Ca\textsubscript{v}1.3 channels (see Fig. 3.7), suggesting that differential potency dependent on the holding potential and frequency of activation can also be observed. Interestingly, the BTZ, diltiazem appeared to inhibit L-VGCCs in a manner distinct from BZs in terms of state- (Tables 3.2 and 3.3) and use-dependence (Fig. 3.3B,C). Moreover, mutation of an amino acid (Ile\textsubscript{1150}) important for diltiazem binding did not affect diazepam affinity, nor was diltiazem able to antagonize diazepam-mediated inhibition (Fig. 3.9). Together the data suggested that BZs interact with L-VGCCs at a site distinct from BTZs despite the structural similarity amongst these compounds. In contrast, pentobarbital potency was reduced 3.5-fold by mutations that}
reduce Ca_{1.2} sensitivity to DHPs (Fig. 3.8), suggesting that the barbiturate site on L-VGCCs may overlap that of DHPs.

It is interesting that all the positive GABA_\text{A} receptor modulators tested enhance currents through GABA_\text{A} receptors, but inhibit L-VGCCs. Thus, the current studies support a common finding amongst drugs that act at both anion and cation channels. In particular, drugs that enhance anion currents inhibit cation currents, and vice versa. Additional examples include the neurosteroid pregnenolone sulfate, a negative modulator at GABA_\text{A} receptors (Akk et al., 2001) but a positive modulator at NMDA receptors (Malayev et al., 2002) and several general anesthetics such as etomidate, isoflurane, propofol, sevoflurane, and nitrous oxide, which are generally positive modulators of GABA_\text{A} and glycine receptors but inhibitors of nicotinic acetylcholine, 5-HT_3, AMPA, kainate, and NMDA receptors (Grasshoff et al., 2006), as well as voltage-gated Na^+ channels (Ouyang et al., 2003). It is interesting to speculate that this effect may be due to intrinsic gating mechanisms that are opposite for anion and cation channels. However, this generalized principle is not true in all cases, and one exception includes DHPs, which inhibit L-VGCCs, as well as GABA_\text{A} receptors (Das et al., 2004).

The mechanisms underlying the neuronal adaptations that occur during treatment and the ensuing CA1 neuron hyperexcitability during withdrawal (Whittington & Little, 1991; Smith et al., 1998; Izzo et al., 2001; Van Sickle et al., 2004) remain unclear, but inhibition of L-VGCCs may contribute. Decreased GABA_\text{A} receptor function and alterations in GABA_\text{A} receptor subunit mRNA and protein expression during long-term treatment may result of chronic enhancement of GABA-mediated inhibition. However, other mechanisms are probably involved, since these changes are not identical between
GABA_A receptor modulators. For example, allosteric uncoupling of GABA and modulator binding sites is a common finding, but differences exist between GABA_A receptor modulators in which sites are uncoupled and the extent of uncoupling (Friedman et al., 1996). These differences may be explained by the varied molecular targets of GABA_A receptor modulators. For example, since GABA_A receptor function is affected by Ca^{2+}-mediated signaling cascades (Houston et al., 2009; Saliba et al., 2009), direct inhibition of L-VGCCs could promote GABA_A receptor dysfunction both acutely and by long-term treatment with barbiturates and ethanol. Moreover, the tight link between L-VGCC-mediated Ca^{2+} influx and gene transcription in neurons (Barbado et al., 2009), allows for the potential regulation of a wide variety of proteins, including GABA_A receptor subunits following long-term L-VGCC inhibition. For example, long-term exposure to both barbiturates and ethanol led to increased α6 subunit expression in the cerebellum (Ito et al., 1996; Sanna et al., 2004a). However, it remains to be determined if long-term inhibition of L-VGCCs alone is sufficient to induce changes in GABA_A receptor subunit expression, or if these changes might involve cross-talk amongst the other molecular targets of barbiturates and ethanol.

Direct inhibition of L-VGCCs by barbiturates and ethanol may also contribute to L-VGCC up-regulation observed following long-term drug exposure (Messing et al., 1986; Grant et al., 1993; Rabbani & Little, 1999; Katsura et al., 2006). In contrast, enhanced L-VGCC function following long-term BZ exposure (Katsura et al., 2007; Xiang et al., 2008) likely occurs via a different mechanism. One possibility is that BZs reduce L-VGCC activity indirectly via enhancement of GABA_A receptor-mediated hyperpolarization. This hypothesis is supported by the fact that 0.1 µM flumazenil was
able to antagonize up-regulated L-VGCC function in cortical cultures chronically exposed to 0.3 µM of diazepam, 1 µM brotizolam, or 1 µM clobazam (Katsura et al., 2007), suggesting that BZ binding to the GABA<sub>A</sub> receptor was required. Although the current studies support antagonism of BZ-mediated inhibition of Ca<sub>v</sub>1.3 channels by flumazenil (see Table 3.1), only a small degree of antagonism was observed and required a relatively high concentration (100 µM) of flumazenil. In addition, BZ antagonism was not observed at Ca<sub>v</sub>1.2 channels in the presence of up to 300 µM flumazenil. Thus, flumazenil likely precluded L-VGCC functional enhancement by preventing BZ binding to GABA<sub>A</sub> receptors in cultured neurons. It would be interesting to determine if flumazenil administration could similarly prevent the enhanced HVA Ca<sup>2+</sup> current density observed in CA1 neurons following long-term FZP treatment in vivo (Xiang et al., 2008).

Co-administration of a DHP with BZs in vitro (Katsura et al., 2007) or with ethanol in vivo (Whittington et al., 1991) also prevented up-regulation of L-VGCCs. This may suggest that increased Ca<sup>2+</sup> influx through L-VGCCs mediates the up-regulation. In hippocampal cultures Ca<sub>v</sub>1.2 trafficking to distal dendrites was enhanced in a Ca<sup>2+</sup>/CaM-dependent fashion upon K<sup>+</sup>-stimulated depolarization (Wang et al., 2007). However, this does not indicate incorporation into the plasma membrane as it was shown that neuronal cells exposed to prolonged depolarizing conditions resulted in a down-regulation of L-VGCC surface expression (DHP binding), which was dependent on Ca<sup>2+</sup> influx and calmodulin and recovered rapidly (DeLorme et al., 1988; Ferrante et al., 1991; Liu et al., 1994; Feron & Godfraind, 1995). The converse is therefore conceivable; that basal Ca<sup>2+</sup> influx through L-VGCCs would serve to maintain their membrane expression at
relatively constant levels, and reduced L-VGCC activity may result in a corresponding increase in surface expression. This would require a Ca\(^{2+}\) sensor capable of responding to lowered [Ca\(^{2+}\)]\(_i\), and/or changes in temporal Ca\(^{2+}\) dynamics. For example, in addition to the Thr\(^{286}\) autophosphorylation site on CaMKII that mediates autonomous activity, autophosphorylation of Thr\(^{305/306}\) prevents Ca\(^{2+}\)/CaM binding and inhibits holoenzyme activity (Hanson & Schulman, 1992). This dynamic regulation allows CaMKII to maintain a short-term molecular memory of past Ca\(^{2+}\) signals, and its activity can therefore be modulated by changes in the frequency and amplitude of Ca\(^{2+}\) oscillations (De Koninck & Schulman, 1998). In addition, Ca\(_{v1.2}\) and Ca\(_{v1.3}\) L-VGCCs themselves are coupled to different intracellular signaling complexes and respond to different stimulation intensities (Zhang et al., 2006), suggesting that changes in neuronal activity can differentially activate L-VGCC subtype signaling cascades. Thus, complete blockade of L-VGCC-mediated Ca\(^{2+}\) influx by a DHP could also prevent a Ca\(^{2+}\)-dependent enhancement of L-VGCC surface expression resulting from reduced (but not abolished) L-VGCC activity.

It is also possible that the BZ-GABA\(_A\) receptor interaction paradoxically increases L-VGCC-mediated Ca\(^{2+}\) influx during FZP treatment, evidenced by the possibly extrasynaptic GABA\(_A\) receptor-mediated depolarization observed during FZP withdrawal (Zeng et al., 1995; Zeng & Tietz, 2000). However, if this same mechanism existed during treatment, it would mediate increased CA1 neuron activity and NMDA-dependent and potentially L-VGCC-dependent plasticity, which seems counter-intuitive. In order for BZs to maintain anxiolytic effects, activity of the neuronal circuits mediating anxiety must still be reduced during treatment. On the other hand, if functional inhibition is
maintained in other parts of the circuit, this may allow for localized dendritic hyperexcitability in CA1 neurons during treatment, while still reducing overall activity through the circuit. Distinguishing these possibilities would require measurement of GABA-mediated Cl\(^-\), HCO\(_3^-\), and Ca\(^{2+}\) fluxes in CA1 neurons during FZP treatment. There is a practical difficulty in doing this due to the continued presence of BZs in the brain during treatment, which would interfere with these measurements. This could potentially be avoided by competitively antagonizing BZs from their GABA\(_A\) receptor binding sites using flumazenil prior to experimental manipulations. Clearly, additional studies are needed to decipher the complex regulation of ion channels by GABA\(_A\) receptor modulators, keeping in mind the importance of interneuron networks on neuronal activity in vivo, which may be lost with in vitro studies.

In conjunction with enhanced voltage-dependent Ca\(^{2+}\) influx, numerous studies detected an increased number of radiolabeled L-VGCC antagonist binding sites on neuronal membranes following long-term exposure to BZs (Katsura et al., 2007), barbiturates (Rabbani & Little, 1999), and ethanol (Messing et al., 1986; Whittington et al., 1991; Katsura et al., 2006). Therefore, the second aim was to determine if expression of L-VGCC subunits is altered during FZP withdrawal. In particular, Ca\(_{\alpha1.3}\) channels, which activate at relatively negative membrane potentials, could mediate the negative shift in the voltage-dependence of HVA Ca\(^{2+}\) channel activation observed following long-term FZP treatment (Xiang et al., 2008). A number of changes in excitatory receptor function also occur during FZP withdrawal. Notably, previous findings suggested that L-VGCC-mediated Ca\(^{2+}\) influx may lead to insertion of homomeric GluA1 AMPA receptors into CA1 neuron PSDs during the first day of FZP withdrawal (Van Sickle et
Further, L-VGCCs maintain AMPA receptor potentiation (Xiang et al., 2008) and may also induce the CaMKII-mediated enhancement of GluA1-containing AMPA receptor conductance during the second day of withdrawal (Shen et al., 2009). However, the extent of CaMKII Thr\textsuperscript{286} autophosphorylation detected in immunoblots was not changed during FZP withdrawal (Shen et al., 2010), suggesting that an alternative mechanism of CaMKII autonomous activity and/or translocation of autophosphorylated CaMKII to the PSD is induced by L-VGCCs. Thus, the final aim of the current studies was to assess the expression level and postsynaptic localization of total CaMKII and its Thr\textsuperscript{286} autophosphorylated form in asymmetric synapses located in the CA1 SR region during FZP withdrawal.

Findings contained in the second manuscript (Chapter 4) indicated that enhanced L-VGCC function during FZP withdrawal was not explained by a simple increase in L-VGCC subunit expression. Other possibilities include enhanced trafficking of channels from intracellular compartments to the plasma membrane and/or post-translational modifications such as phosphorylation, which regulate channel gating. One well-characterized site on the Ca\textsubscript{v}1.2 subunit, Ser\textsuperscript{1928}, enhances time spent in the in open state when phosphorylated by PKA, PKC, or PKG (De Jongh et al., 1996; Yang et al., 2005; Yang et al., 2007). However, there was no change in the Ca\textsubscript{v}1.2 Ser\textsuperscript{1928} phosphorylation state after 2 days of FZP withdrawal. Numerous other phosphorylation sites have also been characterized on both Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunits, including sites regulated by CaMKII, which mediates Ca\textsuperscript{2+}-dependent facilitation of channel function (Hudmon et al., 2004; Song et al., 2007; Xiang & Tietz, 2007; Das et al., 2008; Xiang et al., 2008).
2005; Jenkins et al., 2010). Creation of an antibody to measure phosphorylation at these sites may reveal if L-VGCC function is enhanced in a Ca\(^{2+}\)-dependent manner.

Studies of CaMKII\(\alpha\) and Thr\(^{286}\) autophosphorylated CaMKII expression and synaptic localization at the EM level revealed that CaMKII\(\alpha\), but not pCaMKII expression was decreased in CA1 postsyanpses after 2 days of FZP withdrawal. This resulted in a trend towards an increased ratio of pCaMKII/CaMKII within the PSD, suggesting that a greater proportion of CaMKII molecules are autophosphorylated at Thr\(^{286}\) during FZP withdrawal. The loss of CaMKII\(\alpha\) may relate to the significant decrease in GluN2B-subunit containing NMDA receptors at the same withdrawal time point. While this correlation alone does not indicate an increased interaction, it is possible that drug-induced enhancement of L-VGCC-mediated Ca\(^{2+}\) influx may lead to an increased interaction of CaMKII with GluN2B to increase CaMKII autonomous activity (Bayer et al., 2001). Subsequent to CaMKII-mediated potentiation of AMPA receptor currents and conductance (Shen et al., 2009; Shen et al., 2010), removal of GluN2B-CaMKII complexes from the PSD would serve to homestatically reverse AMPA receptor-mediated hyperexcitability, attenuating withdrawal anxiety. Since similar mechanisms are not observed during barbiturate or ethanol withdrawal, this may explain why the withdrawal syndrome for BZs is much less severe compared to the former non-selective GABA\(A\) receptor modulators.
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