Health Science Campus

FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

Neuronal Adaptations in Rat Hippocampal CA1 Neurons during Withdrawal from Prolonged Flurazepam Exposure: Glutamatergic System Remodeling

Submitted by:
Jun Song

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

Examination Committee

Major Advisor: Elizabeth Tietz, Ph.D.
Academic Advisory Committee:
Howard C. Rosenberg, Ph.D.
L. John Greenfield Jr., Ph.D.
David r. Giovannucci, Ph.D.
Zi-Jian Xie, Ph.D.

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: April 18, 2007
Neuronal Adaptations in Rat Hippocampal CA1 Neurons
during Withdrawal from Prolonged Flurazepam Exposure:
Glutamatergic System Remodeling

Jun Song
Biomedical Sciences PhD Program
Cellular and Molecular Neurobiology Program
College of Medicine
University of Toledo, Health Science Campus
2007
ACKNOWLEDGEMENTS

I would especially like to take the opportunity to thank Dr. Elizabeth I. Tietz, my major advisor, for her meticulous mentoring in my five-year study here. As a teacher and mentor, she has taught me more than I could ever give her credit for. It is her bright ideas and encouragement that provide the power of my research. Her thorough guidance has enabled me to capture opportunities and achieve what I have accomplished. She is the strongest advocate of my success.

I would also like to express my acknowledgement to my committee members, Drs. Howard Rosenberg, John Greenfield, Zi-jian Xie, and David Giovannucci. They are dedicated, considerate, open-minded scientists and teachers, and have great accomplishments in the academic field. Each of them has provided me extensive personal and professional guidance, and taught me a great deal about scientific research and life in general. I also want to extend my gratitude to Dr. Linda Dokas and Dr. William Gunning, for their shared expertise and enthusiasm to contribute to my personal and professional progress.

I’m also very grateful to all of my laboratory partners, Paromita Das, Guofu Shen, Kun Xiang, Yingzhe Li, Krista Pettee, Kathleen Davis, William J. Ferencak III, Margarete Otting and Eugene Orlowski, who have been supportive of my career goals and who work actively to provide me pleased environment for conducting my experiment. This work would not have been possible without the support of my colleagues, including Rebecca Pierson, Christine Brink, Adriane Sumner, Tyler Hendershot, as well as Christian Peters working with Dr. Giovannucci and Nathalie Boulineau from Dr. Greenfield’s lab. They are the persons who afford technical assistance and encouragement whenever I need.
In my last five years, there are many to thank for the persons working in the department of Pharmacology and the department of Neurosciences. They provide valuable opportunities for me to communicate with and learn from other students and scientists. I am especially indebted to Martha Heck, Debbie LeBarr, Ruth Dohse and Shirley Wosniak who have been supportive of my social life and career goals.

Nobody has been more important to me in the pursuit of my PhD degree than the members of my family, my parents and my sister Jie. Their love and support were the source of my strength to pursue my goals and happiness. Most importantly, I wish to thank my loving and supportive husband, Lijun, and my daughter, Carol, who provide me enduring inspiration forever.
# TABLE OF CONTENTS

- **INTRODUCTION** ........................................................................................................... 1
- **LITERATURE REVIEW** .................................................................................................. 12
- **MANUSCRIPT 1** ........................................................................................................... 48
- **MANUSCRIPT 2** ........................................................................................................... 100
- **MANUSCRIPT 3** ........................................................................................................... 134
- **CONCLUSIONS** .......................................................................................................... 194
- **SUMMARY** .................................................................................................................... 196
- **BIBLIOGRAPHY** .......................................................................................................... 201
- **ABSTRACT** ................................................................................................................... 244
INTRODUCTION

In the mid of 1950’s Leo Sternbach’s group synthesized the first 1,4-benzodiazepine, chlordiazepoxide, which proved to have anticonvulsant, muscle relaxant and taming effects in mice and monkeys (Sternbach, 1979, 1994). In 1960, benzodiazepines (BZs) were first prescribed. BZs are extremely effective anxiolytic agents, as well as being used for insomnia, convulsive disorders, muscle relaxation and sedation. Following the introduction of chlordiazepoxide (Librium), many related BZ compounds with varying potency and pharmacokinetic properties were developed, and because of their safety and efficacy, BZs became the most popularly prescribed drugs in the 1960s and 1970s. However, several side effects of these drugs became apparent, like drowsiness, confusion, memory impairment, etc. Furthermore, it became apparent that more serious problems occurred following long-term drug exposure. For example, tolerance occurs, i.e. the original dose of the drug has progressively less effect and a higher dose is required to obtain the original effect; and patients become both physically and psychologically dependent upon BZs, experiencing acute or chronic withdrawal phenomena after abrupt cessation of treatment. Furthermore, BZs have also been classified as drugs of abuse. It was reported that about 80% of BZ abuse is in conjunction with other abused drugs (Griffiths and Johnson, 2005; Wafford, 2005). Because of this, the use of BZs has been reduced in recent years. But they are still among highly prescribed drugs.
BZs act by enhancing the effect of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). They bind directly to a site on the receptor, altering the functional response upon receptor activation by GABA. In 1987 the first GABA_\text{A} receptor subunit was reported and demonstrated to belong to the super-family of ligand-gated ion channels (Schofield et al., 1987). This family has become known as the ‘cys-loop’ family because of the presence of a cysteine loop in their N-terminal domain. GABA_\text{A} receptors exist as pentamers, arranged around a central ion channel, composed of a wide variety of subunit combinations with distinct physiological and pharmacological properties. To date, there are seven different subunit classes with multiple members, including α (1-6), β (1-3), γ (1-3), δ, π, θ and ε (Macdonald and Olsen, 1994). The five subunits of GABA_\text{A} receptors are arranged to form a channel that is selectively permeable to chloride (and bicarbonate) ions. GABA binding extracellularly at the interface of α and β subunits increases the open probability of the channel. The inflow of chloride ions hyperpolarizes the cell, and decreases the likelihood of the neuronal firing and action potential generation. The vast majority of GABA_\text{A} receptors are characterized by their sensitivity to BZs. These receptors contain α1, α2, α3 or α5 subunits, β subunits (mainly β2 or β3) and a γ subunit are sensitive to BZs. GABA_\text{A} receptors comprising two α subunits, two β subunits and an additional γ2 subunit confers a key part of the required binding site for BZs. On the other hand, the binding sites for BZs and GABA on the receptors are “coupled”, i.e. the binding of one ligand will increase the binding of the other. As a consequence, BZs allosterically
increase the affinity of GABA to its receptor, and increase the frequency of chloride ion channel openings (Olsen and Leeb-Lundberg, 1981; Paul et al., 1981).

It is well established that following chronic treatment with BZs, there are alterations in GABAergic neurotransmission, which contribute to tolerance and may be involved in the symptoms of withdrawal and dependence. The nature and mechanism of these changes are still unclear. However, they are closely linked to the dose of BZ, the manner of drug administration and the duration of usage (Allison and Pratt, 2003; Kan et al., 2004). Tolerance is defined as the decreased ability of the drug to produce the same level of pharmacological effects overtime. Since BZs act directly on GABA \(_A\) receptors, downregulation of GABA \(_A\) receptor subunit expression has been thought to be one potential mechanism for tolerance. Studies aimed at testing this hypothesis reported conflicting findings: a reduction of GABA \(_A\) receptor \(\alpha1\) and \(\beta3\) subunit mRNA expression and protein level, as well as an upregulation of \(\beta2\) mRNA were observed after 1-week flurazepam (FZP) exposure (Chen et al., 1999; Tietz et al., 1999a); in another study, prolonged FZP treatment resulted in no changes in the levels of \(\alpha1\)-, \(\alpha2\)-, \(\gamma2\)- or all three \(\beta\)-subunit mRNAs, whereas the \(\alpha3\)- and \(\alpha6\)-subunit mRNAs were sequentially increased over the treatment period (O'Donovan et al., 1992).

BZ binding sites have been reported to be decreased (Rosenberg and Chiu, 1981; Miller et al., 1988; Miller et al., 1989) or unchanged (Gallagher et al., 1984; Farb et al., 1986; Roca et al., 1990) under varied exposure and withdrawal conditions. However, increasing evidence has shown that uncoupling of the allosteric linkage between the
GABA and BZ binding sites, changes in receptor subunit turnover and changes in receptor gene expression are also related to BZ tolerance (Bateson, 2002).

Drug dependence refers to drug-induced adaptations that compensate for drug exposure and lead to a series of withdrawal symptoms after drug cessation. In contrast to BZ tolerance, which has been associated with changes in GABA\textsubscript{A} receptor expression, assembly (Kang and Miller, 1991; Primus and Gallager, 1992; Impagnatiello et al., 1996), and allosteric uncoupling (Klein and Harris, 1996; Primus et al., 1996), the molecular mechanisms underlying dependence after BZ withdrawal have not been well characterized. In addition to the GABAergic system (Allison and Pratt, 2003; Wafford, 2005), the glutamatergic system has been reported to be involved in BZ dependence (Izzo et al., 2001; Allison and Pratt, 2003). Pharmacological experiments with antagonists of specific glutamate receptor subtypes suggested that both \textit{N}-methyl-\textit{D}-aspartate (NMDA) receptor- and \textit{alpha}-amino-3-hydroxy-5- methylisoxazole-4-propionic acid receptor (AMPAR)-dependent mechanisms might underlie the expression of BZ dependence. For example, treatment with NMDAR inhibitors (CPP or CGP 39551) prevented the reduction in pentylenetetrazole-induced seizure threshold, abolished repeat BZ withdrawn-enhanced seizure sensitivity or eliminated the withdrawal symptoms (Steppuhn and Turski, 1993; Koff et al., 1997; Dunworth and Stephens, 1998) suggesting that NMDARs may play a in BZ dependence. Moreover, it was reported that AMPAR GluR1 subunit mRNA and protein was augmented in cortex and hippocampus in association with anxiety-like behavior (Izzo et al., 2001). The amplitude of AMPAR-mediated mEPSCs was enhanced
with the presence of BZ dependence, and pretreatment with the AMPAR specific antagonist, GYKI-52466, abolished BZ withdrawal-induced anxiety in rats (Van Sickle et al., 2004; Xiang and Tietz, 2007).Taken together, these data suggested that the dependence elicited by BZ withdrawal maybe associated with increased glutamatergic strength, especially AMPAR activation.

The remodeling of AMPARs was thought to be an important neural substrate of drug dependence, addiction and other models of activity-dependent plasticity (Malinow and Malenka, 2002; Malenka, 2003; Glass et al., 2005; Nestler, 2005). AMPAR function could be affected by a change in protein expression, a switch in subunit composition, altered phosphorylation status and redistribution of AMPARs to membranes/synapses. The mechanisms underlying enhanced AMPAR expression in activity-dependent plasticity remains unclear. There are several macromolecules have been found to regulate AMPAR synthesis, including brain-derived neurotrophic factor (BDNF), cAMP-dependent protein kinase (PKA) and metabotropic glutamate receptors. In rodent neocortical neurons, BDNF application lead to enhanced expression of AMPAR GluR1 and GluR2/3 subunits via Src-family protein tyrosine kinases (Narisawa-Saito et al., 1999). During hippocampal long-term potentiation (LTP), AMPAR synthesis could be blocked by inhibitors of PKA and transcription, indicating the crucial roles for PKA in regulating AMPAR expression (Nayak et al., 1998). Moreover, pharmacological activation of metabotropic glutamate receptors in the dendrites of neurons in primary hippocampal cell culture result in GluR2
mRNAs being translated into proteins and inserted into the plasma membrane (Kacharmina et al., 2000).

Three of most important neurophysiological characteristics of AMPARs are calcium permeability, polyamine blockade and current rectification, which are all dependent on the presence of the GluR2 subunit (Van Damme et al., 2002). A positively charged arginine at site 586 (Q/R editing site in the intro-membrane loop) instead of glutamine, results in GluR2-containing AMPARs are calcium-impermeable, not susceptible to polyamine blockade and outwardly rectifying (Hume et al., 1991; Van Damme et al., 2002). In contrast, GluR2-lacking AMPARs, such as GluR1 homomers, are calcium-permeable, easily blocked by polyamines and display inward rectification (Hollmann et al., 1991; Verdoorn et al., 1991; Herlitze et al., 1993). An AMPAR subtype switch has been observed following both physiological and pathological states including: development, synaptic activation, ischemia and epilepsy, (Pellegrini-Giampietro et al., 1992; Pellegrini-Giampietro et al., 1997; Liu and Cull-Candy, 2000; Kumar et al., 2002; Krestel et al., 2004; Liu et al., 2006). In hippocampal organotypic slices, BDNF exposure, causing synaptic activation and/or changes in gene expression underlying synaptic plasticity, enhanced synaptic incorporation of GluR1 homomers, which is independent of NMDAR activation (Caldeira et al., 2007). Brief oxygen-glucose deprivation, an in vitro model of brain ischemia, promoted GluR2-containing AMPARs endocytosis from synapses and delivery of GluR2-lacking AMPARs to synaptic sites (Liu et al., 2006).
Protein kinases can also critically regulate AMPAR function and subsequent synaptic plasticity in hippocampus. Calcium/calmodulin-dependent protein kinase II (CaMKII) and PKA have both been implicated in the regulation of AMPAR function. During LTP, CaMKII signaling drives synaptic delivery of AMPARs through interactions between GluR1 and PDZ (PSD-95, Dlg and ZO-1)-domain-containing proteins, such as postsynaptic density 95 (PSD95) and synapse-associated protein 97 (SAP97) (Hayashi et al., 2000; Zhu et al., 2002); AMPAR channel conductance was also elevated by CaMKII activation (Derkach et al., 1999). On the other hand, a fundamental role for PKA has been found in many forms of synaptic plasticity that bring about morphological and physiological alterations, as well as in models of long-term synaptic plasticity. PKA phosphorylation of GluR1 and GluR4 controls synaptic delivery of AMPARs (Esteban et al., 2003); PKA activity is required for AMPAR synthesis and the maintenance of LTP (Nayak et al., 1998); repeated activation of PKA in hippocampal slices enhanced synaptic transmission efficiency, concomitant with an increase in the number of perforated synapses (Urakubo et al., 2006). The mechanisms underlying PKA phosphorylation of AMPARs have been comprehensively investigated. PKA was demonstrated to recruit AMPARs through a membrane-associated guanylate kinase family/A kinase-anchoring protein (MAGUK/AKAP) complex (Colledge et al., 2000). In cultured hippocampal neurons, phosphorylation of AMPARs is enhanced by a SAP97-AKAP79/150 complex that directs PKA to GluR1 via a PDZ domain interaction (Colledge et al., 2000). As a crucial member of the MAGUK family, SAP97 binds specifically to GluR1 but not other
AMPAR subunits, acting as a scaffolding protein (Leonard et al., 1998). In another study, Sans et al. found that interactions between SAP97 and GluR1 could occur early in the secretory pathway, while the receptors were still in the endoplasmic reticulum or Golgi apparatus (Sans et al., 2001). Using light and electron microscopic immunocytochemistry, Valtchanoff et al. demonstrated the cellular and synaptic colocalization of SAP97 and GluR1 in rat cerebral cortex, and showed that SAP97 concentrates at synapses that contain GluR1 subunits, but not necessarily GluR2 or GluR3 subunits (Valtschanoff et al., 2000). Collectively, these findings indicate that SAP97 has dual roles in regulating AMPAR delivery by releasing GluR1 from an intracellular pool and targeting AMPARs to membranes/synapses. Therefore, clarifying the interactions between GluR1-SAP97-AKAP-PKA complexes will be beneficial in understanding the mechanisms underlying AMPAR-involved activity-dependent plasticity, in particular associated with benzodiazepine withdrawal.

To investigate neuronal adaptive remodeling after prolonged benzodiazepine exposure, our laboratory utilizes a water-soluble benzodiazepine, FZP, chronic treatment model. Uncoupling of GABA/benzodiazepine binding sites, reduced benzodiazepine binding and decreases in GABA_A receptor mRNAs and subunit proteins were observed specifically in hippocampus and cortex, associated with the occurrence of anticonvulsant tolerance (Rosenberg et al., 1985; Tietz et al., 1989; Chen et al., 1999; Tietz et al., 1999a; Zeng and Tietz, 1999). In conjunction with GABAergic dysfunction, our recent studies reveal that benzodiazepine withdrawal symptoms, manifest as anxiety-like behavior, depend on
glutamatergic system remodeling and are brain region specific. AMPAR antagonist binding and the amplitude of miniature excitatory postsynaptic currents (mEPSCs) are enhanced in hippocampal CA1 neurons but not in dentate granule cells during FZP withdrawal (Van Sickle and Tietz, 2002); injection of the AMPAR antagonist GYKI-52466 at the onset of withdrawal eliminated increased AMPAR-mediated currents and withdrawal-anxiety (Van Sickle et al., 2004; Xiang and Tietz, 2007). However, the precise mechanisms underlying FZP-withdrawal-induced glutamatergic remodeling in hippocampus remains unresolved.

Therefore, in a series of studies, electrophysiological, immunohistochemical and molecular methods were used to further delineate the mechanisms underlying AMPAR potentiation in CA1 neurons associated with BZ withdrawal. In the first manuscript, whole-cell recordings on acutely dissociated hippocampal CA1 pyramidal neurons and hippocampal slices were used to investigate AMPAR kinetics, rectification and polyamine blockade in control and 2-day FZP-withdrawn rats. Significant increases in the amplitude of both glutamate- and kainate-induced AMPAR currents were observed after FZP withdrawal, in the absence of alterations in receptor kinetics. A reduced rectification index and enhanced spermine blockade suggest that more GluR2-lacking AMPARs were incorporated into membrane/synapse during FZP withdrawal. Immunofluorescence studies showed surface expression of GluR1 but not GluR2 subunit was augmented in response to FZP withdrawal, in agreement with electrophysiological findings and suggested that membrane-inserted AMPARs were shifted toward GluR1-containing
receptors. Additionally, surface colocalization of GluR1 and GluR2 was also increased in CA1 pyramidal neurons. Given that there were no alterations in GluR2 membrane incorporation, these data indicate that membrane-associated AMPAR subunit was switched from GluR2/3 composition to GluR1 homomers and GluR1/2 heteromers. GluR2-lacking AMPARs have higher conductance than GluR2-containing heteromers (Swanson et al., 1997), the increased GluR1 homomers in our study may contribute to BZ-withdrawal-induced AMPAR potentiation.

As mentioned above, increased protein level, enhanced phosphorylation and membrane/synapse trafficking could also result in glutamatergic strength. To clarify the molecular mechanisms underlying AMPAR remodeling after FZP withdrawal, the protein level of GluR1 and GluR2 subunits were investigated in the second manuscript by subcellular fractionation and immunoblotting. Increased expression of GluR1, rather than GluR2, was found in cytosolic, crude membrane and PSD-enriched fractions, which may contribute to upregulation of surface GluR1 subunit incorporation. As AMPAR function and trafficking is fundamentally controlled by scaffolding proteins and protein kinases, immunoblotting assays focusing on phospho-Ser\textsuperscript{845} GluR1, SAP97, AKAP and PKA were conducted on subcellular fractions of CA1 minislices from control and 2-day FZP-withdrawn rats. Notably, consistently significant increases in phospho-Ser\textsuperscript{845} GluR1, SAP97 and AKAP79/150 were observed in crude membrane and PSD-enriched compartments; while no alterations in PKA regulatory and catalytic subunits were observed. The proportionate upregulation of phospho-Ser\textsuperscript{845} GluR1 associated with
enhanced total GluR1 levels, concomitant with increases in SAP97 and AKAP proteins, indicated that FZP withdrawal induced a greater number of AMPAR GluR1 subunits to be recruited by PKA through SAP97-AKAP interactions, and also facilitated AMPAR phosphorylation and subsequent potentiation.

Furthermore, to achieve whole-scale profiling of benzodiazepine-withdrawal-induced GABAergic/glutamatergic remodeling, the third manuscript describes microarray analysis of mRNA derived from CA1 minislices coupled with real-time PCR confirmation. Twenty-seven differentially regulated genes were found by microarray analysis, including a number of genes involved in neuronal plasticity (BDNF, CAMKIIB, HPCA and APP), neurotransmitter release (BDNF, SYN2, STXB1, SYT11 and APP), nervous system development, cytoskeleton protein organization, ion homeostasis etc. Surprisingly, given the previous findings from our laboratory (Tietz et al., 1993; Tietz et al., 1999a; Song and Tietz, 2005, Soc. Neurosci Abstr.), there were no alterations in the expression of GABA receptor or glutamate receptor at mRNA level transcripts by microarray analysis. All but one gene product (hippocalcin) were confirmed by real-time PCR. We then constructed functional pathways involving these regulated genes by using Ingenuity Pathway Analysis. Two major pathways identified included LTP-associated and neurotransmission pathways. Finally, the differentially regulated gene transcripts were validated at the protein level by confocal analysis of immunofluorescence. Expression of CaMKIIB and the colocalization of CaMKIIB and GluR1 were enhanced during FZP withdrawal, indicating that more CaMKIIα may be targeted to GluR1 subunits by CaMKIIB and thus facilitate receptor
phosphorylation. There were no alterations in the levels of hippocalcin or GluR2/3 subunit proteins, consistent with our earlier findings; while more GluR2/3-containing AMPARs were colocalized with hippocalcin in CA1 pyramidal neuron somata during FZP withdrawal. Given that hippocalcin couples with the AP2 adaptor complex and is thought to be involved in GluR2 subunit endocytosis in long-term depression (LTD) (Palmer et al., 2005), the increased colocalization between hippocalcin and GluR2/3 indicates that hippocalcin may play a role in AMPAR subunit switching by internalizing GluR2/3 heteromers.

Taken together, our findings comprehensively define key macromolecules and signaling pathways involved in BZ withdrawal-induced glutamatergic remodeling. To our knowledge, this is the first report suggesting a pivotal role for an AMPAR subunit switch and SAP97-AKAP-PKA interactions in BZ withdrawal. It is also the first investigation of mRNA expression profiling after chronic BZ administration using microarray techniques coupled with Ingenuity Pathway Analysis. Our data indicate possible roles for AMPAR synthesis, protein phosphorylation and a subunit switch in drug dependence and provide evidence that benzodiazepine dependence shares similar mechanisms with neuronal plasticity.
LITERATURE REVIEW

Benzodiazepine effects and GABA$_A$ receptors

BZs were introduced into clinical practice in the 1950s, as a class of effective drugs commonly used as tranquillizers and sleeping pills. The BZs are currently among the most frequently prescribed of all drugs for their anxiolytic, anticonvulsant, and sedative/hypnotic properties. Compared with their non-selective predecessors, BZs exhibit a safer profile by interacting with a specific binding site (alpha/gamma subunit interface) on the GABA$_A$ receptor complex. BZ binding potentiates the activity of this major inhibitory neurotransmitter in mammalian brain, GABA, by increasing the affinity of at least one agonist binding site (alpha/beta interface) of GABA$_A$ receptors. This phenomenon was called allosteric coupling (Costa et al., 1978). BZ binding causes increased frequency of opening of the GABA$_A$ receptor chloride channel and subsequent membrane hyper-polarization, which inhibits neuronal excitation.

GABA$_A$ receptors, made up of many different subtypes, exist as pentamers arranged around a central ion channel. To date, there are 18 different subunits have been isolated not including alternatively spliced variants (Wafford, 2005). The structure of four transmembrane components of each subunit is conserved with an extracellular N-terminal domain, transmembrane domain (TM)1-3 with a N-glycosylated domain of about 220 amino acid residues, and a longer residue chain between TM3 and TM4 that forms an intracellular loop and a small extracellular C terminal tail (Costa et al., 2002). The BZ binding site is located in the extracellular N-terminal portion of the receptor, and both $\alpha$
and γ subunits contribute to this binding locus. Through site-directed mutagenesis, it was demonstrated that several crucial amino acids are involved in BZ binding or function, for example, His101, Tyr159, Gly200, Thr206 and Tyr209 in the α1 subunit; and Phe77, Ala79, Thr81 and Met130 in the γ2 subunit (Wafford, 2005).

BZ actions vary depending upon the specific subunit composition of GABA_\text{A} receptors. On type A GABA receptors, the BZ binding site is composed of γ and α1-3, 5 subunits. The α4 and α6 subunits combine with β and γ subunits to produce receptors those are insensitive to classical BZs. Different subtypes of α subunits mediate the diverse pharmacology of BZs. It was reported that hetero-oligomeric GABA_\text{A} receptors containing α1 subunits mediate the sedative, amnestic, and anticonvulsant activity of the BZs (Rudolph et al., 1999); while the anxiolytic action of BZs was mediated via α2-containing GABA_\text{A} receptors (Rudolph et al., 1999; Low et al., 2000). GABA_\text{A} receptors with α5 subunits and those expressing α2 or α3 subunits that are located on motor neurons and in the dorsal horn of spinal cord may be involved in mediating the muscle relaxant activity of diazepam (Bohlhalter et al., 1996). Furthermore, the three γ-subunit isoforms also confer differing functional properties: BZs with high affinity for γ2-subunit containing receptors show increased efficacy for potentiation of GABA-induced current (Puia et al., 1991; Herb et al., 1992); the neutral modulator, i.e. the antagonist, flumazenil doesn’t recognize γ1-subunit containing receptors (Benke et al., 1996); and classical BZs show higher affinity for γ2- than γ3-subunit containing receptors (Herb et al., 1992).
Benzodiazepine tolerance

Prolonged BZs exposure will cause tolerance and dependence, which has limited the clinical utility of these agents. An explanation for the development of tolerance and physical dependence has been sought for a number of decades. To date, the underlying mechanisms are still unclear.

Tolerance has been defined as the reduction in the ability of a drug on repeated exposure to produce the same level pharmacological effect or the requirement to increase the amount of drug intake to obtain the same effect. Tolerance to the sedative effects of classical benzodiazepines has been widely reported in both animals and humans (Miller et al., 1988; Soldatos et al., 1999). Tolerance to the anxiolytic effects in human is difficult to demonstrate. In animal studies, the plus maze test has been widely used to examine anxiety-like behavior in rats or mice. One of the most important features of tolerance development is the differential timescale of its development. It has been demonstrated that tolerance to the sedative effects of benzodiazepines occurs more rapidly (within 3-5 days) than to the anticonvulsant effects (after 5 days) (File, 1985), which suggest that there are different BZ targets or various signaling pathways involved in the development of tolerance.

Binding of BZs and GABA to GABA_A receptors are reciprocally coupled, that is, binding of one enhances the binding of the other. Therefore, a change in coupling between the binding sites may contribute to the reduced effects of BZ upon chronic treatment. Primary cultured neurons have proven to be an effective tool to analyze the
pharmacological properties of GABA_\textsubscript{A} receptors. In rat cerebellum granule neurons, chronic flunitrazepam exposure reduced the maximal GABA-stimulated increase in extracellular acidification rate and also abolished the flunitrazepam potentiation of the GABA response (Brown et al., 1998). Also in cultured chick cortical neurons, chronic treatment with FZP caused reversible uncoupling of BZ and GABA recognition sites with a t_{1/2} of about 18 hours, similar to the half-time for receptor turnover (Roca et al., 1990). Using animal model, which produced behavioral tolerance in rats with 3 week-diazepam treatment, there is a decreased responsiveness to the stimulation of $^{36}$Cl- influx by GABA and a corresponding decrease in the ability of flunitrazepam to enhance GABA-stimulated $^{36}$Cl-influx in cortical, rather than in cerebellar tissue (Marley and Gallager, 1989). Further evidence in support of this uncoupling theory was produced by a study with FZP (Tietz et al., 1989) in which allosteric coupling between the BZ and GABA recognition sites was compromised in cortex, but not in cerebellum or medulla immediately after 4-week FZP treatment; while acute treatment has no effect on BZ-GABA coupling. These \textit{in vitro} and \textit{in vivo} experiments indicated that prolonged BZ exposure can induce BZ-GABA binding site uncoupling specific to local brain regions.

Given the time course in which BZ-GABA uncoupling occurred and recovered, both post-translational receptor modification and receptor internalization have been investigated. In some studies, the BZ/GABA uncoupling is coordinated with decreased receptor numbers (Lyons et al., 2001), suggesting the uncoupling represents a reduction in GABA_\textsubscript{A} receptor expression or an increase in its internalization. On the other hand,
Uncoupling was found without changes in the number of BZ binding sites or the affinity of BZ binding (Roca et al., 1990; Primus et al., 1996). In order to isolate the post-transcriptional effects on GABA_A receptor uncoupling, a number of groups examined the effects of prolonged BZ exposure on the allosteric linkage between the BZ and GABA binding sites. In one study, mouse Ltk^- cells, stably transfected with GABA_A receptors were treated with flunitrazepam or clonazepam (Klein et al., 1994). They found exposure of cells expressing α1, β1- and γ2L-subunit cDNAs to clonazepam or flunitrazepam caused a time- and dose-dependent reduction in the ability of GABA to potentiate BZ binding, whereas K_D (dissociation constant) and B_max (maximum number of binding sites) were unaffected. This observed uncoupling was also reversible. Their results suggest that the uncoupling observed in this system was not accompanied by receptor internalization; and as the expression of the receptor subunit cDNAs was controlled by the artificial promoter, it is unlikely to be due to changes in receptor subunit composition. Furthermore, using a different expression system (Sf9 cells), researchers (Primus et al., 1996) demonstrated that uncoupling occurs with other GABA_A receptor subtypes. It was reported that expressing GABA_A receptor subunit cDNAs from baculovirus vectors in the insect cell line Sf9 could result in large amounts of receptor incorporated into the plasma membrane that displays appropriate biological properties, and that the level of expression of the GABA_A receptor complex varies with the levels of expression of the individual subunits (Hartnett et al., 1996). Primus et al. examined the effects of chronic exposure to various BZ-site ligands on α1/β2/γ2, α2/β2/γ2 and α5/β2/γ2 subunit combinations.
Primus et al., 1996). They found the appearance of uncoupling was time-dependent and the magnitude of uncoupling at expressed GABA<sub>A</sub> receptor subtypes after chronic exposure was dependent upon the efficacy of the ligand in a subtype-specific manner. Meanwhile, down-regulation of total receptor number or receptor internalization did not appear to occur, since changes in GABA receptor localization was not observed when binding to intact or homogenized membranes. Their findings revealed drug efficacy played an important role in determining the degree of allosteric uncoupling.

On the other hand, alterations in GABA<sub>A</sub> receptor subunit protein was implicated as an underpinning of reduced BZ sensitivity following chronic exposure. Down-regulation in the total number of GABA<sub>A</sub> receptors as a consequence of prolonged BZ administration has been reported as a potential mechanism of the development of BZ tolerance previously (Rosenberg et al., 1985). But the evidence of subunit alterations is confusing, with some reports describing upregulation of particular subunits, while other studies report no effect or downregulations (Rosenberg et al., 1985; Wu et al., 1994; Holt et al., 1996; Tietz et al., 1999a). For example, Chen et al. compared expression of different GABA<sub>A</sub> receptor subunits in hippocampus and cortex after prolonged FZP exposure. In parallel with the localized reduction in α<sub>1</sub> and β<sub>3</sub> subunit mRNA expression detected in previous studies (Tietz et al., 1993; Tietz et al., 1999a), a significant decrease in α<sub>1</sub> and β<sub>3</sub> protein levels was found in FZP-treated rat hippocampal CA1, CA3 and dentate dendritic regions, and in specific cortical layers (Chen et al., 1999). In other studies, 4-week FZP treatment was found associated with an immediate reduction in β<sub>2</sub> and β<sub>3</sub> subunit mRNAs in rat
hippocampus (Zhao et al., 1994); while 3-week diazepam exposure decreased $\alpha_1$, $\alpha_5$ and $\gamma_2$ subunit mRNA levels in hippocampus, cerebral cortex/hippocampus and only in cortex, respectively, without alterations in $\beta_2$ and $\beta_3$ subunit mRNA levels (Wu et al., 1994). A more extensive study of $\text{GABA}_A$ receptor mRNA steady-state levels (O'Donovan et al., 1992) was conducted by O'Donovan and colleagues in rat whole brain following treatment with FZP over a 32 day period. They found no changes in the levels of $\alpha_1$-, $\alpha_2$-, $\gamma_2$- or all three $\beta$-subunit mRNAs, whereas the $\alpha_3$- and $\alpha_6$-subunit mRNAs were sequentially increased over the treatment period. These discrepancies indicate that although regulation of specific subunit genes may contribute to the expression of BZ tolerance, it is not likely required.

To further understand the mechanisms underlying BZ tolerance, some other studies explored $\text{GABA}_A$ receptor recycling. Moss and colleagues confirmed the involvement of clathrin-mediated endocytosis in constitutive $\text{GABA}_A$ receptor turnover and showed that the adapter protein AP-2, which is necessary for recruitment of proteins to clathrin-coated pits, associates with $\text{GABA}_A$ receptor $\beta$ and $\gamma$ subunits (Connolly et al., 1999; Kittler et al., 2000). They also showed that $\text{GABA}_A$ receptor phosphorylation by protein kinase C (PKC) plays a role in regulating receptor recycling, probably via proteins other than the receptor itself (Connolly et al., 1999). These findings suggested recycling of different $\text{GABA}_A$ receptor subunits may be involved in chronic BZ exposure induced tolerance.

In clinical pharmacology, drug tolerance has been divided into two categories: pharmacokinetic tolerance, in which the clearance of a drug is increased resulting in a
reduction of the agent at the site of action; and pharmacodynamic tolerance, an alteration in the amount or properties of drug target (e.g. receptor desensitization, changes in receptor expression, alterations in neurotransmitter release or uptake or altered signal transduction pathways). Given that the mechanisms underlying reduced potentiation of BZ on GABA\(_A\) receptors include uncoupling of BZ with GABA\(_A\) receptors, regulation of mRNAs, increased internalization or degradation of receptors, reduced protein synthesis etc. (Chen et al., 1999; Tietz et al., 1999a; Bateson, 2002), BZ tolerance is primarily pharmacodynamic.

**Benzodiazepine withdrawal symptoms**

The second element of chronic BZ exposure is dependence, manifested as a withdrawal syndrome. Withdrawal signs refer to the unpleasant symptoms when intake of the drug is abruptly stopped, usually apparent as a characteristic withdrawal syndrome, and where the same substance is taken to avoid or relieve these unpleasant symptoms. BZ withdrawal represents a series of physical, psychological and behavior changes upon abrupt cessation of treatment including rebound anxiety, insomnia, seizures etc. There are a number of factors that affect the likelihood of BZ dependence, including the regimen and methodology of BZ administration, the exposure duration, the pharmacokinetic properties of specific BZs administered and individual differences.

Knowledge of the comprehensive mechanisms underlying the development of BZ dependence remains incomplete. Previous studies of the mechanisms underlying BZ withdrawal have focused on altered use-dependent regulation of GABA\(_A\) receptors (Brett
and Pratt, 1995; Pratt et al., 1998). As discussed above, with prolonged BZ exposure, the decreased number or affinity of GABA<sub>A</sub> receptors, an uncoupling of BZ and GABA sites, receptor internalization or subunit alterations likely result in blunting the ongoing enhancement of inhibitory neurotransmission.

In conjunction with the changes in inhibitory neurotransmission, recent evidence suggests that the glutamatergic system may play a key role in BZ withdrawal and dependence on other drugs of abuse (Pratt et al., 1998; Izzo et al., 2001; Van Sickle and Tietz, 2002; Van Sickle et al., 2004). Increased neuronal excitability has been found during BZ withdrawal: AMPAR binding was significantly increased in proximal dendritic fields of CA1 pyramidal neurons at 2 days after cessation of FZP administration (Van Sickle and Tietz, 2002); and the amplitude of AMPAR-mediated mEPSC was significantly increased in hippocampal CA1 neurons from FZP-withdrawn rats, associated with withdrawal-induced anxiety-like behavior (Van Sickle et al., 2004). In addition, it has been observed that many of the behavioral properties of BZs which appear upon withdrawal, are prevented by antagonists of excitatory amino acid receptors: for example, application of AMPAR antagonist, GYKI-52466 eliminated anxiety-like behavior in FZP withdrawn rats (Van Sickle et al., 2004); the administration of GYKI-52466 during the silent phase of withdrawal prevented the subsequent development of withdrawal signs in the active phase in mice, while the administration of CPP, an NMDAR blocker, administered during the active phase of withdrawal abolished the withdrawal symptoms,
suggesting the involvement of NMDA and AMPA receptors at different time points in the development of BZ withdrawal symptoms (Steppuhn and Turski, 1993).

The alterations in AMPAR and NMDAR subunit composition have also been found during BZ withdrawal (Tsuda et al., 1998; Izzo et al., 2001). Izzo et al. (2001) found that tolerance to the anticonvulsant action of diazepam occurred at 6h, but was abated between 72–96 h after diazepam discontinuation. At 96 h after cessation, an increased anxiety was seen, which was characterized by decreased entries into the open arms of an elevated plus maze and an increased susceptibility to pentylenetetrazol-induced seizures, suggesting BZ withdrawal symptoms occurred. These symptoms were accompanied by increases in mRNAs levels of GluR1-4 subunits of cortical AMPARs; while in hippocampus, only GluR1 subunit expression was augmented (Izzo et al., 2001). These data indicated BZ withdrawal-induced AMPAR regulation may be brain-region specific. Similar to BZ withdrawal-induced AMPAR regulation, the NMDAR NR1 and NR2B, but not NR2A, subunits were significantly upregulated in diazepam-withdrawn rat cerebral cortex. Meanwhile, $[^3]H$MK-801 binding was significantly increased in cerebrocortical tissue from diazepam-withdrawn mice (Tsuda et al., 1998). Together, the results suggest that selective upregulation of AMPA and NMDA receptor subunits in the brain are associated with BZ withdrawal symptoms.

Furthermore, withdrawal symptoms may be the result of changes in the activity of different neuronal pathways, since different symptoms are mediated by different neurotransmitter systems (Allison and Pratt, 2003). For example, anxiety-like behavior
observed after 1-week FZP exposure in rats was mediated through AMPARs (Van Sickle et al., 2004); in vervet monkeys, tremors, vomiting, vocalizations, chewing and piloerection were observed associated with an increase in noradrenergic metabolites after precipitated BZ withdrawal, indicating increased central noradrenergic activity may play a role in BZ withdrawal (Grant et al., 1985); the enhanced locomotor activity during the withdrawal of the BZ receptor antagonist, flumazenil, has been thought to be related to the increased ligand binding to GABA/BZ receptor complex (Kulkarni and Ticku, 1990). Taken together, although there is a significant body of literature concerning BZ withdrawal, we are still far from understanding the adaptive mechanisms and neuronal pathways which lead to benzodiazepine dependence.

**Ionotropic Glutamate receptors**

*Classification of ionotropic glutamate receptors.* Ionotropic glutamate receptors which may play a major role in mediating BZ withdrawal symptoms, are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the central nervous system. Native glutamate receptors exhibit various pharmacological, biochemical, and ionic permeability characteristics, governed by receptor subunit composition. The cloning of cDNAs encoding glutamate receptor subunits revealed that AMPARs are assembled from the GluR1-GluR4 subunits (also called GluRA-GluRD). Kainate (KA) receptors are encoded by two gene families, GluR5-GluR7 and KA1/KA2; the latter form functional channels only when assembled with members of the GluR5-GluR7 family. NMDARs are assembled from three gene families, NR1-3. All NMDARs contain obligate
NR1 subunits that serve two roles: including the site of glycine binding and trafficking of heteromeric receptor assemblies to the plasma membrane. NR1 subunits co-assemble with the NR2A-NR2D subunits that encode glutamate binding sites; less commonly they combine with the NR3A/NR3B gene families that encode glycine binding sites distinct from those on the NR1 subunit (Mayer and Armstrong, 2004).

Post-transcriptional editing of glutamate receptors. The functional diversity of ionotropic glutamate receptors is also determined by post-transcriptional alterations: alternative splicing and RNA editing. All four AMPA receptor subunits have two alternatively spliced versions, flip and flop, that are encoded by exons just before the third transmembrane domain. These characteristics impart different pharmacological and kinetic properties on currents evoked by L-glutamate or AMPA, but not those evoked by kainate (Sommer et al., 1990). Flip variants predominate before birth and continue to be expressed in adult rats, whereas flop variants are in low abundance before the eighth postnatal day and are upregulated to about the same level as the flip forms in adult animals (Monyer et al., 1991). The flip forms of most subunits desensitize more slowly and less profoundly than the flop forms (Sommer et al., 1990). Desensitization in the flip forms is more potently attenuated by cyclothiazide, whereas 4-[2(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA) preferentially reduces the desensitization of the flop forms (Dingledine et al., 1999).

C-terminal splice variants are found in GluR2, GluR4, and the kainate receptor subunits GluR5-7. For example, a small percentage of GluR2 protein exhibits a long C
terminus in murine brain (Kohler et al., 1994); cerebellar granule cells and Bergmann glial cells expresses GluR4c subunits, which has a C terminus that is shorter than regular GluR4 subunit and is homologous to the tail of GluR2short (Gallo et al., 1992); GluR6 and GluR7 subunits also have two C-terminal splice variants, with glutamate showing a 5 to 10 fold greater potency on GluR7a-containing receptors than GluR7b-containing receptors (Gregor et al., 1993; Schiffer et al., 1997). Additional functional differences among these C-terminal splice variants remain unclear. But the different C termini may bind to different intracellular proteins and thus influence receptor phosphorylation, trafficking or clustering.

Alternative splicing was also found in exon 5 in the N terminus (also called the N1 cassette) and exons 21 and 22 in the C terminus (also called C1 and C2 cassettes) of the NMDAR NR1 subunit. Recombinant NR1 receptors lacking the N1 cassette have a higher affinity for NMDA and are potentiated by Zn$^{2+}$ when expressed without NR2 subunits in *Xenopus* oocytes; while when expressed with NR2 subunits, they are more sensitive to block by Zn$^{2+}$ and protons, and show stronger potentiation by polyamines through relief of proton inhibition (Durand et al., 1993; Hollmann et al., 1993; Traynelis et al., 1998). The C1 cassette found in NR1-1 and NR1-3 is involved in receptor clustering, which binds to neurofilament and the scaffolding protein yotiao (Ehlers et al., 1998; Lin et al., 1998). Moreover, the C1 cassette contains PKC phosphorylation sites and binds to calmodulin. Clustering and interaction with these regulators can be inhibited by PKC phosphorylation in the C1 cassette (Ehlers et al., 1995).
To date, RNA editing was only characterized in AMPARs and KARs, but not in NMDARs. In the primary transcript of GluR2, GluR5, and GluR6 subunits, a glutamine codon (CAG) in the M2 domain can be edited to an arginine (CIG) at the Q/R site. The arginine in edited versions causes low calcium permeability, low single channel conductance, and an outward or linear rectification in an activity- and voltage-dependent pattern (Hume et al., 1991; Swanson et al., 1996; Washburn and Dingledine, 1996). Furthermore, some of the AMPAR subunits, GluR2-4, are edited at the R/G site, which is located just before the flip/flop exons. The glycine codon (IGA) replacing the genomically encoded arginine (AGA) in GluR3 and GluR4 reduces and speeds up recovery from desensitization, respectively (Lomeli et al., 1994).

**AMPA type ionotropic Glutamate receptors**

*AMPAR structure.* AMPA-type glutamate receptors mediate the majority of fast synaptic transmission in central nervous system. Activation of AMPARs is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as LTP and LTD, phenomena thought to underlie learning and memory. AMPARs are cation-selective tetrameric receptors, primarily composed of either homomers of GluR1 or GluR4 subunits, or heteromers of GluR2 with GluR1, GluR3 or GluR4. In hippocampus, dentate granule, CA1 and CA3 neurons express GluR1, GluR2, and GluR3 subunits; only CA1 pyramidal neurons also express GluR4 subunits (Black, 2005). But the expression of GluR4 is much higher in developing brain as compared to the adult (Sans et al., 2000).
Therefore, in mature rat hippocampus, GluR1 homomers, as well as GluR1/2 and GluR2/3 heteromeric AMPARs are predominantly expressed (Wenthold et al., 1996).

AMPAR subunits are comprised of about 900 amino acids and share similar structures. Each subunit is composed of an N-terminal domain, an agonist-binding domain, pore loop ion channel, three transmembrane segments, one intramembrane loop and a cytoplasmic C-terminal domain. The first ~400 amino acid polypeptide segment of AMPARs forms dimers in solution (Ayalon and Stern-Bach, 2001), most likely related to receptor assembly. The S1 and S2 polypeptide sequences that make up the agonist-binding core are interrupted by two membrane-spanning, most likely α-helical segments, in combination with a pore helix and pore loop. The Q/R editing site is located within the pore loop and contributes to ion permeability. The functional role of the last transmembrane domain of AMPARs has not been clearly defined. In NMDARs, removal of the last transmembrane domain did not affect NR2A surface expression, whereas NMDARs lacking this domain cannot form functional receptors (Meddows et al., 2001; Schorge and Colquhoun, 2003), suggesting a requirement for this domain in receptor function. The C terminus of AMPAR subunits varies in length and interacts with numerous cytoskeletal proteins important for receptor regulation and trafficking.

**AMPAR ion permeability and rectification.** AMPAR ion permeability is chiefly controlled by Q/R editing in intramembrane domains. As mentioned above, during development, the GluR2 Q/R site is the most vigorously edited site and even essential for survival. After E14 in rats, 99% of GluR2 mRNA has arginine at the Q/R site. Removal of
the editing complementary sequence in one GRIA2 allele in mice, which reduced the efficiency of Q/R site editing by about 25%, resulted in epilepsy and early death, demonstrating the importance of efficient GluR2 editing to normal neuronal function and survival (Brusa et al., 1995). This editing site is a major contributor to the ionic selectivity of the pore. AMPARs lacking the GluR2 subunit are highly permeable to Ca\(^{2+}\) and their current-voltage (I-V) relationship is strongly inwardly rectifying. In contrast, AMPAR containing GluR2 subunits exhibit a low permeability to Ca\(^{2+}\) and their I-V relationship is approximately linear or, in particular in CA1 neurons, is outwardly rectifying. Inward rectification means that when the membrane potential (Vm) is more negative than the reversal potential (Vrev), cations are free to move into the cell through AMPAR channels, but if Vm becomes more positive than Vrev, cations are unable to move out of the cell through AMPAR channels, despite the outward voltage gradient. The inward rectification is known to be caused by the presence of endogenous intracellular polyamines, particularly spermine, but also spermidine. However, if Vm is made sufficiently positive, the driving force on the polyamine molecules is sufficient such that they begin to permeate the channel. At this point relief of block by polyamines is observed and thus some outward current is observed. AMPARs containing GluR2 subunits introduce a positively charged arginine (R) residue at the Q/R site in the channel pore, which prevents blockade by intracellular polyamines. Thus GluR2-containing AMPAR do not exhibit inward rectification (Stromgaard and Mellor, 2004).
AMPAR phosphorylation. Like many other proteins, AMPAR function is under tight control by various protein kinases (Roche et al., 1994; Tan et al., 1994). Neuronal AMPAR activation can be potentiated by PKA, PKC, CaMKII and other unspecified kinases (Tan et al., 1994; Nakazawa et al., 1995; Roche et al., 1996; Mammen et al., 1997; Carroll et al., 1998; Banke et al., 2000; Vinade and Dosemeci, 2000; Esteban et al., 2003b). The potentiation of native AMPARs in cultured neurons by PKA appears to be due to an increase in channel open probability (Banke et al., 2000) or open time (Greengard et al., 1991). Ser\(^{845}\) of GluR1 is a PKA target, because the C-terminus of the GluR1 (S845A) mutant could not be phosphorylated after incubation with PKA (Roche et al., 1996). Moreover, increased phosphorylated Ser\(^{845}\) was detected by immunoblotting in hippocampal slices after forskolin treatment (Mammen et al., 1997); and PKA activation appears to increase the open probability of recombinant GluR1 receptors but not of GluR1 (S845A) mutant receptors (Banke et al., 2000). All these findings sufficiently demonstrate that GluR1 Ser\(^{845}\) site is a phosphorylation target of PKA. Changes in the phosphorylation status of Ser\(^{845}\) are specifically associated with synaptic plasticity. During chemically induced LTD, a decrease of phosphorylated Ser\(^{845}\), but not phosphorylated Ser\(^{831}\), was found (Lee et al., 1998). On the other hand, LTP induction stimulates phosphorylation of Ser\(^{845}\) and Ser\(^{831}\) (Lee et al., 2000), followed by increased AMPAR function and surface/synapses trafficking (Banke et al., 2000; Esteban et al., 2003b; Du et al., 2004). This suggests the critical roles of PKA phosphorylation of GluR1 subunit in synaptic
depression and potentiation. To date, functional roles of PKA phosphorylation of GluR2 and GluR3 subunits are unclear.

GluR1 and GluR2/3 in the postsynaptic density (PSD) of asymmetric, excitatory synapses could also be phosphorylated by endogenous kinases in the presence of calcium and calmodulin (Hayashi et al., 1997). Phosphorylation of AMPARs by CaMKII and possibly PKC was produced in CA1 region of hippocampal slices with theta burst stimulation that induced LTP; and autophosphorylation of CaMKIIα was detected utilizing a specific antibody. This phosphorylation of AMPARs is correlated with the activation and autophosphorylation of CaMKII and was blocked by the CaMKII inhibitor KN-62 (Barria et al., 1997b). To identify the CaMKII regulatory phosphorylation site on AMPARs, HEK-293 cells were transfected with site-specific mutants of GluR1. Only mutations affecting Ser\textsuperscript{831} altered the \textsuperscript{32}P peptide maps of GluR1 from HEK-293 cells coexpressed with an activated CaMKII (Barria et al., 1997a). Meanwhile, this phosphorylation site is specific to GluR1, as CaMKII did not phosphorylate or potentiate currents in cells expressing GluR2 subunits. The idea that phosphorylation of AMPARs by CaMKII might contribute to synaptic plasticity is supported by the finding that the Ser\textsuperscript{831} to Ala mutant failed to show potentiation of the GluR1 current (Barria et al., 1997a). Conversely, mice lacking the α subunit of CaMKII are devoid of LTP and spatial learning (Silva et al., 1992a; Silva et al., 1992b). To further examine the roles of CaMKII in neuronal plasticity, Pettit et al. (Pettit et al., 1994) utilized an approach of transfecting the hippocampal region CA1 with vaccinia virus expressing a constitutively activated
CaMKII. Slices expressing active CaMKII showed a 4-fold increase in synaptic transmission, compared to slices transfected with control virus. They subsequently tested whether this CaMKII infection enhanced synaptic transmission by the same mechanisms as those involved in the physiological induction/expression of LTP. Consistent with their hypothesis, LTP was occluded in slices transfected with activated CaMKII.

Activation of CaMKII has been shown to affect some of the properties of AMPARs. Coexpression in HEK-293 cells of activated CaMKII with GluR1 did not affect the glutamate affinity of the receptor, the kinetics of desensitization and recovery, channel rectification, open probability, or gating. Single-channel recordings identified the contribution of CaMKII to higher-conductance AMPARs, indicating that CaMKII can mediate plasticity at glutamatergic synapses by increasing single-channel conductance of existing functional AMPARs or by recruiting new high-conductance-state AMPARs to synapses (Derkach et al., 1999). In addition, AMPAR-mediated EPSCs were specifically potentiated in active CaMKIIα-infected cells, compared with nearby non-infected neurons. This potentiation was associated with a reduction in the proportion of synapses devoid of AMPARs (silent synapses), and expression of activated CaMKII increased the quantal size of AMPAR-mediated responses (Poncer et al., 2002). On the other hand, when Andrasfalvy and Magee (2004) used non-stationary fluctuation analysis to investigate AMPAR properties on hippocampal CA1 apical dendrites, they found that intracellular application of Ca\(^{2+}\)/CaM induced similar effects on AMPARs as LTP induction, that is, AMPAR channel number was nearly two-fold larger than in controls, while single channel
conductance and maximum open-probability were unchanged. These findings suggest that whether receptor phosphorylation by CaMKII is involved in altered channel conductance is still uncertain. As Ser\textsuperscript{831} is not a consensus site for either PKC or CaMKII and is only poorly phosphorylated by CaMKII after LTP induction (Barria et al., 1997a), the significance of its phosphorylation remains unclear.

Other protein kinases, like PKC have also been found to play important roles in controlling AMPAR function. Phosphorylation by PKC could occur on GluR1, GluR2/3 and GluR4 subunits. Administration of the PKC activator, phorbol-12, 13-dibutryate, caused a significant increase in mEPSC amplitude and frequency on hippocampal slices (Carroll et al., 1998). It was noted that at least 2 phosphorylation sites on PKC were observed on GluR1 subunit, Ser\textsuperscript{831} and Ser\textsuperscript{818}. Although the mechanisms of PKC activation on AMPAR function are still under investigation, increased phosphorylation of GluR1 by PKC at Ser\textsuperscript{818} was observed during LTP and critical for LTP expression. Acute phosphorylation at GluR1 Ser\textsuperscript{818}, as well as a phospho-mimetic mutation, promotes GluR1 synaptic incorporation. Conversely, preventing GluR1 Ser\textsuperscript{818} phosphorylation reduces LTP and blocks PKC-driven synaptic incorporation of GluR1 (Boehm et al., 2006). Protein kinase Mzeta (PKMzeta), a constitutively active, atypical PKC isoform, enhances AMPAR-mediated synaptic transmission, and this enhancement fully occludes the following LTP induction. In addition, inhibition of PKMzeta activity 1 hr after tetanization reverses the maintenance of AMPAR-mediated synaptic potentiation (Ling et al., 2002). Persistently increased phosphorylation by PKMzeta also maintains...
late phase LTP possibly by establishing and maintaining spine structural changes (Serrano et al., 2005).

The PKC phosphorylation of GluR2 is a critical event in the induction of LTD (Xia et al., 2000; Chung et al., 2003). Synaptic AMPAR clusters in dendritic spines of Purkinje cells are disrupted upon PKC-mediated phosphorylation of Ser\textsuperscript{880} in the C-terminal domain of GluR2. Phosphorylation of this residue causes significant reduction in the affinity of the GluR2 C-terminus for glutamate receptor interacting protein (GRIP), a molecule known to be crucial for AMPAR clustering. Consequently, AMPARs on the synaptic membrane are destabilized and internalized by endocytosis (Seidenman et al., 2003). Moreover, transfection with a point mutant of GluR2 that mimicked phosphorylation at Ser\textsuperscript{880} could occlude subsequent LTD (Chung et al., 2003).

In addition to the protein kinases PKA, CaMKII and PKC, which phosphorylate serine and threonine residues, protein kinases which phosphorylate tyrosines are also involved in synaptic plasticity. Of the various non-receptor tyrosine kinases which are present in hippocampus, evidence indicates the involvement of members of the fyn and Src families of tyrosine kinases (Grant et al., 1992; Kojima et al., 1997; Lauri et al., 2000). Although the target proteins for these tyrosine kinases are likely to be NMDARs, Lu et al. found that directly activating Src in the postsynaptic neuron enhanced excitatory synaptic responses, occluding LTP, and Src-induced enhancement of AMPAR-mediated synaptic responses required raised intracellular Ca\textsuperscript{2+} and activation of NMDA receptors (Lu et al., 1998).
AMPAR dephosphorylation. As AMPARs are crucial to bidirectional regulation of synaptic plasticity, such as in LTP and LTD, the phosphorylation and dephosphorylation status of AMPARs are thought to be centrally involved in maintaining normal synaptic function, as well as in learning and memory (Colbran, 2004). AMPAR dephosphorylation at the Ser^{845} site has been observed in NMDAR-mediated chemical-LTD (Kameyama et al., 1998); while low frequency stimulation-induced depotentiation is associated with a persistent dephosphorylation of the GluR1 subunit at Ser^{831} (Huang et al., 2001). The dephosphorylation of AMPARs is controlled by a series of protein phosphatases, including PP1, PP2A, PP2B and PP2C.

Similar to protein kinases, protein phosphatase complexes are formed by assembly of catalytic subunits coupled to regulatory subunits. PP1 and PP2A catalytic subunits are constitutively active, whereas PP2B (also known as calcineurin, CaN) is activated by binding of Ca^{2+}/calmodulin, and PP2C is Mg^{2+}-dependent (Colbran, 2004). A CaMKII/PP1 switch to CaMKII has been found to potentiate synaptic transmission by anchoring AMPARs at synapses (Lisman and Zhabotinsky, 2001). The roles of PP2A and PP2C in synaptic regulation are poorly understood. Within these protein phosphatases, PP2B is the only phosphatase directly modulated by a second messenger (Ca^{2+}/calmodulin) and has relatively restricted substrate specificity. PP2B could be activated at lower Ca^{2+}/calmodulin concentrations than CaMKII. Therefore, weak synaptic stimulation may preferentially activate PP2B, dephosphorylating inhibitor-1 and thereby activating PP1; whereas stronger stimulation also recruits CaMKII activation (Lisman, 1989). PP2B can
also be targeted to PSD by binding to AKAP79/150 (Coghlan et al., 1995). Additional interactions between AKAP79/150 and the scaffold proteins, SAP97 or PSD95 mediate association of PP2B with AMPA- and/or NMDA-type glutamate receptors (Colledge et al., 2000). In hippocampal neurons, activation of PP2B disrupts the association of AKAP79/150 with PSD95 and cadherins, leading to AMPAR dephosphorylation and internalization from synapses, indicating that a shift in the balance of kinase to phosphatase activity may play an important role in synaptic plasticity.

**AMPAR trafficking.** Many of the known protein-protein interactions of AMPARs involved in receptor trafficking are related to intracellular PDZ domain-containing proteins. GluR1 appears to interact specifically with the PDZ protein, SAP97; while GluR2 and GluR3 interact through a different class of PDZ domains with GRIP, AMPAR binding protein (ABP) and protein interacting with C kinase-1 (PICK1). Although many details remain to be clarified, the interactions of AMPARs with these various PDZ proteins appear to be important in both the targeting and clustering of AMPARs to membrane and subcellular regions, as well as the stabilizing of AMPARs both on the neuronal cell surface and in intracellular pools. It appears that AMPAR trafficking and their basal rate of insertion and removal, as well as the activity-dependent delivery are dependent upon their subunit composition. GluR2/3 oligomers are continuously delivered into synapses in a manner primarily independent from synaptic activity (Passafaro et al., 2001; Shi et al., 2001); whereas GluR1/2 and GluR4-containing receptors are added into synapses in a manner dependent upon NMDAR activation.
According to this scenario, this constitutive trafficking would serve to maintain synaptic strength despite protein turnover, and act in a relatively rapid manner (half-time of minutes); the regulated pathway would act transiently upon induction of plasticity, leading to the long-lasting enhancement of synaptic strength also known as LTP. To date, the precise trafficking steps controlled by these PDZ proteins are not well characterized. But it was found that SAP97 interacts with immature GluR1 subunits early in the secretory pathway, probably while the receptor is still in the endoplasmic reticulum (ER) (Sans et al., 2001). Meanwhile, the SAP97-interacting region of GluR1 is necessary for the receptor to reach its synaptic targets (Hayashi et al., 2000). PDZ domain-containing proteins, GRIP1/ABP interact directly with the heavy chain of conventional kinesin and C-terminal PDZ motif of GluR2 and GluR3 (Dong et al., 1997; Srivastava et al., 1998; Setou et al., 2002). It is reported that the GluR2/GRIP1/kinesin complex can be immunoprecipitated from brain lysates, and expression of dominant-negative versions of kinesin reduced the presence of AMPAR at synapses (Setou et al., 2002). Internalization of GluR2-containing receptors is modulated by their interaction with PICK1, which is highly dependent on the activation of PKC. PICK1 targets PKC to GluR2 and subsequent PKC phosphorylation of GluR2 facilitates the release of GluR2 from the synaptic anchoring proteins ABP and GRIP (Perez et al., 2001). Subsequently, a switch of GluR2 binding partners from GRIP1/ABP to PICK1 results in decreased synaptic function (Chung et al., 2000; Kim et al., 2001), suggesting that the PICK1/GluR2 complex is critical for LTD.
Another protein that plays important roles in AMPAR trafficking is NEM-sensitive factor (NSF), which was originally identified as an ATPase that is required for the membrane fusion process during intracellular protein trafficking and presynaptic vesicle exocytosis (Rothman, 1994). NSF interacts with the intracellular C-terminus of GluR2 and is thought to be important for the delivery of AMPARs to the synaptic plasma membrane and/or their stabilization within the membrane (Malinow and Malenka, 2002; Song and Huganir, 2002). The controlled removal of AMPARs from synapses is as important as the delivery of new AMPARs for synaptic function and plasticity. A marked progressive decrement of AMPAR-mediated synaptic transmission was found, when the interactions between NSF and GluR2 are prevented by intracellular application of an anti-NSF antibody (Nishimune et al., 1998). Although the mechanisms for GluR2/3 AMPARs constitutive cycling in and out of synapses are unknown, NSF is thought play a critical role in this process by regulating the interactions between GluR2 and PICK1 (Hanley et al., 2002).

The movement of AMPARs from the cytosol to the dendritic plasma membrane and subsequently to the synapse may involve an additional critical protein, stargazin. Stargazin was originally identified as the mutant protein that is responsible for the neurological deficits in the stargazer mutant mouse. Cerebellar cells from these mice lack functional synaptic and extrasynaptic AMPARs on plasma membrane. Acute expression of stargazin in these cells rescues both synaptic responses and the responses to exogenous glutamate, indicating stargazin is required for the surface expression of
AMPARs (Chen et al., 2000). Stargazin regulates AMPAR trafficking through direct binding of the first two PDZ domains of synaptic PSD-95 (Schnell et al., 2002). Over-expression of stargazin extensively increases the number of extrasynaptic AMPARs, but fails to alter synaptic currents if synaptic PSD-95 levels are kept constant. In addition, a mutant form of stargazin lacking the PDZ-binding domain is still capable of delivering AMPARs to the cell surface but not to the synapse (Schnell et al., 2002). These results suggest that stargazin-AMPAR interactions are critical for the delivery of AMPARs to the plasma membrane, and a subsequent interaction between stargazin and PSD-95 is required for AMPAR diffusion into synapses.

The signaling cascades that seem to be central to the regulated trafficking of AMPARs include PKA and CaMKII pathways for the synaptic delivery of AMPARs, as well as PKC and RAP-p38/MAPK pathways for the removal of AMPARs from synapses. Recent findings have offered a clearer picture of how the activation of CaMKII pathway leads to the addition of new AMPARs into synapses. LTP has been proposed to be mediated by the activation of CaMKII, which in turn activates Ras by inhibiting a synapse-localized ras GTPase-activating protein (SynGAP); active Ras would then lead to synaptic delivery of AMPARs via activation of its downstream effectors p42–44 MAPK and/or phosphatidylinositol 3-kinase (PI3K) (Esteban, 2003a). In addition to CaMKII, it is also known that phosphorylation of GluR1 by PKA is required for AMPAR synaptic delivery, and controls the recycling of receptors between the plasma membrane and endosomal compartments (Ehlers, 2000; Lee et al., 2000; Esteban et al., 2003b).
Interestingly, the role that CaMKII and PKA signaling cascades play in the control of the synaptic delivery of AMPARs changes during the course of postnatal development. Early in the postnatal development of the hippocampus, the regulated delivery of AMPARs involves PKA-mediated phosphorylation of GluR4-containing receptors (Zhu et al., 2000; Esteban et al., 2003b); whereas later in postnatal development, the regulated delivery of AMPARs requires PKA phosphorylation of GluR1; but this event is no longer sufficient, and the activation of CaMKII-Ras-MAPK pathway is also required for delivery (Esteban, 2003a). Therefore, in the juvenile and adult brain, PKA-mediated phosphorylation of GluR1 and the activity of CaMKII are both necessary for AMPAR synaptic incorporation. As mentioned above, the removal of AMPARs during LTD correlates with phosphorylation of GluR2 by PKC and a switch from an interaction with the GluR2-ABP/GRIP complex to a GluR2-PICK1 interaction, which pulls GluR2 subunits away from synapses (Chung et al., 2000; Kim et al., 2001; Perez et al., 2001). Additionally, the activation of p38 MAPK can also lead to AMPAR endocytosis. During LTD, Ca\textsuperscript{2+} entry is triggered by NMDAR channel opening, leading to the activation of Rap1, a small GTPase known to activate p38 MAPK, and subsequently promote the removal of GluR2-containing AMPARs from synapses (Esteban, 2003a).

**AKAP-PKA complex structure and function in LTP**

*PKA signaling proteins.* PKA is one of the best characterized members of the protein kinase superfamily. It is assembled with two active catalytic (C) subunits and a dimer of regulatory (R) subunits, and is enzymatically inactive in the absence of cAMP. Activation
is achieved by co-operative binding of four molecules of cAMP with high affinity to the regulatory subunits. When cAMP binds, the PKA dissociates, releasing free active C subunits that will phosphorylate serine and threonine residues on specific substrate proteins. Two different isozymes of PKA, termed type I and II (PKAI and PKAII, respectively), were identified (Corbin et al., 1975). Cloning of cDNAs for R subunits have identified two RI subunits termed RIα and RIβ and two RII subunits termed RIIα and RIIβ as four separate gene products (Lee et al., 1983; Jahnsen et al., 1986; Sandberg et al., 1987; Scott et al., 1987; Clegg et al., 1988). Despite the fact that RIα and RIβ subunits are transcribed from different genes, they reveal a high homology at the amino acid level (81% identity), as do the RIIα and RIIβ subunits (68% identity). Both the RI and RII subunits may be divided into three functional domains, consisting of a dimerization/docking domain (D/D domain) at the N-terminus, a flexible linker region responsible for interaction with the C subunit and a carboxy terminus where two tandem cAMP-binding domains termed sites A and B are located (Corbin et al., 1978). Binding of cAMP to the B site enhances binding of cAMP to the A site in a positively co-operative manner, as a result of a conformational change in the molecule and the activation of catalytic subunit. Three distinct C subunits were identified by molecular cloning, and were designated Cα, Cβ and Cγ (Showers and Maurer, 1986; Uhler et al., 1986; Beebe et al., 1992; Foss et al., 1992).

X-ray crystallography demonstrates that the regulatory isoforms of PKA (RIα, RIIα, and RIIβ) exhibit major differences in their overall shape and conformational changes,
when they form a holoenzyme complex with the catalytic subunits (Kim et al., 2005). This change involves the ordering of the flexible linker and reorganization of the helical motif of the cAMP binding domain. The catalytic subunit acts as a scaffold for protein-protein interactions. Three regions of the C subunit contribute to the docking of RIα: the inhibitor peptide docks to the active site cleft; the αG helix binds to a portion of the phosphate binding cassette and creates an extended hydrophobic interface, and the outward facing surface of the activation loop interacts with the αB/αC helical region of RIα subunit. In the process of binding to the catalytic subunit, the RIα subunit undergoes major conformational changes, including ordering of the inhibitor site and the linker region as well as a major reorganization of the helical subdomain of cAMP-binding site A (Kim et al., 2005).

Although PKA subunits share high homology at the amino acid level, it has been reported that PKAI and PKAII holoenzymes have distinct biochemical properties. PKAI holoenzymes are more readily dissociated by cAMP in vitro than PKAII holoenzymes (Dostmann et al., 1990). Furthermore, when overexpressed RII subunits in NIH 3T3 cells, which contain approximately equal amounts of both holoenzymes, PKAI and PKAII, the C subunit preferentially binds to RII subunits and thereby the RI isozyme level is reduced. In contrast, overexpression of the type I regulatory subunit did not alter PKA isozyme levels (Otten and McKnight, 1989). This indicates that PKAII holoenzymes are assembled preferentially with PKAI under physiological conditions. The mechanism for this phenomena is unknown, but may involve several features such as lower sensitivity of
PKAI\textsubscript{II} to cAMP compared to PKAI and differential kinetics of association/dissociation influenced by salt and MgATP between the two holoenzyme types. In addition, RII\textalpha\ and RII\textbeta\ holoenzymes also exhibit different affinities for catalytic subunit. RII\textalpha\ and RII\textbeta\ are eluted from DEAE-cellulose columns at different positions in the PKAI\textsubscript{II} area, and RII\textalpha\ expressed at high levels will compete with RII\textbeta\ in binding the C subunit, indicating that RII\textalpha\ subunit has a higher affinity for the C subunit (Otten et al., 1991).

PKA isozymes are also located in different structures and bind differentially to signaling proteins. The RII\textalpha\ and RII\textalpha\ subunits are ubiquitously expressed, whereas the expression of RII\textbeta\ and RII\textbeta\ is more tissue-specific expression with the highest levels in brain (Boundy et al., 1998). PKARI was found to be soluble and preferentially located in the cytosol (Meinkoth et al., 1990); whereas PKARI\textsubscript{II} was distributed both in the cytosol and associated with the cytoskeleton, and enriched at nerve endings (Mori et al., 1998; Liu et al., 2004). It was demonstrated that RII\textalpha\ binds to the adapter protein Grb2, an association that allows PKARI to interact with the epidermal growth factor receptor in epithelial MCF-10A cells (Tortora et al., 1997). In addition, an Caenorhabditis Elegans AKAP (AKAPCE) was found only to binds to RI subunit (Angelo and Rubin, 1998). Apart from these two reports, no other evidence has been provided on PKAI-specific binding proteins. Other AKAPs (i.e. AMAP79/150, AKAP140, D-AKAP, AKAP82) have been reported to exhibit dual-specificity for both RI and RII subunits (Huang et al., 1997; Miki and Eddy, 1998; Brown et al., 2002; Smith et al., 2006).
Only two C subunits have been found in mice hippocampus, C\(\alpha\) and C\(\beta\) (C\(\gamma\) only expressed in humans) (Brandon et al., 1997). C\(\beta\) is expressed at low levels in many tissues, but has been found at high levels only in the nervous system. The C\(\beta\) gene is encoded by three different splice variants, termed C\(\beta\)1, C\(\beta\)2 and C\(\beta\)3 (Brandon et al., 1997). Besides its expression in non-neural tissues, C\(\beta\)1 is highly enriched in hippocampus and cortex; C\(\beta\)2 is only expressed in the nervous system with highest levels in cortex and limbic structures; the expression of C\(\beta\)3 is low throughout the brain. Mice lacking the C\(\beta\)1 subunit showed deficits in LTP maintenance (Huang et al., 1995). Given the observation that the PKA catalytic subunit was translocated from the cytoplasm to the nucleus during transcriptional activation (Meinkoth et al., 1993), these data indicate the crucial physiological roles of PKA catalytic subunits in mediating protein synthesis in activity-dependent plasticity.

**AKAPs and their binding proteins.** The AKAP family consists of more than 50 structurally diverse, but functionally similar members that are classified by their ability to co-purify with PKA catalytic activity from tissues. Despite their diversity, all AKAPs share certain common properties: a PKA-anchoring domain; unique localization signals; and the ability to form complexes with other signaling molecules. AKAPs contain a conserved amphipathic helix motif that binds to the N-terminus of PKA regulatory subunit dimers with high affinity. This AKAP-PKA complex is then localized through unique targeting domains on AKAPs to specific locations. In addition to anchoring PKA, many AKAPs serve as multi-functional signal integrators that bind other signaling, scaffold, and
membrane receptor proteins. To date, AKAPs have been found to be involved in ion channel modulation, G-protein-coupled receptor desensitization, vesicular secretion, actin cytoskeletal dynamics, cell division, as well as gene transcription regulated by cAMP/Ca\(^{2+}\) and lipid second messengers (Michel and Scott, 2002).

The AKAP79/150 family comprises a group of three structurally similar orthologues: human AKAP79, murine AKAP150 and bovine AKAP75. AKAP79/150 is enriched in the PSD fractions in the neuronal fractions and present in the dendritic spines of neurons (Carr et al., 1992), which determines their important roles in regulating synaptic receptor function. Consistent with their role in mediating PKA interactions with ionotropic glutamate receptors, AKAP79/150 was found at asymmetric synapses, but not symmetric, i.e. inhibitory synapses in both human (AKAP79) (Sik et al., 2000) and rat (AKAP150) (Lilly et al., 2005) hippocampus. Recruitment of AKAP79/150 to NMDARs and AMPARs is achieved by association with the membrane/actin cytoskeleton in a PKC/CaMKII-regulated manner and by binding to PSD membrane-associated guanylate kinase (MAGUK) scaffold proteins PSD95 and SAP97 (Colledge et al., 2000; Gomez et al., 2002). The SH3 and GK regions of the MAGUKs mediate direct binding to the AKAPs. Additionally, AKAP79/150 contains binding sites for PKC, calcineurin catalytic A subunit, actin and phospholipids (Dell'Acqua et al., 1998; Dell'Acqua et al., 2006). Recent fluorescence resonance energy transfer (FRET) microscopy demonstrated that PKA-AKAP-CaN complex assembly with SAP97 at the plasma membrane of living cells (Oliveria et al., 2003). Recruitment of kinases and phosphatase to the AMPAR by AKAP
indicate the crucial roles of AKAP in regulating AMPAR function. In support of this, Tavalin et al. demonstrated that AKAP79 not only promoted basal phosphorylation of Ser\(^{845}\) but also conferred a calcium- and CaN-mediated downregulation to GluR1 receptor currents. In addition, disruption of AKAP-PKA anchoring or inhibition of PKA activity with synthetic peptides in hippocampal neurons leads to a CaN-dependent, LTD-like down-regulation of AMPAR currents and loss AMPAR surface expression (Rosenmund et al., 1994; Tavalin et al., 2002; Hoshi et al., 2005; Snyder et al., 2005). Together, this evidence reveals the dual effects of AKAP79/150 to regulate AMPAR function: anchoring PKA to receptor to facilitate AMPAR phosphorylation and trafficking in activity-dependent plasticity; or recruitment of CaN to dephosphorylate AMPARs in LTD.

Another feature of AKAP is that it can link F-actin, phosphatidylinositol-4, 5-bisphosphate (PIP2), MAGUKs and cadherins together. AKAP79/150 recruitment of MAGUK-glutamate receptor complexes depends on proper polymerization of actins (Gomez et al., 2002). AKAP79 and cadherins localized within the PSD but were also enriched perisynaptically, where cadherins were found in an adhesion ring around the PSD (Gorski et al., 2005; Dell'Acqua et al., 2006). Positioning MAGUK-AKAP79-PKA/CaN complexes in the adhesion ring would provide an optimal geometry for regulating AMPAR phosphorylation by PKA. Under basal activity, AKAP-anchored PKA is partially active and CaN is inhibited (Dell'Acqua et al., 2006). Thus AKAP-cadherin complexes might define an area of high PKA and low CaN activity. In addition, MAGUK-AKAP-cadherin complexes might also be part of a physical barrier keeping
AMPARs in the synapse. Thus, interaction between AKAP79/150, MAGUKs, and cadherins may coordinate synaptic form and function by linking adhesion complexes with signaling proteins that control AMPAR phosphorylation and trafficking.

**AKAP-PKA complex function.** The roles of AKAP-anchored PKA in modulating synaptic activity was first demonstrated by Rosenmund et al., who showed that perfusion of peptides derived from the conserved kinase binding region of AKAPs prevented the PKA-mediated regulation of AMPA/kainate currents as well as fast excitatory synaptic events (Rosenmund et al., 1994). Since then, efforts have been put forth to understand the molecular mechanisms of this regulation. It is now clear that regulation of PKA phosphorylation of AMPARs plays important roles in establishment of LTP and LTD. The N-terminal PDZ domains of PSD95 and SAP97 associate with the C-terminal tails of NMDA and AMPA glutamate receptors, respectively; and the C-terminal SH3 and GK regions of the MAGUKs bind to AKAP79/150. In this way, through simultaneous association with the MAGUKs, glutamate receptors and PKA are recruited into a macromolecular signaling complex at excitatory synapses (Colledge et al., 2000).

In recent years, attention has focused on the regulation of AMPAR phosphorylation and trafficking by PKA and CaN (PP2B) pathways coordinated by the AKAP79/150-MAGUK protein complex. A series of molecular, biochemical and electrophysiological experiments established that AKAP79/150-anchored PKA phosphorylates Ser$^{845}$ on GluR1 resulting in regulation of channel activity (Colledge et al., 2000; Tavalin et al., 2002). They also identified PP2B as the predominant phosphatase
opposing modulation of hippocampal AMPARs by anchored PKA. This implies the balance between kinase (PKA) and phosphatase (PP2B) activity is an important determinant in the regulation of both native and recombinant AMPA receptor currents and may govern synaptic plasticity.

Meanwhile, AKAP-directed kinase/phosphatase complexes have been implicated in the regulation of AMPA receptor trafficking. The phosphorylation of GluR1 by PKA has been observed to be necessary for AMPAR synaptic incorporation during learning and memory (Lee et al., 2003a; Esteban, 2003a); PKA phosphorylation of AMPAR interacting protein, stargazin, was also implied to play a role in regulated trafficking events (Chetkovich et al., 2002; Schnell et al., 2002). By using in vitro peptide phosphorylation assays and immunoblot analysis with phospho-stargazin-specific antibodies, the critical threonine within the stargazin PDZ binding site was found to be phosphorylated by PKA (Chetkovich et al., 2002). Uncoupling stargazin from the AMPAR or from PSD95, events that would subsequently result in the uncoupling of the AKAP signaling complex from the receptor, led to a decrease in the surface expression of AMPARs (Schnell et al., 2002). In another study, Gomez et al. reported that a NMDAR-CaN signaling pathways control AMPAR trafficking and also negatively regulated the localization and association of the endogenous AKAP79/150-PKA complex with PSD95 in neurons (Gomez et al., 2002). These findings suggest that AKAP anchored PKA and PP2B can play a major role in regulating AMPA receptor surface expression and in turn synaptic plasticity.
**Excitatory neurotransmission and other drug abuse**

Recently, a prominent hypothesis in the addiction field is that cellular mechanisms, such as synaptic plasticity, which are crucial during adaptive forms of experience-dependent plasticity, may also play important roles in the neural adaptations generated by exposure to drugs of abuse (Hyman and Malenka, 2001; Everitt and Wolf, 2002). Ungless et al. examined excitatory synaptic responses recorded from the ventral tegmental area (VTA) dopamine neurons in slices prepared from cocaine-treated animals, and observed that a single \textit{in vivo} exposure to cocaine caused AMPAR-mediated LTP at excitatory synapses onto dopamine cells (Ungless et al., 2001). More recently, not only administration of cocaine but also exposure to multiple classes of drugs of abuse (amphetamine, morphine, ethanol, etc.) are thought to enhance excitatory synaptic strength on midbrain dopamine neurons when administered \textit{in vivo} (Malenka, 2003). These results suggest that plasticity at excitatory synapses on dopamine cells may be a key neural adaptation contributing to addiction.

Though the detailed molecular mechanisms that are responsible for synaptic changes related to addiction are unknown, regulations of GluR1- and GluR2-containing AMPARs were observed. Repeated cocaine treatment caused the persistent expression of highly stable isoforms of deltaFosB, which increased the responsiveness of animals to the rewarding and locomotor-activating effects of cocaine. Interestingly, these effects of deltaFosB appear to be mediated partly by induction of the GluR2-containing AMPARs in the nucleus accumbens (NAc), as expression of deltaFosB significantly increased GluR2
by more than 50%. No other glutamate receptor subunits were regulated by deltaFosB. Furthermore, deltaFosB is also induced in the nucleus accumbens by repeated exposure to other drugs of abuse, including amphetamine, morphine, nicotine and phencyclidine (Kelz et al., 1999).

On the other hand, considerable evidence demonstrates that the GluR1 AMPAR subunit is involved in neuronal adaptations which occur during administration or withdrawal from of drugs of abuse. Long-term ethanol exposure augmented levels of immunoreactivity of NR1 and GluR1 subunits in VTA (Ortiz et al., 1995). Using immunoblotting methods, Fitzgerald et al. found repeated cocaine treatment increased the expression of GluR1 and NR1, but not GluR2 or NR2 subunits in VTA; and this effect was not observed in other regions of the mesolimbic or nigrostriatal dopamine pathways, including the substantia nigra, suggesting chronic cocaine administration-induced adaptive effects on AMPARs and NMDARs is brain-region specific (Fitzgerald et al., 1996). Given the purported role for the VTA and NAc in the development of sensitization, the molecular mechanisms underlying neuronal adaptations in these regions have been well defined. A significant increase in GluR1 levels in VTA and NAc was associated with behavioral sensitization; and no upregulation of GluR1 was observed in animals which did not show behavioral sensitization (Churchill et al., 1999). Further demonstration of GluR1 subunit involvement in drug sensitization was found by the Nestler group (Carlezon et al., 1997; Carlezon et al., 1999). After microinjecting a viral vector expressing GluR1 into VTA, morphine's stimulant and rewarding properties were intensified (Carlezon et al.,
1997); pretreatment with AMPAR antagonist, LY293558 attenuated the acute locomotor-stimulating actions of morphine and eliminated the sensitization after repeated morphine treatment (Carlezon et al., 1999). Together, these data indicated that the GluR1 AMPAR subunit is sufficient to trigger sensitization in this specific brain area.

More recently, a new quantitative proteomic analysis technique with the cleavable isotope-coded affinity tag (ICAT) reagent was applied to measure relative changes in protein levels of mouse hippocampal PSD in response to morphine administration (Moron et al., 2006). The majority of differentially regulated proteins were signaling, trafficking and cytoskeletal proteins involved in synaptic plasticity, learning and memory. A substantial reduction in the association levels of GluR1 with clathrin was also found in this area, supporting the former findings that GluR1 levels are upregulated in drug-induced plasticity. However, to date, altered levels of AMPAR subunits in different brain regions have not been replicated by all groups (Lu et al., 1997; Lu et al., 2002). Different treatment regimens, the time after drug cessation and the methodologies used to investigate changes may be responsible for the inconsistent findings.

**Regulation of both inhibitory and excitatory systems in rats administered flurazepam**

Consistent with reports from other laboratories following treatment with other BZs, both GABAergic and glutamatergic systems are regulated by the 1-week oral FZP administration utilized in our lab. During 1-week FZP treatment, rats ingest an average of 100mg/kg for 3 days and 150 mg/kg for 4 days to achieve the desired brain BZ levels
(~160 ng/g brain tissue), which is comparable to that achieved with 5 mg/kg diazepam injection (~144 ng/g brain tissue) (Brett and Pratt, 1995; Pratt et al., 1998). In contrast to diazepam, FZP is a water-soluble BZ, allowing chronic oral administration via the drinking water without the problems inherent in daily intraperitoneal injections. Chronic FZP treatment elicits *in vitro* and *in vivo* manifestations of BZ anticonvulsant tolerance, such as a reduction in GABA-mediated inhibitory postsynaptic potentials in hippocampal CA1 pyramidal neurons (Zeng et al., 1995); and with abrupt cessation of treatment after 1-week FZP exposure, rats also expressed a BZ withdrawal symptom that is manifested as increased anxiety-like behavior (Van Sickle et al., 2004). The changes associated with tolerance and withdrawal symptom are also brain-region specific. Altered synaptic function was observed mainly in hippocampal CA1 area, but not in adjacent regions, such as the dentate gyrus or CA3 regions.

The rat hippocampus exhibits a high density of BZ-sensitive GABA\textsubscript{A} receptors and exerts *in vitro* effects related to anxiolytics and anticonvulsant actions (Oliver et al., 1977; Ashton and Wauquier, 1985; McNaughton and Gray, 2000). Chronic FZP exposure alters the ability of BZs to modulate hippocampal neurotransmission, uncouples GABA/BZ binding sites and reduces GABA\textsubscript{A} receptor density and subunit expression in hippocampus, which contributes to the expression of BZ tolerance (Rosenberg and Chiu, 1981; Rosenberg et al., 1985; Tietz et al., 1989; Zeng and Tietz, 1999; Tietz et al., 1999a; Tietz et al., 1999b). There are also changes in excitatory neurotransmission in hippocampal CA1 pyramidal neurons, including CA1 pyramidal neuron hyperexcitability,
increases in AMPAR-mediated mEPSC amplitude and enhancement of AMPA antagonist binding correlated with increased anxiety-like behavior (Van Sickle and Tietz, 2002; Van Sickle et al., 2004). Therefore, 1-week FZP administration provides an excellent model in which to study BZ tolerance and dependence both in vivo and in vitro.
Benzodiazepine Withdrawal-Induced Glutamatergic Plasticity Involves
Upregulation of GluR1-Containing AMPA Receptors in Hippocampal CA1

Neurons

Jun Song1,3, Guofu Shen1,3, L. John Greenfield, Jr.1,2,3 and Elizabeth I. Tietz1,3*

1Department of Physiology and Pharmacology, 2Department of Neurology, and 3the Cellular and Molecular Neurobiology Program,
University of Toledo College of Medicine, Toledo, OH 43614

Running title: Benzodiazepine Withdrawal-Induced AMPAR Regulation

ABBREVIATIONS: AMPA, alpha-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GABA, gamma-amino butyric acid type A; NMDA, N-methyl-D-aspartate; CON, control; FZP, flurazepam; AKAP, A kinase-anchoring protein; PKA, cAMP-dependent protein kinase A; CaMKII, Ca2+/calmodulin dependent protein kinase II; LTP, long-term potentiation; KA, kainate; NAS, 1-naphthylacetyl spermine; APV, DL-2-amino-5-phosphopentanoic acid; TTX, tetrodotoxin; RMP, Resting membrane potential; PDZ, PSD-95, Dlg and ZO-1; mEPSCs, miniature excitatory postsynaptic currents; GluR, glutamate receptor; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; PSD, postsynaptic density.

Footnotes: This work was supported by Department of Health and Human Services grants from the National Institute on Drug Abuse: R01-DA-04075-15 and R01-DA18342-02 to EIT; and predoctoral fellowships from the University of Toledo, College of Medicine to JS and GS.
ABSTRACT
Modification of glutamatergic synaptic function, a mechanism central to neuronal plasticity, may also mediate long-term drug effects, including dependence and addiction. Benzodiazepine withdrawal results in increased glutamatergic strength, but whether AMPARs are functionally and structurally remodeled during benzodiazepine withdrawal is uncertain. Whole-cell recordings of rat hippocampal CA1 neurons, either acutely-dissociated or in hippocampal slices, revealed that AMPAR function was enhanced up to 50% during flurazepam (FZP) withdrawal, without changes in whole-cell channel kinetic properties. Agonist-elicited AMPA currents showed a negative shift in rectification in the presence of spermine, suggesting augmented membrane incorporation of GluR2-lacking AMPARs. As GluR1-containing AMPARs are critical for activity-dependent alterations in excitatory strength, we sought to determine whether changes in GluR1 subunit distribution in CA1 neurons occurred during benzodiazepine withdrawal. Confocal image analysis revealed that FZP withdrawal promoted GluR1 subunit incorporation into somatic and proximal dendritic membranes of CA1 neurons, without GluR2 subunit alterations. Findings of immunoblot studies were consistent with immunofluorescent studies indicating increased GluR1, but not GluR2, subunit protein levels in cytosolic, crude membrane and PSD-enriched fractions from CA1 minislices. As with LTP, the FZP-withdrawal-induced GluR1 incorporation into CA1 neuron membranes may require the GluR1-trafficking protein, SAP97, which was also elevated in membrane-associated fractions. Together, our findings provide evidence that during
FZP withdrawal, increased membrane incorporation of GluR1-containing AMPARs and associated upregulation of AMPAR function in hippocampal CA1 pyramidal neurons share fundamental similarities with the mechanisms underlying LTP. This implies that glutamatergic neuronal remodeling observed in LTP also subserves physiological adaptations to drug withdrawal.
Introduction

Remodeling of glutamatergic neurotransmission has recently been associated with dependence on and addiction to various drugs of abuse (Nestler, 2005), including the benzodiazepines (Izzo et al., 2001; Van Sickle et al., 2004; Xiang and Tietz, 2007). Benzodiazepines are widely prescribed anxiolytics and hypnotics, but have more limited clinical use as anticonvulsants due to the development of tolerance, largely mediated by dysfunction of gamma-aminobutyric acid type A (GABA$_A$) receptors, the site of benzodiazepine allosteric actions (Zeng and Tietz, 1999; Bateson, 2002). Abrupt benzodiazepine withdrawal causes anxiety and insomnia, which can lead to drug self-administration and contribute to benzodiazepine misuse and abuse (Griffiths and Johnson, 2005). Recent evidence suggests that the benzodiazepine withdrawal syndrome is related to the regulation of the glutamatergic system (Izzo et al., 2001; Bateson, 2002; Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Xiang and Tietz, 2007). The amplitudes of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR)-mediated miniature excitatory postsynaptic currents (mEPSCs) and AMPAR specific binding were increased in hippocampal CA1 pyramidal neurons after FZP withdrawal (Van Sickle and Tietz, 2002), associated with anxiety-like behavior in the elevated plus maze (Van Sickle et al., 2004; Xiang and Tietz, 2007). The strengthening of CA1 neuron excitatory synaptic function and the expression of anxiety were mitigated by prior pharmacological antagonism of AMPAR upregulation, underscoring the association of these neurophysiological and behavioral changes (Van Sickle et al., 2004;
However, the exact mechanisms by which glutamatergic neurotransmission is enhanced during benzodiazepine withdrawal are not well understood.

Enhanced glutamatergic neurotransmission in CA1 neurons also occurs during long-term potentiation (LTP). The molecular mechanisms underlying LTP have been thoroughly studied and similar mechanisms may play a role in long-term drug effects, including dependence and addiction (Nestler, 2005). Increased glutamatergic strength during LTP primarily results from enhanced function of ionotropic AMPARs (Hayashi et al., 2000; Liu and Cull-Candy, 2000; Soderling and Derkach, 2000). Activity-dependent AMPAR trafficking between the plasma membrane and intracellular compartment (Malinow and Malenka, 2002) and rapid translocation between synaptic and extra-synaptic regions (Borgdorff and Choquet, 2002) are two mechanisms crucial to this form of plasticity. In addition, increased AMPAR expression, phosphorylation status and switches in AMPAR subunit composition can also contribute (Hayashi et al., 2000; Liu and Cull-Candy, 2000; Soderling and Derkach, 2000).

In rat hippocampus, three AMPAR subpopulations are expressed: GluR1 homomers, GluR1/2 and GluR2/3 heteromers (Wenthold et al., 1996). The AMPAR GluR1 subunit is essential for neuronal plasticity, since the presence of a GluR1 subunit alone was sufficient to sustain plasticity in GluR2/3 double knockout mice (Meng et al., 2003). GluR1 homomers lacking GluR2 subunits and therefore are permeable to calcium, susceptible to spermine blockade and show inward rectification at positive holding.
potentials (Lerma et al., 1994). Recombinant AMPARs show a graded inward rectification proportional to the number of functionally expressed non-GluR2 to GluR2 subunits (Washburn and Dingledine, 1996). Glutamate-activated AMPAR currents in hippocampal CA1 pyramidal neurons with a heterogeneous complement of primarily GluR2-containing AMPARs (Wenthold et al., 1996) show predominantly outward rectification (Lerma et al., 1994).

To further explore the molecular mechanisms underlying enhanced glutamatergic strength in CA1 neurons during benzodiazepine withdrawal, we used whole-cell electrophysiological recordings, confocal immunofluorescent imaging and immunoblot approaches to examine synaptic and extrasynaptic AMPAR channel properties and AMPAR subunit composition and distribution, using our well-established FZP withdrawal model (Van Sickle et al., 2004; Xiang and Tietz, 2007). We show that FZP withdrawal enhances GluR1 subunit expression, directs GluR1-containing AMPARs, including both GluR1 homomers and GluR1/2 heteromers, to extrasynaptic and synaptic sites on hippocampal CA1 pyramidal neurons. Moreover, this delivery may be promoted by synapse-associated protein 97 (SAP97), directing GluR1 subunit redistribution (Leonard et al., 1998; Sans et al., 2001).

The findings implicate enhanced AMPAR function and associated CA1 neuron hyperexcitability during FZP withdrawal (Van Sickle et al., 2004; Xiang and Tietz, 2007), as a potential substrate of physical dependence, a mechanism that shares fundamental characteristics with other forms of neuronal plasticity.
Materials and Methods

**Chronic FZP treatment.** All methods were approved by the University of Toledo College of Medicine (formerly the Medical University of Ohio), Institutional Animal Care and Use Committee (IACUC) and conformed to National Institutes of Health guidelines. One-week FZP treatment was carried out as previously established (Van Sickle et al., 2004). Male Sprague–Dawley rats (Harlan, Indianapolis, IN), P36-42 at the time of study, were first adapted 2-4 days to 0.02% saccharin vehicle, then offered FZP (provided by the National Institute on Drug Abuse Drug Supply Program) in the vehicle for 1 week (100 mg/kg for 3 days, 150 mg/kg for 4 days) as their sole source of drinking water, followed by 2 days of drug withdrawal. Daily water consumption was monitored to adjust drug concentration. Rats that did not achieve a weekly average of 120 mg/kg/day were excluded. During drug withdrawal, rats received saccharin water. Control rats received saccharin vehicle in parallel. All electrophysiological and immunochemical studies were conducted with the observer blind to the experimental treatment. This dosing regimen reliably induces manifestations of both benzodiazepine tolerance and dependence (Tietz et al., 1999b; Zeng and Tietz, 1999; Van Sickle et al., 2004; Xiang and Tietz, 2007). Rats treated in this way show a progressive increase in AMPA mEPSC amplitude from day 1 (~15%) to day 2 (~30%) after benzodiazepine withdrawal. Both the increase in AMPAR function and the associated anxiety-like behavior are transient and return to control levels within 4 days after treatment cessation (Van Sickle et al., 2004).
FZP is prescribed as a hypnotic at doses 6 times lower than diazepam, reflecting differences in its clinical potency and bioavailability (Chouinard, 2004) and is a full agonist at recombinant GABA<sub>A</sub> receptors with a relative potency ~10 times lower than diazepam (Downing et al., 2005). Thus, the concentration of FZP (100-150 mg/kg) offered to rats is appropriate to its relative potency. When expressed in diazepam equivalents, 1-week FZP treatment results in brain levels of FZP and its active metabolites equivalent to 0.6 mM diazepam, as determined by radioreceptor assay (Xie and Tietz, 1992), comparable to that reported for other typical chronic diazepam treatments (Izzo et al., 2001). Due to the short half-life of FZP in rat brain (<12 hr), the FZP level is negligible in the hippocampus 2 days after FZP withdrawal (Xie and Tietz, 1992; Van Sickle et al., 2004).

**Hippocampal tissue preparation for electrophysiology.** For acutely isolated neurons, coronal half-brain sections (400 μm) were prepared on a vibraslicer (Campden Instrument, Ltd. Lafayette, IN) in ice-cold, pre-gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) Ca<sup>2+</sup>-free PIPES solution containing (mM): NaCl 120; KCl 2.5; MgCl<sub>2</sub> 1; glucose 25; PIPES 20 (pH 7.4, 305 mOsm). For *in vitro* recordings, transverse hippocampal slices (400 μm) were prepared on a vibratome (Ted Pella, Inc., Redding, CA) as previously described (Van Sickle et al., 2004) in low Ca<sup>2+</sup>-high Mg<sup>2+</sup> dissection buffer then maintained ≥ 1 hr in artificial cerebral spinal fluid (ACSF, in mM: NaCl, 119; KCl, 2.5; CaCl<sub>2</sub>, 1.8; MgSO<sub>4</sub>, 1.0; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; NaHCO<sub>3</sub>, 26; D-glucose, 10; pH 7.4) prior to whole-cell recording at room temperature.
Acute neuron isolation for electrophysiology. Coronal slices were maintained at room temperature for >1 hr in gassed PIPES then digested in 1.4 units/ml protease XIV (Sigma-Aldrich Corp., St Louis, MO) in PIPES buffer at 32 °C for 40 min. After a wash in 0.1 % BSA and then in PIPES, the hippocampus was dissected on ice. CA1 minislices were notched on the dorsal and ventral edges, and followed by triturating in 100 μl PIPES using a 22, then 30 gauge pipette. The supernatant was removed and plated onto acid-washed coverslips. Cells were allowed to attach 15 min before use.

Whole-cell recording in isolated neurons. AMPAR currents were recorded in dissociated neurons in external solution, including (mM) NaCl 154; CaCl₂ 2; KCl 2.5; MgCl₂ 2; glucose 10; HEPES 10 (pH 7.35, 315 mOsm) with 200 μM CdCl₂ and 0.5 μM tetrodotoxin (TTX, Sigma) to block voltage-gated Ca²⁺ and Na⁺ channels. Neurons were selected by their relative size and pyramidal morphology as previously described (Tietz et al., 1999a). Elliptical shaped, very large or bipolar neurons were excluded. The borosilicate micropipette (4-6 MΩ, Sutter Instruments, Novato, CA) internal solution contained (mM): CsCH₃SO₃ 115; CsCl 20; HEPES 10; MgCl₂ 2.5; EGTA 0.6; Na₂ATP 4; NaGTP 0.4; Na₂phosphocreatine 10; as well as 50 units/ml creatine phosphokinase (pH 7.25, 295 mOsm). Bath application of 100 μM DL-2-amino-5- phosphopentanoic acid (APV, Sigma) was used to block N-methyl-D-aspartate receptor (NMDAR) currents activated by glutamate. Whole-cell recordings with ultra-fast drug application were performed at 22 °C on lifted, isolated CA1 pyramidal cells, similar to Mayer et al. (Partin and Mayer, 1996). Drugs were applied to lifted cells positioned near the adjacent opening.
of a three-barrel square-tip pipette, coupled to a fast-stepper device (Warner Instruments, Hamden, CT). Each barrel is ~7 times larger (~160 μm) than the dissociated CA1 neuron allowing for sufficient drug exposure. Transition time (20-80%, 0.09-0.47 ms) was estimated with the open-tip patch electrode response to a rapid solution change from normal (2.5 mM) to 100 mM external KCl at the end of the experiment. These application rates were previously shown to be sufficient for detection of whole-cell AMPAR currents and evaluation of AMPAR current kinetics (Partin and Mayer, 1996).

The degree of inward rectification of currents elicited by glutamate or kainate application to acutely isolated neurons or by endogenous glutamate release onto CA1 neurons in hippocampal slices, was used to evaluate the effect of benzodiazepine withdrawal on the proportion of GluR2-lacking to GluR2-containing AMPARs at extrasynaptic and synaptic sites (Washburn et al., 1997). In hippocampal neurons as in heterologous expression systems, GluR2-containing AMPARs, due to the Q residue at the Q/R RNA editing site in the intra-membrane domain, are resistant to spermine blockade and show linear I-V relationships or outward rectification in a voltage- and activity-dependent manner. Conversely, AMPARs lacking the GluR2 subunit are susceptible to spermine blockade and show inward rectification at positive holding potentials (Lerma et al., 1994; Koike et al., 1997). All of the widely used AMPAR agonists, including glutamate and AMPA, evoke strong desensitization of the receptor, with the exception of kainate (Patneau and Mayer, 1991). Importantly kainate responses in CA1 neurons can also be completely blocked by the AMPA antagonist, GYKI 53655 (Seifert et
al., 2000). Therefore, rectification studies were carried out with ultra-rapid application of glutamate onto lifted cells or during prolonged exposure to kainate to avoid AMPAR desensitization. During 1 ms application of 2 mM glutamate ($V_H = -60$ and $+40$ mV) or a voltage-ramp during 300 mM kainate application ($V_H = -100$ through $+80$ mV, 1 mV/ms ramp), 100 μM spermine (Sigma) was contained in the micropipette solution. Voltage-ramp currents were recorded on the same cell before and during kainate application. The kainate-induced I-V curve was obtained by subtracting the former from the latter currents.

Polyamines, like 1-naphthylacetyl spermine (NAS), which carry a bulky hydrophobic head group, either too large to permeate the narrow part of the pore and/or bind to a hydrophobic region in the channel, are much more potent AMPAR inhibitors than spermine itself (Stromgaard and Mellor, 2004). Therefore, NAS (100 μM, Sigma) was co-applied (2 s) extracellularly to inhibit currents elicited by sustained (6 s) application of kainate (300 μM, Ocean Produce International, Canada).

Currents were low-pass filtered at 5 kHz (Axoclamp 200A, Axon Instruments) and digitized at 10-20 kHz (Digidata 1322, Axon Instruments, Foster City, CA). The series resistance was compensated at least >85%. Whole-cell responses from isolated neurons (Table 1) were analyzed off-line from digitized current traces using Clampfit (Axon Instruments Inc., Foster City, CA) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Peak current (pA) was defined as the initial maximal negative deflection from the baseline value determined immediately before the onset of the drug response.
To avoid current variances due to differences in cell size, current density (pA/pF) was set as peak current amplitude (pA) divided by membrane capacitance (pF) from the same cell. The rise and decay kinetics of the AMPA currents were measured following application of a 1 ms pulse of 2 mM glutamate, while a 180 ms application was delivered for the measurement of desensitization kinetics. The rise and decay times were determined from 10% to 90% of the AMPA current rising phase or decay phase, respectively. The time constant ($\tau$) of desensitization was fit by a one component exponential equation $[f(t) = \sum_{i=1}^{n} A_i \exp(e \exp(-t / \tau_i)) + C]$. Concentration-response curves were fit by non-linear regression [sigmoidal dose-response (variable slope), I = $I_{\text{min}} + (I_{\text{max}} - I_{\text{min}})/(1 + 10^{((\text{LogEC}_{50} - \text{Log}[\text{glutamate}]) \times \text{HillSlope}))$. The rectification index was defined as peak current amplitude at a holding potential of +40 mV divided by that at -60 mV. For analysis of voltage ramps during kainate application, the ratio of the ramp slope measured around +40 (+35 to +45) and -60 (-65 to -55) mV was calculated. A linear I-V relationship yielded a ratio = 1; while ratios > 1 or < 1 were indicative of outward or inward rectification, respectively. Kainate-induced current density was also measured at $V_{\text{H}} = -60$ mV.

**Whole-cell recording in hippocampal slices.** For recording of action potential-independent, AMPAR-mediated miniature EPSCs, hippocampal slices were perfused (2.5 ml/min) at room temperature with gassed ACSF. Patch pipettes (5–9 MW) were filled with internal solution containing (in mM): CsCH$_3$SO$_3$, 132.5; CsCl, 17.5;
HEPES, 10; EGTA, 0.2; NaCl, 8; Mg-ATP, 2; Na₃-GTP, 0.3; QX-314, 2; spermine, 0.1, pH 7.2 adjusted with CsOH. Resting membrane potential (RMP) was measured immediately upon cell break-in and was invariant between groups (Table 2). Cells in which the holding current changed by more than 20% or the seal degraded were abandoned. Neurons were voltage-clamped ($V_H$ -60 to +40 mV) in continuous mode (cSEVC) using an Axoclamp 2A amplifier (Axon Instruments Inc.). Baseline mEPSC activity was recorded for at least 8 min with 1 μM TTX, 25 μM CGP-35348, 50 μM picrotoxin and 50 μM APV. Current output, monitored online (PClamp 9.0 Software, Axon Instruments Inc.), was low-pass filtered (10 KHz), amplified 100×, DC offset and then digitized at 20 kHz (Digidata 1320A, Axon) for later analysis.

Miniature EPSC events were detected and averaged using MiniAnalysis software (Synaptosoft Inc., Leonia, NJ). Peak current amplitude was derived from averaged events aligned by rise time (< 5 ms). Peak mEPSC amplitude and current kinetics were estimated using a single exponential function: $y(t) = a \exp(-t/t)$. The rectification index in CA1 neurons from hippocampal slices was defined as in isolated neurons.

**Immunofluorescence studies.** For confocal imaging of surface GluR1 and GluR2 subunits, and SAP97 antibody immunofluorescence, 5 mm hippocampal blocks were post-fixed in 4% paraformaldehyde in 0.1 M PB at 4°C overnight after decapitation. For dual labeling of surface N-GluR1/N-GluR2, non-permeablizing conditions were used on free-floating coronal slices (40 μm). After blocking with 10% donkey serum, signals were detected with a rabbit antibody against the amino terminal of the GluR1 subunit.
(N-GluR1 antibody, 1:5, Calbiochem, Germany) and a mouse antibody directed against the amino terminal of the GluR2 subunit (N-GluR2 antibody, 1:250, Chemicon), and then visualized with an Alexa 488-conjugated donkey anti-rabbit antibody for N-GluR1 (1:500, Molecular Probes, Eugene, OR) and an Alexa 594-conjugated donkey anti-mouse secondary antibody for N-GluR2 (1:500, Molecular Probes). For SAP97 analysis, tissue was permeabilized with 0.1% Tween20. After blocking with 10% donkey serum, slices were incubated at first with a rabbit anti-SAP97 antibody (1:250, Affinity BioReagents, Golden, CO), and followed by incubation with an Alexa 488-conjugated donkey anti-rabbit antibody (1:500). Experimental controls to verify antibody specificity included antigen pre-absorption, omission of primary antibodies, host-matched γ-globulin or incubation with detergent (data not shown). This penetration with detergent has been reliably applied for detecting total antibody signal, including that associated with cytoplasmic and membrane compartments. Omission of detergent was designed to assess surface signal. Sections were visualized and quantified using an Olympus BX51WI microscope (Olympus America Inc., Melville, NY), coupled to a Radiance 2000 laser scanning confocal system (Bio-Rad, Hercules, CA) with constant laser power, gain, iris and offset settings between samples. All data collection and analysis were conducted with the observer unaware of experimental group.

Immunofluorescence labeling on confocal images was assessed using ImageJ software (National Institutes of Health, Bethesda, MD). To avoid cross-talk between channels, we acquired green and red signals separately within a given area of the same
sections using different filters. The intensity of immunofluorescence was determined from the average grey scale value. Appropriate areas of interest were used to analyze the immunofluorescence signals: stratum pyramidale (sp) GluR1 and GluR2, somatic membrane area; stratum radiatum (sr) GluR1 and GluR2, dendrite area; sp SAP97, entire cell body. Mean density (integrated density/μm²) measurements were obtained using ImageJ Software. N-GluR1/2 colocalization signals were highlighted with the colocalization tool. The threshold was optimized to highlight only the area of colocalization and was held constant among samples and experimental groups. Particle mean density and particle number were standardized to cell circumference (sp) or dendrite length (sr) (μm).

**Subcellular fractionation and immunoblotting.** Fractionation methods were modified from those of (Smith et al., 2006), to yield P2 and P3 fraction relatively enriched in PSDs, as confirmed by PSD-95 immunoblotting (data not shown). All procedures were conducted at 0-4°C. Hippocampi were rapidly dissected from matched pairs of control and FZP-withdrawn rats and the CA1 region microdissected from 2-3 mm hippocampal slices, was immediately submerged in ice-cold homogenization buffer [10 mM Tris, pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 μM CsA, 0.5 μM okadaic acid, 1% protease inhibitor (Sigma)]. Homogenates were centrifuged at 960 X g for 10 min to remove large debris. The supernatant (S1) was then centrifuged at 10,000 X g for 30 min to obtain the crude membrane pellet (P2) and cytosol (S2). The PSD-enriched fraction (P3) was obtained by
incubating P2 pellets in Triton-homogenate buffer on ice for 20 min and then centrifuging at 32,000 X g for 1 hr. Proteins in S2 were precipitated with acetone at -20 °C for more than 2 hr, centrifuged at 3,000 X g for 30 min and air dried. Final pellets were sonicated in resuspension buffer (10 mM Tris, pH 8, 1 mM EDTA, and 1% SDS). Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Ten or fifteen micrograms of protein per well was mixed with sample buffer [Laemmli sample buffer (Bio-RAD, Hercules, CA) plus 5% β-mercaptoethanol] and running buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS) then loaded on a 10% polyacrylamide gel. Protein was wet-transferred to a nitrocellulose membrane. Primary antibodies were incubated with membranes overnight at 4°C. The antibody signal was detected with horseradish peroxidase (HRP)-coupled secondary antibodies (1:10,000/20,000, Jackson ImmunoResearch Inc, West Grove, PA), followed by enhanced chemiluminescence (ECL) (Denville Scientific Inc., Metuchen, NJ). All P2 and S2 fractions were conducted under the same conditions simultaneously to obtain P2/S2 density ratios. P3 fractions were run separately. Primary antibodies used included: anti-GluR1 (1:400, Santa Cruz biotechnology, CA), anti-GluR2 (1:500 or 1:1000, Molecular Probes) and anti-SAP97 (1:4000, Stressgen Biotechnologies, Canada) anti-actin (1:20,000, Chemicon, Temecula, CA). Images of immunoblots were scanned and immunoreactivity quantified with UN-SCAN-IT (ver 6.1, Silk Scientific, Orem, UT). Each antibody signals were normalized to the corresponding actin signal.
**Statistical analyses.** Data are reported as mean ± SEM and the significance level was set at $p < 0.05$. Error bars represent the standard error of the mean (SEM). Deviations from normality and homogeneity of variance were determined by Shapiro-Wilk test and F test, respectively. Statistical differences were determined by unpaired two-tailed Student’s $t$-test, or alternately Mann-Whitney U test when samples were not normally distributed.
Results

Synaptic and extrasynaptic AMPAR function is increased in response to FZP withdrawal. To investigate AMPAR channel properties, acutely isolated CA1 pyramidal neurons and hippocampal slices were prepared from control and 2-day FZP-withdrawn rats. AMPARs desensitize rapidly in the continued presence of agonist (Patneau and Mayer, 1991), limiting kinetic analysis. To avoid this problem, whole-cell recordings were first performed on lifted, isolated CA1 neurons (Fig. 1A), using ultra-rapid application of a saturating concentration (2 mM) of glutamate for 1 ms. Neurons from FZP-withdrawn rats showed enhanced AMPAR function, an increase in current density (pA/pF), i.e. current amplitude normalized by cell size, compared to controls. In some studies, excised patches have been used to investigate AMPAR kinetics. However, outside-out patch recordings only detect currents from isolated somatic or primary dendritic membranes. Therefore, whole-cell recordings rather than outside-out patch recordings were preferable to determine the kinetic characteristics of available surface AMPARs. There were no significant alterations in membrane capacitance, rise time or decay time between CON and FZP groups (Fig. 1B and C; Table 1). These results suggest that the function of both extrasynaptic and synaptic AMPARs may be upregulated in response to FZP withdrawal, though the contribution of synaptic AMPARs is uncertain.

To better address whether synaptic AMPAR function is augmented, whole-cell recordings were also performed in intact pyramidal neurons in hippocampal slices. Consistent with the former findings in isolated neurons, we also observed a significant
increase in AMPAR mEPSC amplitude at $V_H = -60$ mV, without a change in current kinetics (Table 2). These results are also compatible with previous mEPSCs recordings in CA1 neurons in hippocampal slices from FZP-withdrawn rats (Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Xiang and Tietz, 2007). Collectively, these findings suggest FZP withdrawal facilitates both extrasynaptic and synaptic AMPAR function.

Molecular mechanisms underlying the progressive increase in AMPAR function observed during FZP withdrawal could include one or several of the following AMPAR modifications: 1) altered channel kinetic properties, 2) increased glutamate affinity or efficacy, 3) increased membrane insertion of receptors, or 4) changes in AMPAR subunit composition or phosphorylation status. To further investigate channel kinetic properties, AMPAR desensitization was measured using prolonged glutamate application (180 ms) on lifted isolated neurons. There was no significant difference in tau of desensitization ($\tau_{\text{des}}$: CON 41.4 ± 4.0 vs. FZP 40.8 ± 4.4) (Fig. 1E and F), suggesting that altered whole-cell channel kinetics may not account for potentiation of AMPA currents during FZP withdrawal.

To address changes in glutamate affinity and efficacy at AMPARs during FZP withdrawal, glutamate concentration-response studies were conducted on lifted, isolated CA1 neurons. At saturating concentrations, from 500 μM to 3000 μM, neurons from the FZP-withdrawn group exhibited significantly increased AMPAR current density (Fig. 1G and H). Analysis of individual fits of concentration-response data revealed that average maximal current density ($I_{\text{max}}$) was significantly augmented during FZP withdrawal.
withdrawal (CON: 161.7 ± 24.2 pA/pF; FZP 233.4 ± 19.3 pA/pF, p < 0.05) in the absence of a change in glutamate EC₅₀ (CON: 210 ± 36 mM; FZP 282 ± 36 mM, p > 0.05) suggesting there was no change in glutamate affinity. Since the increased AMPAR current density was observed at saturating glutamate concentrations, an increase in the number of functional AMPARs at the cell surface rather than altered glutamate affinity or efficacy, is chiefly responsible for the enhanced AMPAR function.

**Functional incorporation of GluR2-lacking AMPARs in response to FZP withdrawal.** A switch in AMPAR subunit composition has been observed after many forms of neuronal activation (Liu and Cull-Candy, 2000). To determine whether such changes occur during FZP withdrawal, the current-voltage relationship (I-V) of CA1 neuron AMPA currents was evaluated in both isolated CA1 neurons and neurons in hippocampal slices. Inward rectification in the presence of intracellular spermine has been used as a measure of the proportion of GluR2-lacking AMPARs on CA1 neurons, both in culture and in organotypic hippocampal slices following tetanic stimulation of Schaffer collaterals (Shi et al., 1999; Esteban et al., 2003b). Isolated neurons from the FZP-withdrawn group showed a moderate decrease in the rectification index (peak amplitude at +40 mV/ peak amplitude at -60 mV) following rapid (1 ms, 2 mM) glutamate application (CON: 0.56 ± 0.04 vs. FZP: 0.46 ± 0.03, p < 0.05) (Fig. 2A and B), consistent with the degree of change in other pathological conditions (Washburn et al., 1997), though smaller than that reported in CA1 neurons following transient overexpression of GluR1 (or GluR4) subunits (Shi et al., 1999; Esteban et al., 2003b).
Miniature synaptic currents recorded from CA1 neurons in hippocampal slices from FZP-withdrawn rats also showed a decreased +40/-60 rectification index (CON: 0.76 ± 0.03 vs. FZP: 0.60 ± 0.04) (Fig. 2C and D). Together, the findings suggest a relative increase in both extrasynaptic and synaptic GluR2-lacking AMPARs during FZP withdrawal, which likely represent GluR1 homomers.

Since kainate is the only AMPAR agonist with a minimal capacity to induce desensitization and since at this age (P36-42) more than 95% of kainate-induced currents in CA1 neurons are due to AMPAR activation (Seifert et al., 2000), we applied kainate on CA1 neurons to further characterize the I-V properties of altered AMPA currents. There was a clearly observable (Fig. 2E and F) negative shift in the slope ratios derived from individual pyramidal neurons responses to a voltage ramp (-100 to +80 mV) during kainate application (CON slope ratio: 1.44 ± 0.08, n= 14; FZP slope ratio: 1.15 ± 0.03, n=21, p < 0.001). Eleven of fourteen (71%) control neurons were outwardly rectifying (1.2-2.1) while the remainder (4/14) had rectification indices between 1.1 and 1.2. In contrast, 4 of 21 FZP-withdrawn neuron responses were inwardly rectifying with slope ratios < 1.0 (0.83-0.95), while 16 had rectification indices between 1.0 and 1.2. One FZP-withdrawn cell exhibited outward rectification with a slope ratio of 1.39.

Acutely dissociated hippocampal neurons, most but not all with a pyramidal shape, were previously classified into three types: Type I (RI ≈ 1.61, 20.5%), outward rectifying; Type II (RI ≈ 0.5, 23%), inwardly rectifying; Type III (RI ≈ 1.13, 56.4%), linear I-V relationship (Lerma et al., 1994). In that report, the degree of kainate-induced
AMPAR-mediated current rectification and Ca\(^{2+}\) permeability in the whole cell population was inversely correlated.

The distribution of rectification indices in control neurons in the present study is consistent with that found by Lerma et al. (1994). The shift towards primarily linear or inwardly rectifying kainate-induced responses during FZP withdrawal suggests that in most of these CA1 neurons, AMPARs were transformed from principally GluR2-containing to a mixture of GluR2-containing and GluR2-lacking, Ca\(^{2+}\) -permeable AMPARs, reinforcing the findings observed in neurons from FZP-withdrawn rats after glutamate application and or synaptic glutamate release.

GluR1-containing AMPARs are specifically blocked by polyamines and several toxins. Due to the absence of a bulky hydrophobic head group, spermine is much less potent than the joro-spider toxin analogue, NAS and the digger wasp venom derivative, PhTX-343 (Stromgaard and Mellor, 2004). Thus in the current study, the presence of GluR1-containing AMPARs was further assessed pharmacologically with NAS. NAS was applied extracellularly during prolonged kainate exposure on acutely isolated neurons. Consistent with its relatively greater potency to block GluR2-lacking AMPARs (Koike et al., 1997; Stromgaard and Mellor, 2004), neurons from the FZP-withdrawn group showed a sizeable (71%) NAS blockade of kainate-induced AMPA currents compared with a small, though detectable (16%) blockade in neurons from the control group (Fig. 2G and H). Though the relative potency of spermine to block applied and exogenous glutamate can not be directly compared to NAS blockade of 300 \(\mu\)M kainate
currents, this observation is also consistent with the findings of rectification studies employing spermine in the micropipette. Together, these findings are all in agreement with a major contribution of GluR2-lacking AMPARs, possibly GluR1 homomers to the augmented AMPAR response during benzodiazepine withdrawal.

**Delivery of synaptic and extrasynaptic CA1 neuron GluR1 subunits in response to FZP withdrawal.** Rectification studies suggested an increase in membrane incorporation of GluR1 homomers during FZP withdrawal. However, the relative contribution of an increase in surface GluR1 homomers or GluR1/2 heteromers to AMPAR potentiation is unclear. Moreover, GluR1 subunit membrane incorporation may result from the regulation of receptor expression or redistribution of existing receptors. To address these questions, confocal immunofluorescence analysis was performed in hippocampal slices.

To detect whether GluR1 and/or GluR2 subunit surface incorporation was altered in response to FZP withdrawal, dual-immunofluorescence confocal imaging studies of anti-amino terminal (N-terminal) GluR1 and GluR2 antibodies were carried out under non-permeabilizing conditions, the most practical available approach to label surface AMPAR subunits. A marked increase in GluR1 signal as well as GluR1/2 colocalization was observed in CA1 neuron somata (sp) and apical dendrites (stratum radiatum, sr), but not basal dendritic regions (stratum oriens, so) (Fig. 3; so GluR1/2 co-localization data not shown). The increased GluR1 signal in sr, but not in so agrees with recent preliminary studies that showed a differential distribution of GluR1 homomers in apical
vs. basal dendrites (Bagal et al., 2005, Soc. Neurosci Abstr.). No differences were detected in surface GluR2 signal in somatic or dendritic areas (Fig. 3), suggesting that a change in GluR2 subunit surface expression is not involved in AMPAR potentiation during FZP withdrawal. Quantification of signal intensity (Fig. 3E and F) revealed that surface GluR1 signal was augmented more than GluR1/2 colocalization in sp (mean density increase: 64% vs. 26%) and sr (mean density increase: 71% vs. 45%), indicating that GluR1 subunits were inserted in somatic or apical dendritic membranes during FZP withdrawal as GluR1 homomers and GluR1/2 heteromers. Given the absence of alterations in surface GluR2 labeling, the observation that GluR1/2 colocalization was also enhanced suggests that membrane-inserted AMPARs might be reassembled from the GluR2/3 AMPAR subpopulation into GluR1/2 heteromers. Nevertheless, other possibilities such as synaptic delivery of homomeric GluR1 AMPARs adjacent to existing GluR2-containing receptors cannot be excluded.

To corroborate immunofluorescence studies, immunoblot analyses of subcellular CA1 neuron fractions were undertaken. Three subcellular fractions were collected from hippocampal CA1 minislices, including S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions. There were significant increases in GluR1 immunoreactivity in cytosolic and crude membrane fractions in response to FZP withdrawal (Fig. 4A), indicating that total GluR1 protein expression was augmented and may contribute to the elevated surface GluR1 incorporation. Moreover, a 57% increase in the amount of GluR1 in the PSD-enriched, P3 fraction was found in membranes from FZP-withdrawn as
compared to control rats (Fig. 4A), which support the electrophysiological findings and suggest that more GluR1 homomers may be inserted in synaptic membrane. In contrast to the increased GluR1 signal density, no alterations in the amount of GluR2 protein were found in any fraction after FZP withdrawal (Fig. 4B), in agreement with the lack of change in GluR2 immunofluorescence labeling. Meanwhile, the P2/S2 ratio of both GluR1 and GluR2 subunits was unaltered in CA1 minislices from the FZP vs. the control group (GluR1 P2/S2: CON 1.09 ± 0.11, FZP 1.14 ± 0.18; GluR2 P2/S2: CON 1.58 ± 0.30, FZP 2.23 ± 0.58, p > 0.05). Collectively, our immunochemical data suggest that enhanced AMPAR GluR1 subunit expression but not redistribution from the membrane P2 fraction to a more cytosolic fraction, contribute to AMPAR membrane incorporation and subsequent receptor potentiation during FZP withdrawal. Taken together, the findings suggest that enhanced GluR1 subunit expression and subsequent membrane delivery, rather than elevated GluR2 degradation, may result in increased GluR1 homomer incorporation into extrasynaptic and synaptic membranes, since neither total GluR2 protein level nor its P2/S2 ratio were changed after FZP withdrawal.

**SAP97 may promote GluR1 membrane delivery in response to FZP withdrawal.**

The increase in GluR1-containing cell surface AMPARs observed with FZP withdrawal is similar to that observed with LTP, and we thus sought to determine whether other similar mechanisms might be involved. In LTP, GluR1-containing AMPAR trafficking depends on interactions between the C-terminus of the GluR1 subunit and type I (PSD-95, Dlg and ZO-1) PDZ domain-containing proteins, such as SAP97 (Leonard et al., 1998; Hayashi et
SAP97, a 97 kDa postsynaptic PDZ protein, demonstrated to specifically associate with GluR1, but not other AMPAR subunits (Leonard et al., 1998). SAP97 was shown to act in the early secretory pathway of membrane-targeted GluR1-containing AMPARs (Sans et al., 2001). To investigate if SAP97 may be involved in the control of GluR1-containing AMPAR trafficking during FZP withdrawal, immunofluorescence studies with an anti-SAP97 antibody were performed following membrane permeabilization. Cytoplasmic SAP97 clusters were denser than observed in dendrites, and a significantly increased SAP97 signal (20%) was apparent in sp, but not sr of the FZP-withdrawn group (Fig. 5A). There was no change in SAP97 signal in basal dendrites (so) during FZP withdrawal (data not shown).

SAP97 protein level was further investigated by subcellular fractionation and immunoblotting. As seen in Fig. 5B, a significant upregulation of SAP97 expression was found in CA1 neuron P2 and P3 membrane components from FZP rats, with no apparent regulation of S2. Interestingly, the P2/S2 density ratio of anti-SAP97 labeled protein was significantly elevated in response to FZP withdrawal, suggesting a shift of SAP97 from the cytosolic to the membrane fraction. This shift corresponded to a ~45% increase in the amount of SAP97 in P2 relative to the S2 fraction during FZP withdrawal. As S2 SAP97 was unaltered, the increased in SAP97 protein associated with crude membranes (P2) and those enriched in asymmetric synapses (P3), may indicate a possible role for SAP97 in facilitating GluR1-containing AMPAR trafficking to extrasynaptic and synaptic membranes during benzodiazepine withdrawal.
Discussion

AMPAR-mediated current potentiation and a switch in AMPAR subunit composition at the postsynaptic membrane are critical to activity-dependent changes in synaptic strength. The nature of regulated AMPAR trafficking in various forms of synaptic plasticity is well characterized (Shi et al., 1999), while its role in mediating long-term drug effects, in particular FZP withdrawal-induced glutamatergic remodeling, is unclear. In our previous studies, anxiety-like behavior was associated with increased CA1 neuron AMPAR function in FZP-withdrawn rats (Van Sickle et al., 2004; Xiang and Tietz, 2007). The current electrophysiological studies demonstrate that extrasynaptic and synaptic AMPAR currents were both significantly enhanced during benzodiazepine withdrawal with no change in AMPAR channel properties. The relative shift towards inward rectification of AMPAR-mediated currents in the presence of polyamines indicates a substantial contribution of GluR2-lacking AMPARs to the increased AMPAR potentiation during FZP-withdrawal. Immunofluorescence studies and immunoblot analysis revealed increases in extrasynaptic and synaptic GluR1 signals with an increase in the total amount GluR1 protein, collectively suggesting that FZP-withdrawal promotes the expression of GluR1 subunits, as well as the delivery of GluR1 homomers into somatic and apical dendritic membranes and synapses. Concomitant with the increased level of GluR1 in P2 and P3 fractions, FZP withdrawal also promotes SAP97 expression, which may favor GluR1 subunit release from the endoplasmic reticulum (ER) and drive GluR1 subunits into membranes/synapses. Moreover, the significant increases in membrane association of
SAP97 and a shift of SAP97 from the S2 to the P2 fraction further supports the notion that SAP97 redistribution may be an important step for GluR1 membrane/synapse delivery during FZP withdrawal, as with other forms of activity-dependent plasticity. This is the first demonstration that FZP withdrawal promotes AMPAR membrane/synapse trafficking and subunit remodeling, leading to potentiation of AMPAR function, a mechanisms that is also central to LTP (Zamanillo et al., 1999; Hayashi et al., 2000; Shi et al., 2001; Malinow and Malenka, 2002). Moreover, the findings suggest that the mechanisms underlying activity-dependent hippocampal glutamatergic plasticity may be highly conserved and subserve the physiological adaptations that occur during drug withdrawal as well as other adaptive behaviors.

**AMPAR channel characteristics and increased glutamatergic strength in response to FZP withdrawal.** Enhancement of AMPAR function is the main phenomenon defining activity-dependent plasticity. LTP studies indicate that augmented AMPAR function could be related to alterations in AMPAR trafficking, receptor number, subunit composition, phosphorylation status, channel conductance and kinetics (Soderling and Derkach, 2000), among other possibilities. Both extrasynaptic and synaptic AMPAR-mediated currents elicited by exogenous and endogenous glutamate were augmented during FZP withdrawal. To explore the underlying mechanisms and evaluate the complement of AMPARs on cell membranes, AMPAR channel properties were investigated at the whole-cell level. Given that AMPARs desensitize very rapidly (in milliseconds), an ultra-rapid stepper system, which allowed millisecond agonist
application onto cells lifted, was used to ensure nearly simultaneous receptor activation without desensitization. No evidence of alterations in AMPAR channel kinetic properties (rise time, decay and desensitization rates) was found after FZP withdrawal, consistent with other LTP studies (Rammes et al., 1999). Therefore, channel kinetic properties are not a likely basis for increased glutamatergic strength induced by FZP withdrawal. Further single channel studies of somatic and dendritic membranes could help to clarify whether fundamental changes in AMPAR channel conductance or gating underlies AMPA current potentiation.

**AMPAR membrane delivery increases glutamatergic strength in response to FZP withdrawal.** Considerable evidence indicates that native AMPAR subunits have different trafficking patterns; GluR1-containing AMPARs are inserted into membranes in an activity-dependent manner, determined by interactions between GluR1 and type I PDZ proteins (Leonard et al., 1998; Sans et al., 2001), while GluR2-containing AMPARs are continuously cycled in and out of membranes, replacing existing receptors (Shi et al., 2001; Malinow and Malenka, 2002). These subunit-specific properties govern membrane delivery of AMPARs in response to neuronal activity. As in LTP, GluR1 membrane delivery requires the interaction between the PDZ binding domain of GluR1 and SAP97, the only PDZ protein known to interact specifically with the GluR1, but not other AMPAR subunits (Leonard et al., 1998). Our immunofluorescence studies demonstrated that surface GluR1 signal was augmented in sp, associated with increased SAP97 expression in the cell body, suggesting that SAP97 may be involved in facilitating release of GluR1
subunits from the ER and their further delivery to membranes/synapses. Importantly, enhanced GluR1 levels in P2 and P3 fractions were concomitant with the upregulation of SAP97 and its redistribution from cytosolic to membrane fractions. The membrane/synaptic relocalization of SAP97 was suggested to enable its participation in the regulation of AMPAR trafficking (Wu et al., 2002) and GluR-Ser\textsuperscript{845} phosphorylation (Colledge et al., 2000). Therefore, similar mechanisms may be operative during FZP withdrawal resulting in potentiation of AMPAR function and the associated anxiety-like behavior (Van Sickle et al., 2004; Xiang and Tietz, 2007).

The relative contribution of extrasynaptic GluR1-containing AMPARs to glutamatergic strength is uncertain. Several studies have suggested that GluR1-containing AMPARs at non-synaptic sites may also be important for plasticity, because functional AMPARs are expressed on CA1 somata and dendrites consistent with immunolabeling studies (Wenthold et al., 1996). Accordingly, large glutamate-evoked currents from nucleated CA1 somatic patches were nearly abolished in GluR1-deficient mice, reflecting a significant functional role for GluR1-containing AMPARs on CA1 cell bodies (Zamanillo et al., 1999). Despite the marked reduction in somatic AMPAR currents, these GluR1-deficient mice had normal AMPAR-mediated synaptic transmission; however, they could not sustain LTP (Zamanillo et al., 1999), confirming the importance of this subunit for activity-dependent plasticity and suggesting an important role for somatic GluR1-containing AMPARs in mediating increases in glutamatergic strength. Based on recent studies using photo-inactivation of AMPARs to measure real-time receptor
trafficking, it was proposed that AMPARs from a large internal store exchange rapidly with extrasynaptic somatic AMPARs and subsequently travel to dendrites via lateral diffusion where they are incorporated into synapses (Adesnik et al., 2005). Thus, in the present study, increased SAP97 may “prime” synaptic incorporation of GluR1-containing AMPARs by targeting GluR1 subunits to somatic membranes or by directly driving GluR1 subunit into synapses in response to FZP withdrawal, potentiating glutamatergic synaptic transmission.

Other candidates implicated in “priming” AMPAR trafficking, such as cAMP-dependent protein kinase A (PKA)-mediated phosphorylation, may also be involved. It has been reported that direct phosphorylation of GluR1 subunit by PKA permits AMPAR translocation from intracellular pools to the extrasynaptic membrane (Esteban, 2003a; Oh et al., 2006). Indeed, we have observed increased expression of phospho-GluR1 Ser\textsuperscript{845}, a specific site for PKA, in preliminary studies in FZP-withdrawn rats, supporting the hypothesis that PKA-mediated phosphorylation may be associated with GluR1 synaptic remodeling (Oh et al., 2006). Moreover, SAP97 could target A kinase-anchoring protein (AKAP)/PKA complex to GluR1 subunits via a PDZ domain interaction to facilitate phosphorylation of AMPAR GluR1 subunits by PKA (Colledge et al., 2000). Thus, SAP97 and PKA signaling pathways may be cooperatively involved in FZP-withdrawal-induced AMPAR remodeling. Detailed studies on AKAP-PKA complex distribution and activity in response to FZP withdrawal will be helpful. On the other hand, new evidence from our laboratory suggests that CaMKII signaling pathways, linked to
LTP induction and maintenance (Soderling and Derkach, 2000) also play a role in enhanced AMPAR function during FZP-withdrawal (Shen and Tietz, 2005, Soc. Neurosci Abstr.).

Another scenario mediating enhanced glutamatergic strength in LTP is an increase in the proportion of GluR1 subunit-containing synapses. In the adult hippocampus, up to 25% of excitatory synapses do not contain AMPARs and are termed “silent synapses". Upon LTP induction there is a decrease in the number of synaptic failures, indicating a conversion of silent to non-silent synapses, an effect that depends on increasing synaptic incorporation of GluR1-containing AMPARs (Shi et al., 1999; Malinow and Malenka, 2002). Whether there is a reduction in the proportion of silent synapses in FZP-withdrawn rats remains unclear, and will be resolved with more detailed electrophysiological studies and electron microscopic analysis of the relative distribution of GluR1 and GluR2 subunits in relation to the NMDA receptor NR1 subunit.

**Switch in AMPAR composition and its role in FZP withdrawal.** Previous studies have indicated that the presence of AMPAR GluR1 subunit alone was sufficient to sustain plasticity, since LTP could still be established in GluR2/3 double knockout mice (Meng et al., 2003). In contrast, a critical role for the AMPAR GluR2 subunit was also found in some studies (Bagal et al., 2005; Oh and Derkach, 2005). A switch in AMPAR composition has been observed in other forms of plasticity, such as developmental strengthening of excitatory synapses, and after synaptic activation (Liu and Cull-Candy, 2000). Thus, it is important to clarify whether a switch in AMPAR composition involved
in glutamatergic remodeling during FZP withdrawal. The relative shift in the degree of rectification in CA1 neurons suggests that the composition of the AMPAR population changed during FZP withdrawal: more GluR2-lacking AMPARs, possibly GluR1 homomers, were inserted into the membrane, resulting in AMPAR current potentiation. It appears that the removal of membrane-associated GluR2 subunits was not responsible for the shift in rectification, as there was no evidence of an alteration GluR2 subunit expression at any level examined. Meanwhile, surface GluR1 signal and GluR1/2 co-localization were significantly increased in the sp and sr area during FZP withdrawal. These findings suggest increased delivery of GluR1 homomers to the membrane and also a possible switch in AMPAR composition from GluR2/3 to GluR1/2 heteromers. Immunofluorescence analyses revealed a detectable increase in GluR1 subunit expression in apical (sr) dendritic areas, rather than in basal dendrites (so). Further electron microscopic studies focusing on GluR1 expression in apical and basal dendritic regions, as well as the relative proportion of GluR1 and GluR2 or GluR3 subunits will be beneficial to further elucidate changes in the synaptic composition of AMPARs during benzodiazepine withdrawal.

**Increased glutamatergic strength as a basis for addictive and abusive behaviors.**

Our studies suggest that an LTP-like increase in glutamatergic strength contributes to CA1 hyperexcitability during benzodiazepine withdrawal; however, the stimulus for membrane delivery of GluR1 subunits remains uncertain. The GABA-mediated depolarization observed in FZP-withdrawn rats (Zeng et al., 1995) might provide the Ca$^{2+}$ influx required
to initiate AMPA receptor membrane insertion (Malinow and Malenka, 2002; Oh and Derkach, 2005) via voltage-gated Ca$^{2+}$ channels (Xiang and Tietz, 2007). Moreover, theta activity increases during FZP withdrawal, consistent with an essential role for septo-hippocampal theta activity in the expression of withdrawal-anxiety (McNaughton and Gray, 2000). Drug-induced enhancement of CA1 neuron output through increased theta activity may provide the neurophysiological basis for contextual memories associated with drug-related environmental cues, implicated in re-instatement of drug-seeking behavior (Nestler, 2005). Thus, overactivation of hippocampal glutamatergic efferent circuits may provide a substrate for the reinforcement of addictive and abusive behaviors elicited by a variety of drugs of abuse, despite diverse pharmacologic sites of action (Malinow and Malenka, 2002; Nestler, 2005). Understanding the role of enhanced glutamatergic function in withdrawal-anxiety is clinically significant, as these symptoms can lead to overuse of benzodiazepines prescribed for anxiety and insomnia, a rising form of prescription drug misuse, and may also be a factor in the increase in benzodiazepine abuse among polydrug abusers (Griffiths and Johnson, 2005).

The finding that FZP withdrawal facilitates extrasynaptic and synaptic AMPAR function and promotes membrane and synapse targeting of GluR1-containing AMPARs in CA1 pyramidal neurons is novel, and similar to the central mechanism underlying LTP. SAP97 may prime GluR1 subunit redistribution from the cytosol to the plasma membrane, leading to AMPAR current potentiation. Increased membrane incorporation of GluR1 AMPARs, and the consequent potentiation of AMPAR function, may represent a common
regulatory mechanism for synaptic remodeling and neuronal signal processing associated with a variety of adaptive behaviors including drug dependence, learning and memory.
References


Malinow R and Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. 


Figure 1. AMPAR current density is increased in dissociated CA1 neurons after FZP withdrawal. Currents were recorded at $V_H = -60$ mV with 0.5 μM TTX, 200 μM CdCl$_2$ and 100 μM APV in isolated neurons from control (CON) and 2-day FZP-withdrawn (FZP) rats. (A) Photomicrograph of an acutely dissociated neuron. Scale bar: 40 μm. (B) Evoked AMPAR-mediated currents after ultra-rapid, 1 ms application of 2 mM glutamate (Glu). (C) Glutamate-induced current density (pA/pF) was significantly increased after 2-day FZP withdrawal (n = 21 cells/6 rats per group). (D) Currents evoked by the AMPAR agonist kainate (KA, 300 μM) during a 1mV/ms voltage ramp as shown in Figure 2E. KA-induced current density (pA/pF) at $V_H = -60$ mV was significantly increased after 2-day FZP withdrawal (CON: n = 14 cells/3 rats; FZP: n = 21 cells/3 rats, $p < 0.02$). (E) Currents evoked by 180 ms application of 2 mM glutamate. (F) No significant difference in tau of desensitization between CON and FZP groups (CON: n = 15 cells/5 rats; FZP: n = 16 cells/5 rats). (G) Representative currents elicited by a 10 ms application of increasing glutamate concentrations (30 μM to 3000 μM). (H) Concentration-response curve representing average AMPAR current density (CON: solid circles; FZP: open circles). Current densities (pA/pF) were significantly increased in the FZP group at glutamate concentrations from 500 μM to 3000 μM (CON: n = 10 cells/7 rats; FZP: n = 13 cells/7 rats). Data are represented by mean ± SEM; Error bars in this and all other figures represent the SEM. Asterisks: $p < 0.05$ by unpaired Student’s $t$-test.
Figure 2. Shift towards inward rectification and spermine blockade of agonist-evoked currents in CA1 neurons after FZP withdrawal. (A) AMPAR-mediated currents were recorded at -60 and +40 mV holding potentials in isolated neurons after 1 ms application of 2 mM glutamate with 0.5 μM TTX, 200 μM CdCl2 and 100 μM APV in the bath. Spermine (100 μM) was included in the micropipette to replace endogenous spermine lost during dialysis. (B) The rectification index (+40/-60 mV) for glutamate was significantly decreased after 2-day FZP withdrawal (CON: 18 cells/6 rats; FZP: 20 cells/6 rats). (C) Representative averaged AMPAR-mediated mEPSCs recorded at -60 and +40 mV holding potentials in hippocampal slices in the presence of 1 μM TTX, 50 μM APV, and 50 μM picrotoxin, 25 μM CGP35348 and with spermine in the micropipette. (D) The mEPSC rectification index (+40/-60 mV) was significantly decreased after 2-day FZP withdrawal (CON: 9 cells/8 rats; FZP: 11 cells/6 rats). Asterisk in B and D: $p < 0.05$ by unpaired Student’s t-test. (E) Representative pair of voltage-ramps at holding potentials from -100 to +80 mV generated at 1 mV/ms during 300 μM KA application. The current density from the FZP neuron was normalized to the CON neuron. At positive holding potentials, the CON neuron showed outward rectification (slope ratio = 1.46), while the FZP neuron rectification was nearly linear (slope ratio = 1.08). (F) The distribution of slope ratios from individual CON (closed circles) and FZP neurons (open circles). The slope ratios (+35 to +45 mV/-65 to -55 mV) derived from the voltage ramp during KA application were significantly decreased after 2-day FZP withdrawal (CON: 14 cells/3 rats; FZP: 21 cells/3 rats). Horizontal bar.
represents the mean. Asterisks: \( p < 0.001 \) by unpaired Student’s \( t \)-test. (G)

Representative currents elicited at -80 mV by 6 s 300 \( \mu \)M KA and 2 s co-application of 100 \( \mu \)M NAS without intracellular spermine. (H) Cells from the FZP group exhibited significantly increased NAS blockade compared to the CON group, (CON: 16 cells/3 rats; FZP: 13 cells/3 rats). Asterisks: \( p < 0.001 \) by Mann-Whitney U test.
Figure 3. Surface GluR1, but not GluR2, subunit immunofluorescence is increased in CA1 neurons after FZP withdrawal. GluR1 and GluR2 surface expression were detected with anti-N-terminal antibodies. (A) Surface GluR1 subunit staining in hippocampal CA1 area (so: stratum oriens; sp: stratum pyramidale; sr: stratum radiatum). Scale bar: 50 μm. (B) Merged images of GluR1/2 subunit staining in so from both groups. There are no significant differences in surface GluR1, GluR2 (data not shown) or GluR1/2 colocalization. Scale bar: 20 μm. (C) Representative photomicrographs of GluR1 (left), GluR2 (middle) and colocalization of GluR1/2 staining (right) in sp from the CON (upper panels) and FZP (lower panels) groups. Scale bar: 20 μm. Arrows within inset images (enlarged 200%) depict examples of co-localized signal at the membrane, quantified in E as a function of cell circumference. (D) Representative photomicrographs of GluR1 (left), GluR2 (middle) and colocalization of GluR1/2 staining (right) in the first 100 μm of the apical dendritic area (sr) immediately adjacent to sp, from the CON (upper panels) and FZP (lower panels) groups, Scale bar: 20 μm. (E) Quantification of GluR1 (left), GluR2 (middle) and colocalization of GluR1/2 (right) immunofluorescence signals in sp. The FZP group showed a significant increase in surface GluR1 signal (mean density) compared with the CON group by Mann-Whitney U Test. No significant differences were found in GluR2 signal between groups. Quantification of GluR1/2 colocalization signal between groups showed a significant increase in particle mean signal density and particle number/cell in the FZP group by unpaired Student’s t test. (F) Quantification of GluR1 (left), GluR2 (middle) and colocalization of GluR1/2 (right)
immunofluorescence signal in sr. The FZP group showed a significant increase in surface GluR1 signal (mean density) by unpaired Student’s t test. No significant differences were found in GluR2 signal between groups. Quantification of GluR1/2 colocalization signal between groups showed a significant increase in particle mean signal density and particle number/dendrite length by unpaired Student’s t test. CON: n = 19-27 cells/4 rats; FZP: n = 19-28 cells/4 rats. Asterisk: $p < 0.05$; double asterisks: $p < 0.01$; and triple asterisks: $p < 0.001$. 
A

S2

GluR1

Actin

% of Control Density Ratio

CON   FZP

*  

P2

CON   FZP

*  

P3

CON   FZP

*  

B

S2

GluR2

Actin

% of Control Density Ratio

CON   FZP

CON   FZP

CON   FZP
Figure 4. Total GluR1, rather than GluR2, protein levels were enhanced in CA1 neurons after FZP withdrawal. CA1 minislices from pairs of control (CON) and FZP-withdrawn groups were fractionated to obtain S2 (cytosolic), P2 (crude membrane) and P3 (PSD-enriched) fractions. GluR1 and GluR2 signal densities were normalized to the respective actin signal. (A) Total GluR1 signal density was significantly increased in S2, P2 and P3 fractions derived from of FZP-withdrawn rats. Representative blots are illustrated in the top panel; quantitative analyses of signal density are shown in the histograms below (single asterisk: $p < 0.05$, n = 7/group). GluR1 subunit protein did not redistribute during 2-day FZP withdrawal with P2/S2 ratio $105 \pm 17\%$ of control ($p > 0.05$). S2 and P2: 15 μg protein/well; P3: 10 μg protein/well. (B) GluR2 signal density did not change in any fraction in response to FZP withdrawal ($p > 0.05$, n = 7/group). Although there was a slight increase in GluR2 P2/S2 ratio in FZP rats ($41 \pm 37\%$), no significant difference was found between groups. All data were analyzed by Student’s $t$ test. All fractions loaded with 15 μg protein/well.
Figure 5. SAP97 signal increased in CA1 neurons after FZP withdrawal. SAP97 immunoreactivity was detected with confocal imaging (A) and immunoblotting (B). (A) Representative images of SAP97 signal from control (CON) and FZP-withdrawn groups (sp: upper panels; sr: lower panels). Scale bar: 50 μm or 20 μm for sp or sr, respectively. Quantifications of SAP97 signal is shown in the panel at right. Compared with the CON group, the FZP group showed a significant increase in sp SAP97 signal (mean density) by Mann-Whitney U Test. No significant differences in sr SAP97 signal were found between groups. CON and FZP: n = 19 cells/4 rats per group. Single asterisk: *p < 0.05. (B) SAP97 signal density was normalized to the respective actin signal. There were significant increases in SAP97 signal density in the crude membrane (P2) and PSD-enriched (P3) fractions from CA1 minislices of FZP-withdrawn rats compared to the CON group. Representative immunoblots are illustrated in the top panel; quantitative analysis of signal density is shown in the histograms below. Redistribution of SAP97 was also observed in the FZP-withdrawn group with a P2/S2 ratio 144 ± 15% of the CON group. Single asterisk: *p < 0.05, n = 6-7 pairs of rats. P3 SAP97 signal was analyzed by Mann-Whitney U Test; other fractions were analyzed by Student’s t test. S2 and P2: 15 μg protein/well; P3: 10 μg protein/well.
**Table 1.** AMPAR kinetics of 1 ms glutamate (2 mM) application. Whole-cell recordings were made on lifted CA1 pyramidal neurons with ultra-fast drug application system. Current density was significantly increased during FZP withdrawal in the absence of alterations of AMPAR channel kinetics. $C_m$: membrane capacitance; Rise time: 10-90% rise time; $I_{peak}$: peak amplitude of current. Data were listed as mean ± SEM (number of cells). $C_m$, $I_{peak}$ and current density were analyzed by two-tailed unpaired Student’s $t$ test; 10-90% rise time and decay time were analyzed by Mann-Whitney $U$ test. Single asterisk: significant difference ($p < 0.05$).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>$C_m$ (pF)</th>
<th>Rise time (ms)</th>
<th>$I_{peak}$ (pA)</th>
<th>Current density (pA/pF)</th>
<th>Decay time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CON</strong></td>
<td>20.8 ± 0.6 (21)</td>
<td>1.3 ± 0.1 (20)</td>
<td>4159.6 ± 421.3 (21)</td>
<td>199.8 ± 19.3 (21)</td>
<td>16.2 ± 1.1 (18)</td>
</tr>
<tr>
<td><strong>FZP</strong></td>
<td>19.4 ± 0.5 (21)</td>
<td>1.3 ± 0.1 (20)</td>
<td>5073.6 ± 480.0 (21)</td>
<td>260.5 ± 22.7 (21)*</td>
<td>17.3 ± 1.0 (19)</td>
</tr>
</tbody>
</table>
Table 2. Characteristics of mEPSCs. Miniature EPSCs were recorded in CA1 pyramidal neurons in hippocampal slices at $V_H = -60$ mV and $+40$ mV. There was a significant increase in mEPSC peak current amplitude in FZP-withdrawn neurons at $V_H = -60$ mV. RMP: resting membrane potential. Peak current amplitude ($I_{peak}$) was derived from the averaged events with a rise time $< 5$ ms. Data listed as mean ± SEM (number of cells) were analyzed by two-tailed unpaired Student’s $t$ test. Single asterisk indicates a statistically significant difference ($p < 0.05$).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>V$_H$ = -60 mV</th>
<th>V$_H$ = +40 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{peak}$ (pA)</td>
<td>Rise time (ms)</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>67.3 ± 0.8</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(9)</td>
</tr>
<tr>
<td>FZP</td>
<td>64.8 ± 1.3</td>
<td>8.3 ± 0.3</td>
</tr>
</tbody>
</table>
MANUSCRIPT 2

GluR1-SAP97-AKAP-PKA interactions implicated in benzodiazepine withdrawal-induced AMPA receptor-associated plasticity

Jun Song and Elizabeth I. Tietz

Department of Physiology and Pharmacology,

Cellular and Molecular Neurobiology Program,

University of Toledo, College of Medicine, Health Science Campus, Toledo, OH 43614
Abstract

Glutamatergic system adaptive remodeling is emerging as an important neural substrate of drug dependence. Benzodiazepines, widely used as anxiolytics, hypnotics and anticonvulsants, have their actions as allosteric modulators of GABA<sub>AR</sub>s; while withdrawal symptoms such as anxiety, insomnia and seizures may be associated with enhanced AMPAR function. Previous electrophysiological and immunofluorescent studies revealed that the number of GluR1 subunits inserted into CA1 neuron membranes/synapses was increased in FZP-withdrawn rats, which may contribute to AMPAR potentiation. In LTP, AMPAR function and trafficking can be controlled by kinase-mediated phosphorylation, including PKA which can target GluR1 subunit membrane incorporation through direct AKAP79/150-SAP97 interactions. Since expression and membrane/synapse redistribution of SAP97 is augmented in CA1 neurons during FZP withdrawal, PKA thus, GluR1 subunits may be recruited through a similar AKAP79/150-SAP97 interaction. In the current study, immunoblotting was performed on subcellular fractions of CA1 minislices in order to investigate the localization and benzodiazepine withdrawal- associated redistribution of phospho-Ser<sup>845</sup> GluR1, PKA subunits and AKAP79/150. In agreement with increases in SAP97 immunoreactivity, concomitant elevated levels of phospho-Ser<sup>845</sup> GluR1 and AKAP79/150 proteins were found in both crude membrane and PSD-enriched compartments of CA1 minislices from FZP-withdrawn rats. There were no alterations in the density of PKA regulatory and catalytic subunits. Collectively, these findings suggest
an increased interaction between SAP97-AKAP-PKA, rather than enhanced PKA expression or activity, may result in proportionate increases in phosphorylated GluR1-containing AMPARs associated with enhanced total GluR1 expression, and subsequent receptor potentiation.
Introduction

Benzodiazepines, as positive modulators of type A gamma-aminobutyric acid receptors (GABA<sub>A</sub>Rs), have been used clinically as anxiolytics, hypnotics and anticonvulsants. But their utility is limited by the development of functional tolerance and dependence (Bateson, 2002). Uncoupling of the allosteric linkage between the GABA and benzodiazepine sites and downregulation of GABA<sub>A</sub>R subunit expression are mechanisms central to benzodiazepine tolerance (Tietz et al., 1999a; Izzo et al., 2001; Bateson, 2002). However, benzodiazepine dependence has been reported to depend on both GABAergic (Lader, 1994; Bateson, 2002; Wafford, 2005) and glutamatergic systems (Izzo et al., 2001; Van Sickle et al., 2004; Xiang and Tietz, 2007).

Regulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, ionotropic glutamate receptors (AMPARs) plays an important role in benzodiazepine dependence. Benzodiazepine withdrawal symptoms manifest as anxiety-like behavior was associated with AMPAR potentiation; and pretreatment with the AMPAR antagonist, GYKI-52466, completely blocked withdrawal-anxiety (Van Sickle et al., 2004; Xiang and Tietz, 2007). The amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) and glutamate elicited EPSCs was elevated in CA1 pyramidal neurons in response to flurazepam (FZP) withdrawal (Van Sickle et al., 2004; Song and Tietz, 2005, Soc. Neurosci Abstr.), which could arise from increased expression of AMPARs, alterations in AMPAR phosphorylation status, a subunit switch or regulated AMPAR trafficking. Upregulation of GluR1 subunit mRNA and protein was observed in
hippocampal CA1 neurons during benzodiazepine withdrawal (Izzo et al., 2001; Song and Tietz, 2006, Soc. Neurosci Abstr.). Furthermore, the significant increase in both intracellular and extracellular spermine blockade in CA1 neurons supports the possibility of a switch toward GluR1 homomer incorporation into the population of membrane-incorporated AMPARs during FZP withdrawal (Song and Tietz, 2005, Soc. Neurosci Abstr.).

Additionally, during activity-dependent plasticity, AMPAR trafficking and/or function is also controlled by AMPAR phosphorylation (Banke et al., 2000; Esteban et al., 2003b; Oh et al., 2006). There are several important phosphorylation sites on the C-terminus of GluR1 subunits, including Ser$^{831}$, a site for Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII)- and protein kinase C-mediated phosphorylation and Ser$^{845}$, a site for cAMP-dependent protein kinase A (PKA)-mediated phosphorylation (Barria et al., 1997b; Banke et al., 2000; Menard et al., 2005). In our earlier studies, AMPAR channel conductance was increased during FZP withdrawal, an effect eliminated by pre-incubation with the CaMKII antagonist, KN93 (Shen and Tietz, 2005, Soc. Neurosci Abstr.), indicating that CaMKII activity may be required for AMPAR potentiation. PKA is another protein kinase critical for mediating AMPAR potentiation associated with hippocampal long-term potentiation (LTP) (Frey et al., 1993; Weisskopf et al., 1994; Blitzer et al., 1995). In hippocampal CA1 slices, repeated high-frequency stimulation induces long-lasting LTP that requires PKA activity (Huang et al., 1994); PKA inhibitors were shown to block hippocampal LTP and cAMP analogs induced a potentiation that
occluded tetanus-induced LTP (Frey et al., 1993). Although the PKA dependence of hippocampal LTP-induced AMPAR potentiation has been characterized through various pharmacological and genetic approaches (Frey et al., 1993; Abel et al., 1997), whether analogous mechanisms to those underlying activity-dependent plasticity may be involved in benzodiazepine withdrawal-induced glutamatergic remodeling remains unresolved. Thus, the present study focused first on whether PKA-mediated phosphorylation of AMPAR GluR1 subunits is enhanced in the hippocampal CA1 area during FZP withdrawal.

Specificity of PKA signaling cascades could arise from association with A kinase-anchoring proteins (AKAPs), which function to target PKA to specific substrates at precise subcellular locations (Colledge and Scott, 1999). In rat hippocampal CA1 pyramidal neurons, AKAP150 was observed only at the postsynaptic density (PSD) of excitatory, rather than inhibitory synapses (Lilly et al., 2005). AKAP79/150 (human/rodent) is a scaffolding protein, which binds to PKA and to members of the membrane-associated guanylate kinase (MAGUK) protein family, such as PSD95 and SAP97, and subsequently targets PKA to glutamate receptors. In cultured hippocampal neurons, phosphorylation of AMPARs at Ser\(^{845}\) site was enhanced by a SAP97-AKAP complex that directed PKA to GluR1 via a PDZ domain interaction (Colledge et al., 2000). Therefore, understanding the interactions between SAP97-AKAP-PKA complexes will also be helpful to clarify the mechanism underlying CA1 neuron AMPAR potentiation in our FZP-withdrawal model.
To address these issues, immunoblot studies were conducted using modified subcellular fractionation techniques designed to clarify the localization and redistribution of the AMPAR GluR1 subunit, various PKA subunits and AKAP79/150 following FZP withdrawal. Immunoreactivity of phospho-Ser$^{845}$ GluR1, total GluR1 subunit protein, and AKAP79/150 were enhanced in crude membrane and PSD-enriched fractions, concomitant with SAP97 upregulation. No alterations in the density of PKA regulatory and catalytic subunits were observed. Therefore, the increased interaction between PKA, AKAP79/150 and SAP97, rather than an elevation in PKA protein levels or activity, may contribute to the increased amount of phosphorylated GluR1-containing AMPARs proportionate to the enhanced total GluR1 subunit and subsequent plasticity.
Materials and methods

Animal model. All methods were approved by the University of Toledo College of Medicine, Institutional Animal Care and Use Committee (IACUC) and conformed to National Institutes of Health guidelines. One-week FZP treatment and withdrawal was carried out as previously established (Van Sickle et al., 2004; Song et al., 2007 submitted). In short, P36-42 male Sprague–Dawley rats (Harlan, Indianapolis, IN) were first adapted 2-4 days to a 0.02% saccharin vehicle, then offered FZP (provided by the National Institute on Drug Abuse Drug Supply Program) in the vehicle for 1 week (100 mg/kg for 3 days, 150 mg/kg for 4 days) as their sole source of drinking water. Daily water consumption was monitored to adjust drug concentration. Rats that did not achieve a weekly average of 120 mg/kg/day were excluded. During drug withdrawal, rats received saccharin water for 2 days. Control rats received saccharin vehicle in parallel.

Subcellular fractionation and immunoblotting. Fractionation methods were modified from those of (Smith et al., 2006), to yield P2 and P3 fraction relatively enriched in PSDs, as confirmed by PSD-95 immunoblotting (data not shown). All procedures were conducted at 0-4°C. Hippocampi were rapidly dissected from matched pairs of control and FZP-withdrawn rats and the CA1 region microdissected from 2-3 mm hippocampal slices, was immediately submerged in ice-cold homogenization buffer [10 mM Tris, pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 μM CsA, 0.5 μM okadaic acid, 1% protease inhibitor (Sigma)]. Homogenates were centrifuged at 960 X g for 10 min to remove large debris. The
supernatant (S1) was then centrifuged at 10,000 X g for 30 min to obtain the crude membrane pellet (P2) and cytosol (S2). The PSD-enriched fraction (P3) was obtained by incubating P2 pellets in Triton-homogenate buffer on ice for 20 min and then centrifuging at 32,000 X g for 1 hr. Proteins in S2 were precipitated with acetone at -20°C for more than 2 hr, centrifuged at 3,000 X g for 30 min and air dried. Final pellets were sonicated in resuspension buffer (10 mM Tris, pH 8, 1 mM EDTA, and 1% SDS). Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Ten or fifteen micrograms of protein per well was mixed with sample buffer [Laemmli sample buffer (Bio-RAD, Hercules, CA) plus 5% β-mercaptoethanol] and running buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS) then loaded on a 10% polyacrylamide gel. Protein was transferred to a nitrocellulose membrane. Primary antibodies were incubated with membranes overnight at 4°C. The antibody signal was detected with horseradish peroxidase (HRP)-coupled secondary antibodies (1:10,000/20,000, Jackson ImmunoResearch Inc, West Grove, PA), followed by enhanced chemiluminescence (ECL) (Denville Scientific Inc., Metuchen, NJ). All P2 and S2 fractions were conducted under the same conditions simultaneously to obtain P2/S2 density ratios. P3 fractions were run separately. For the phospho-Ser$^{845}$GluR1/GluR1 ratio, after initial detection with phospho-GluR1 antibody, the membranes were stripped at 60°C for 1 hr using Restore stripping buffer (Pierce, Rockford, IL), followed by through washes in Tris-buffered saline with 0.1% Tween 20.
Total-GluR1 antibody was applied after blocking as described above. Primary antibodies used included: anti-GluR1 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Ser^{845} GluR1 (1:400 or 1:800, PhosphoSolutions, Aurora, CO), anti-AKAP150 (1:200, Santa Cruz Biotechnology), anti-PKARII$\alpha$ (1:2000, BD Biosciences, San Jose, CA), anti-PKARII$\beta$ (1:1000, Chemicon, Temecula, CA), anti-PKARI (1:250, BD Biosciences), anti-PKA catalytic subunit (1:1000, BD Biosciences), anti-actin (1:20,000, Chemicon) and anti-GAPDH (1:20,000, Chemicon). Images of immunoblots were scanned and immunoreactivity quantified with UN-SCAN-IT (ver 6.1, Silk Scientific, Orem, UT). Each antibody signal was normalized to the corresponding actin or GAPDH signal.

**Statistical analyses.** Data are reported as mean ± SEM and the significance level was set at $p < 0.05$. Error bars represent the standard error of the mean (SEM). Deviations from normality and homogeneity of variance were determined by Shapiro-Wilk test and F test, respectively. Statistical differences were determined by unpaired two-tailed Student’s $t$-test.
Results

FZP-withdrawal increases GluR1 expression and a proportionate amount of PKA-phosphorylated GluR1

In previous, electrophysiological studies the amplitude of AMPAR-mediated mEPSCs and glutamate-elicited EPSCs was elevated in response to FZP withdrawal (Van Sickle et al., 2004; Song and Tietz, 2005, Soc. Neurosci Abstr.), associated with increased levels of AMPAR GluR1 subunit immunoreactivity (Song et al., 2007 submitted). However, whether other mechanisms, such as AMPAR phosphorylation also contribute to potentiation of AMPAR currents is unknown. In activity-dependent plasticity, PKA-mediated phosphorylation of GluR1-containing AMPARs is necessary for AMPAR potentiation, by increasing receptor open probability (Banke et al., 2000) and/or promoting AMPAR trafficking to synapses (Esteban, 2003a). Therefore, a similar mechanism i.e. increased CA1 neuron AMPAR phosphorylation by PKA may also play a role to increase glutamatergic strength in our FZP withdrawal model.

A modified subcellular fractionation procedure was used to enrich PSDs in P2 and P3 fractions. As shown in Fig. 1A, 2-day FZP-withdrawal an increase in phospho-Ser$^{845}$ GluR1 (pGluR1) immunoreactivity in P2 (crude membrane, ~23%) and P3 (PSD-enriched, ~60%) fractions. The slight change in pGluR1 signal in S2 (cytosol, ~14%) was not significant. No redistribution of phospho-Ser$^{845}$ GluR1 from S2 to P2 was observed (FZP P2/S2 ratio was 97 ± 18% of control). Meanwhile, total AMPAR GluR1 subunit signal density was investigated on the same membrane after stripping (Fig. 1B).
There were significant increases in total GluR1 expression in all fractions, concomitant with our previous observations that GluR1 immunofluorescent labeling was elevated in somata and dendrites of CA1 neurons (Song and Tietz, 2006, Soc. Neurosci Abstr.). As with the phospho-Ser\textsuperscript{845} GluR1 signal, there was also no redistribution of total GluR1 protein between cytosolic and membrane fractions (FZP P2/S2 ratio 95 ± 4% of control).

To investigate whether the enhancement of phosphorylation at the Ser\textsuperscript{845} site was correlated with increases in total GluR1 proteins, the pGluR1/GluR1 ratio was quantified. As shown in Fig. 1C, there were no significant differences in the pGluR1/GluR1 ratio between CON and FZP groups in any fraction, indicating that during FZP withdrawal PKA-mediated GluR1 phosphorylation was due to proportional increases in total GluR1 expression, rather than enhanced PKA levels or activity.

**FZP-withdrawal increases AKAP expression in crude membrane and PSD-enriched fractions**

At the PSD of asymmetric synapses, AKAPs have been characterized that anchor PKA holoenzymes through binding RI or RII regulatory subunits, as well as organize functional ion channels by binding with scaffolding proteins, such as SAP97 and PSD95 (Colledge et al., 2000; Dell'Acqua et al., 2006). As for AMPARs, it was reported that the GluR1 subunit and PKA were recruited into a signaling complex through direct interactions between membrane-associated guanylate-like kinase (MAGUK) proteins, SAP97 and AKAP79/150 (Colledge et al., 2000). In previous studies, SAP97 immunoreactivity and SAP97 signal density on immunoblots was redistributed from
cytosolic (S2) to crude membrane (P2) fractions after FZP withdrawal (Song et al., 2007 submitted). These findings suggest that, as with activity-dependent plasticity, increased levels of SAP97 may interact with AKAP, recruiting PKA to GluR1 subunits at plasma membranes and synapses where it enhances GluR1 Ser$^{845}$ phosphorylation and thus contributes to AMPAR potentiation.

To test this hypothesis, the expression of AKAP79/150 was examined within all subcellular fractions. Significant upregulation of AKAP79/150 signal was observed in P2 (54%) and P3 (89%) fractions from FZP-withdrawn rats (Fig. 2). This enhancement is in agreement with previous findings that SAP97 protein levels were upregulated in crude membranes and PSD-enriched compartments of CA1 neurons from FZP-withdrawn rats (Song et al., 2007 submitted). Although the AKAP P2/S2 ratio in fractions from FZP-withdrawn CA1 minislices was $127 \pm 12\%$ of CON, there was no significant difference between groups. Since enhanced phosphorylation of GluR1 at the Ser$^{845}$ site and increases in levels of SAP97 and AKAP79/150 occurred within the same time-frame, these data support our hypothesis that increased SAP97-AKAP-PKA interactions may contribute to the elevated phosphorylation of GluR1 subunits and subsequent AMPAR plasticity.

**No alterations in the levels of PKA subunits after FZP withdrawal**

To further clarify the roles of different PKA subunits in the facilitation of GluR1 phosphorylation, different antibodies detecting various PKA regulatory and catalytic subunits were used in immunoblot studies. PKA is a tetrameric enzyme composed of two
catalytic and two regulatory subunits (Reimann et al., 1971). At least two catalytic subunits (PKA α and β) and four different regulatory subunits (PKARI α, β and PKARII α, β) are expressed in hippocampus (Cadd and McKnight, 1989). In contrast to PKARI, which is most abundant in cytosol, PKARII subunits are largely associated with organelles and have a higher affinity for AKAP79/150 (Liu et al., 2004; Gold et al., 2006). Both Type I and Type II regulatory subunits of PKA can also be classified into two subgroups: α and β subunits. In contrast to the α subunit, which distributes ubiquitously, the β subunit displays greater tissue specific expression, being highest in brain (Leiser et al., 1986; Boundy et al., 1998; Smith et al., 2006). Therefore, PKARIIβ immunoreactivity was first examined in CA1 subcellular fractions. As seen in Fig. 3, PKARIIβ expression was not differentially regulated after FZP withdrawal, since there was no significant difference in signal density between CON and FZP in any fraction. The P2/S2 ratio of the PKARIIβ signal in the FZP-withdrawal group was also similar to that of controls (103 ± 11%), indicating no redistribution of this subunit. Meanwhile, no alterations in PKARIα signal density (Fig. 4) or redistribution (FZP 97 ± 65 of control) were observed in CA1 subcellular fractions after FZP withdrawal. Together, these findings indicate the increased phosphorylation of GluR1 Ser845 was not related to increased levels of PKARII subunits.

As mentioned above, the PKARI subunit can also bind to AKAP79/150, though with lower affinity. Therefore, the anti-PKARI subunit antibody was also used to evaluate the different subcellular fractions. There were slight increases in PKARI level in S2 (12%)
and P2 (12%) which did not reach statistical significance (Fig. 5). Compared with control rats, no obvious redistribution was found in the FZP group (100 ± 23%).

In addition, a pan antibody for PKA catalytic (PKA cat) subunits was used under the same conditions to examine the level of these subunits among groups. FZP-withdrawal resulted in a slight, non-significant change in the level of PKA cat in P3 fraction (~119% of control) (Fig. 6). There were no alterations in PKA cat expression in S2 or P2 fractions, and no observable shift from S2 toward P2 after FZP withdrawal.
Discussion

Accumulating evidence suggests that the glutamatergic neuronal remodeling observed in models of activity-dependent plasticity also subserves physiological adaptations to drug withdrawal. AMPAR-mediated hyperexcitability in hippocampal CA1 neurons is an essential part of a functional anatomic circuit which contributes to benzodiazepine-withdrawal anxiety. During withdrawal from 1-week FZP treatment, rats exhibit anxiety-like behavior strongly correlated to an increase in hippocampal AMPAR-mediated mEPSC amplitude (Van Sickle et al., 2004); (Xiang and Tietz, 2007). Electrophysiology and immunofluorescent studies showed increases in GluR1-containing AMPARs incorporated into somatic membranes and synapses in response to FZP withdrawal; and immunoblot analysis of cytosolic, membrane and synaptic compartments, revealed a significant upregulation of AMPAR GluR1 subunit protein level in all fractions. Nevertheless, the possibility that phosphorylation of receptors by other kinase candidates may also contribute to the AMPAR potentiation cannot be ruled out, since considerable evidence suggests that altered AMPAR phosphorylation status has significant effects on its function, trafficking and synaptic strength (Derkach et al., 1999; Banke et al., 2000; Esteban, 2003a; Esteban et al., 2003b; Oh et al., 2006).

Among numerous signaling molecules, PKA plays critical roles in controlling gene expression/protein synthesis, AMPAR function and synaptic strength in activity-dependent plasticity (Frey et al., 1993; Abel et al., 1997; Nguyen and Woo,
In hippocampal slices, inhibition of PKA activity with Rp-cyclic adenosine 3’, 5’-monophosphorothioate (Rp-cAMPS) totally blocked the late phase of LTP (Frey et al., 1993); synaptic incorporation of AMPAR subunits GluR4 and GluR1 was directly controlled by PKA phosphorylation of AMPARs in organotypic slices from rat hippocampus (Esteban et al., 2003b); and dephosphorylation of AMPAR GluR1 subunit at Ser\(^{845}\) was observed in long-term depression (Kameyama et al., 1998). Therefore, the current study focused on whether PKA phosphorylation is involved in benzodiazepine withdrawal-induced AMPAR regulation.

Since it was previously shown that FZP withdrawal-induced GluR1 incorporation into CA1 neuron membranes may require the GluR1-trafficking protein, SAP97 (Leonard et al., 1998; Sans et al., 2001; Wu et al., 2002), we employed subcellular fractionation and immunoblotting techniques to examine the localization and redistribution of numerous proteins of interest, including the AMPAR GluR1 subunit, the MAGUK protein, SAP97, the PKA scaffolding protein AKAP79/150 and various PKA subunits. The increased levels of GluR1 subunits and Ser\(^{845}\) phosphorylated GluR1 without an alteration in the pGluR1/GluR1 ratio suggests that benzodiazepine withdrawal activated GluR1 subunit synthesis, thereby causing a proportionate increase in phosphorylated AMPARs to be incorporated into hippocampal CA1 pyramidal neuron membranes/synapses.

PKA is targeted to glutamate receptors not by direct binding but through a MAGUK-AKAP complex interaction (Colledge et al., 2000). The AMPAR GluR1
subunit and PKA are recruited into a macromolecular signaling complex through direct interaction between the MAGUK proteins, PSD-95 and SAP97, and AKAP79/150. The SH3 and GK regions of the MAGUKs mediate binding to AKAP, and the PDZ domain interacts directly with the C-terminus of the GluR1 subunit (Colledge et al., 2000). Therefore, in the current study, the concomitant upregulation of phospho-Ser$^{845}$ GluR1, SAP97 and AKAP in P2 and P3 membrane fractions suggests that AMPAR phosphorylation by PKA may be enhanced through facilitation of interactions among SAP97-AKAP-PKA complexes. Co-immunoprecipitation studies with anti-AKAP79/150 and anti-PKA regulatory subunit antibodies can clarify the direct interactions among these interaction partners.

Protein phosphorylation status is controlled by reciprocal actions of protein kinases and protein phosphatases. AKAP79/150, is a multivalent anchoring protein that binds not only PKA, but also protein kinase C and protein phosphatase-2B (PP2B/calcineurin) at the postsynaptic membrane of excitatory synapses (Dell'Acqua et al., 2002). Therefore, the equilibrium between the activity of protein kinases and phosphatases anchored on AKAP79/150 will dynamically control the phosphorylation status of substrates. In this case, increased PKA activity and/or decreased PP2B activity may contribute to the elevated phosphorylation of AMPAR GluR1 subunit at Ser$^{845}$. Some studies have shown that PP2B binds to AKAP79/150 resulting in an inhibition of PP2B activity (Coghlan et al., 1995; Kashishian et al., 1998); and our preliminary data in whole-cell homogenates of CA1 minislices revealed a decrease in CaN activity after FZP withdrawal. Thus, the
FZP-withdrawal-induced upregulation of AKAP79/150 could also lead to additional PP2B binding, inhibiting PP2B activity, subsequently increasing AMPAR phosphorylation. On the other hand, PKA can also be activated via phosphorylation of catalytic subunits by phosphoinositide-dependent protein kinase I (Cheng et al., 1998). Although, so far, there is no evidence that PKA regulatory or catalytic subunits expression was augmented in our FZP-withdrawal model, it is not clear whether PKA activity or levels of the phospho-PKA catalytic subunit might be increased at asymmetric synapses.

The increase in glutamatergic synaptic strength observed in hippocampal neurons during FZP withdrawal coincides with a downregulation of GABA<sub>A</sub>R function (Zeng et al., 1995; Poisbeau et al., 1997; Zeng and Tietz, 1999). It has been reported that PKA can increase or decrease GABA<sub>A</sub> receptor function, which is likely dependent on the expression of β subunits, the extent of receptor phosphorylation and the manner of PKA activation (McDonald and Moss, 1997; McDonald et al., 1998; Nusser et al., 1999; Poisbeau et al., 1999; Brandon et al., 2000). Given that CA1 area homogenates are a mixture of excitatory and inhibitory synapses, the role of PKA-mediated phosphorylation in the regulation of synaptic ion channel function remains unclear. PSD95-immunoprecipitation of CA1 minislice might provide the needed separation of excitatory from inhibitory synapses, optimal for evaluation of the effects of PKA phosphorylation on different ion channels.
The involvement of PKA activity and its role in phosphorylation of AMPARs has also been investigated in mediating changes in glutamatergic strength associated with dependence on and addiction to other drugs of abuse. For example, chronic exposure of rats to morphine or heroin, which induced severe drug tolerance and dependence, markedly occluded the capacity of hippocampal CA1 LTP during drug withdrawal; and the impaired LTP could be restored by the administration of a PKA inhibitor (Pu et al., 2002). Dopamine receptor stimulation by amphetamine or cocaine exposure triggers AMPAR phosphorylation at Ser\textsuperscript{845} and increases GluR1 surface incorporation (Wolf et al., 2003). Accordingly, as with other forms of experience-dependent plasticity, changes in AMPAR phosphorylation status and/or PKA signaling pathways may also represent an important neuronal adaptive modification induced by persistent drug exposure that is able to elicit long-lasting changes in synaptic weights in crucial brain circuits.

Taken together, FZP withdrawal induced significant increases in the level of phosphorylated AMPAR GluR1 subunit at a well-characterized PKA phosphorylation site, responsible for GluR1 subunit targeting, in both crude membranes and a PSD-enriched compartment in proportion to the amount of upregulated total GluR1 subunit levels. The expression of an important PKA scaffolding protein, AKAP79/150 was also elevated in the same subcellular fractions. Although no significant differential regulation of PKA RII, RI or PKA catalytic subunits were found, the elevation of phospho-GluR1 subunit in conjunction with enhanced total GluR1 subunit protein, and of AKAP79/150 together with the enhancement of SAP97 protein levels within the same
post-withdrawal time frame, suggests that an increase in the interactions between SAP97-AKAP-PKA complexes may promote GluR1 phosphorylation by PKA during benzodiazepine withdrawal. The data also lend further support to the notion that such neuroadaptive changes in signal transduction may be characteristic modifications underlying changes in glutamatergic strength, fundamental to dependence on drugs of abuse, as well as associated with other forms of activity-dependent plasticity, thus are highly conserved in the central nervous system.
References


Tietz EI, Huang X, Chen S, Ferencak WF, 3rd (1999a) Temporal and regional regulation of alpha1, beta2 and beta3, but not alpha2, alpha4, alpha5, alpha6, beta1 or gamma2 GABA(A) receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience 91:327-341.


**Figure 1.** Total GluR1 and phospho-Ser\(^{845}\) GluR1 protein levels were enhanced in CA1 minislice fractions after FZP withdrawal. CA1 minislices from pairs of control (CON) and FZP-withdrawn groups were fractionated to obtain S2 (cytosolic), P2 (crude membrane) and P3 (PSD-enriched) fractions. GluR1 and phospho-Ser\(^{845}\) GluR1 signal densities were normalized to the respective actin signal. Representative immunoblots are illustrated in the top panel; quantitative analyses of signal density are shown in the histograms below. (A) Phospho-Ser\(^{845}\) GluR1 signal density was upregulated in P2 and P3 fractions in response to FZP withdrawal (\(p < 0.05\), \(n = 7\)/group). Although there was a slight increase in S2 phospho-Ser\(^{845}\) GluR1 in FZP rats (14%), no significant difference was found between groups. A P2/S2 ratio 97 ± 18% of control (\(p > 0.05\)) indicated no redistribution of phospho-Ser\(^{845}\) GluR1 during 2-day FZP withdrawal. (B) Total GluR1 signal density on stripped membranes was significantly increased in the same S2, P2 and P3 fractions (single asterisk: \(p < 0.05\), \(n = 7\)/group). A P2/S2 ratio 95 ± 4% of control (\(p > 0.05\)) indicated that GluR1 subunit protein also did not redistribute during 2-day FZP withdrawal. (C) Phospho-Ser\(^{845}\) GluR1/GluR1 ratios were not differentially regulated in any fraction after FZP withdrawal. S2 and P2: 15 μg protein/well; P3: 10 μg protein/well. All data were analyzed by Student’s \(t\) test.
Figure 2. AKAP79/150 signal increased in CA1 minislice fractions after FZP withdrawal. Representative blots (top panel) illustrating AKAP79/150 immunoreactivity in S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions from CA1 minislices of CON and FZP-withdrawn rats. Quantitative analyses of signal density are shown in the histograms below. AKAP79/150 signal densities were normalized to the respective actin signal. There were significant increases in AKAP79/150 immunoreactivity in P2 and P3, rather than in S2 fraction after FZP withdrawal. Redistribution of AKAP79/150 was not observed in the FZP-withdrawn group with a P2/S2 ratio 126 ± 12% of the CON group. Single asterisk: p < 0.05, n = 7 pairs of rats. All data were analyzed by Student’s t test.
Figure 3. PKARIIβ signal was not differentially regulated in CA1 minislice fractions after FZP withdrawal. Representative blots (top panel) illustrating PKARIIβ immunoreactivity in S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions from CON and FZP-withdrawn rats. Quantitative analyses of signal density are shown in the histograms below. PKARIIβ signal densities were normalized to the respective GAPDH signal. There were no significant alterations of PKARIIβ immunoreactivity between CON and FZP-withdrawal groups (p > 0.05, n = 7 pairs of rats). Redistribution of PKARIIβ was not observed in the FZP-withdrawn group with a P2/S2 ratio 103 ± 11% of the CON group. All data were analyzed by Student’s t test.
Figure 4. PKARIΙα signal was not differentially regulated in CA1 minislice fractions after FZP withdrawal. Representative blots (top panel) illustrating PKARIΙα immunoreactivity in S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions from CON and FZP-withdrawn rats. Quantitative analyses of signal density are shown in the histograms below. PKARIΙα signal densities were normalized to the respective GAPDH signal. There were no significant alterations in PKARIΙα immunoreactivity between CON and FZP-withdrawal groups ($p > 0.05$, $n = 7$ pairs of rats). Redistribution of PKARIΙα was not observed in the FZP-withdrawn group with a P2/S2 ratio $97 \pm 6\%$ of the CON group. All data were analyzed by Student’s $t$ test.
Figure 5. PKARI signal was not differentially regulated in CA1 minislice fractions after FZP withdrawal. Representative blots (top panel) illustrating PKARI immunoreactivity in S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions from CON and FZP-withdrawn rats. Quantitative analyses of signal density are shown in the histograms below. PKARI signal densities were normalized to the respective GAPDH signal. There were no significant alterations of PKARI immunoreactivity between CON and FZP-withdrawal groups ($p > 0.05$, CON: $n = 7$ rats, FZP: $n = 5$ rats). Redistribution of PKARI was not observed in the FZP-withdrawn group with a P2/S2 ratio $100 \pm 23\%$ of the CON group. All data were analyzed by Student’s $t$ test.
Figure 6. PKA cat signal was not differentially regulated in CA1 minislice fractions after FZP withdrawal. Representative blots (top panel) illustrating PKA cat immunoreactivity in S2 (cytosol), P2 (crude membrane) and P3 (PSD-enriched) fractions from CON and FZP-withdrawn rats. Quantitative analyses of signal density are shown in the histograms below. PKA cat signal densities were normalized to the respective stripped GAPDH signal. There were no significant alterations of PKA cat immunoreactivity between CON and FZP-withdrawal groups ($p > 0.05$, $n = 7$ pairs of rats). Redistribution of PKA cat was not observed in the FZP-withdrawn group (P2/S2 ratio $108 \pm 8\%$ of the CON group). All data were analyzed by Student’s $t$ test.
Gene Expression Profiling of Plasticity Pathways in Rat Hippocampus During Benzodiazepine Withdrawal: From Gene Transcripts to Proteins

Jun Song and Elizabeth I. Tietz

Department of Physiology and Pharmacology, Cellular and Molecular Neurobiology Program,
University of Toledo, College of Medicine, Health Science Campus, Toledo, OH 43614

Running title: Gene profiling during benzodiazepine withdrawal

ABBREVIATIONS: CON, control; FZP, flurazepam; AP-2, adaptor protein complex 2; LTP, long-term potentiation; LTD, long-term depression; qPCR, quantitative real-time PCR; Ct values, threshold cycle values; IPSCs, inhibitory post-synaptic currents; mEPSCs, miniature excitatory postsynaptic currents; mIPSCs, miniature inhibitory postsynaptic currents; IPA, Ingenuity pathway analysis; DMT, data mining tool; ISPKB, Ingenuity System Pathways Knowledge Base; GluR, glutamate receptor; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; PSD, postsynaptic density; SNARE soluble NSF attachment receptor

Keywords: drug abuse, addiction, glutamate receptor, GABA_A receptor, hippocampal CA1 neuron, microarray
Abstract

The molecular mechanisms underlying benzodiazepine tolerance and dependence were explored by microarray analysis of hippocampal CA1 tissue from 2-day flurazepam-withdrawn rats using Affymetrix GeneChip Rat Genome 230 2.0 arrays. Twenty-seven known gene transcripts were differentially expressed in response to FZP withdrawal, including 19 significantly upregulated transcripts and 8 downregulated mRNAs. Among these transcripts, a number of genes involved in neuronal plasticity (BDNF, CAMKIIB, HPCA and APP), neurotransmitter release (BDNF, SYN2, STXBP1, SYT11 and APP), nervous system development, cytoskeleton protein organization and ion homeostasis were elevated. Surprisingly, no alterations in GABA<sub>A</sub> receptor subunit mRNAs were detected. Following validation with quantitative real-time PCR, Ingenuity pathway analysis suggested one network and two canonical pathways, i.e. LTP and neurotransmission pathways, were regulated during benzodiazepine withdrawal. Gene expressions of select proteins were further explored by confocal analysis of immunofluorescence labeling. CaMKIIβ and AMPA receptor GluR1 subunit immunolabeling, as well as their colocalization were elevated in CA1 cell bodies and apical dendrites after FZP withdrawal, indicating the possible enhancement of GluR1 subunit phosphorylation by CaMKII. The colocalization between hippocalcin, a calcium sensor protein, and the GluR2/3 subunit was also upregulated in CA1 pyramidal neuron somata, suggesting that hippocalcin may facilitate the endocytosis of GluR2/3 heteromers and promote AMPAR reassembly in response to FZP withdrawal. Together,
the data indicate possible roles for AMPAR protein phosphorylation and endocytosis in drug dependence and suggest that benzodiazepine dependence shares similar mechanisms with neuronal plasticity.

Benzodiazepines, among the most commonly used psychoactive class of drugs, have their anticonvulsant, anxiolytic and hypnotic actions by acting on gamma-aminobutyric acid type A (GABA_\text{A}) receptors. Tolerance to and dependence upon prescribed benzodiazepines are recognized as major clinical problems. Tolerance is typically manifest by reduced anticonvulsant effectiveness and dependence by the appearance of withdrawal symptoms such as anxiety and insomnia. Understanding the molecular mechanisms underlying benzodiazepine tolerance and dependence are of clinical significance as these phenomena can lead to misuse of benzodiazepines and may also be a factor in the increase in benzodiazepine abuse among polydrug abusers (Griffiths and Johnson, 2005).

Considerable evidence demonstrates that chronic benzodiazepine exposure results in tolerance, which has been associated with an uncoupling of benzodiazepine and GABA binding site, a reduction in benzodiazepine binding sites, downregulation of specific (e.g. \(\alpha_1, \beta_2\) and \(\beta_3\)) GABA_\text{A}R subunit mRNA and protein, associated with a decrease in GABA-mediated miniature inhibitory post-synaptic currents (mIPSC) amplitude (Poisbeau et al., 1997; Chen et al., 1999; Zeng and Tietz, 1999; Tietz et al., 1999b; Bateson, 2002; Wafford, 2005).
In conjunction with the changes in inhibitory neurotransmission associated with benzodiazepine tolerance, recent evidence suggests that the glutamatergic system may be crucial to mechanisms underlying benzodiazepine dependence (Izzo et al., 2001; Van Sickle et al., 2004; Wafford, 2005; Xiang and Tietz, 2007). Moreover, recent evidence indicates that drug dependence may share common mechanisms with activity-dependent plasticity. In neuronal plasticity, the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ionotropic glutamate receptor (AMPAR) plays an important role in modifying glutamatergic synaptic strength (Malenka, 2003; Esteban, 2003a). Similar mechanisms have also been implicated in dependence on drugs of abuse (Malenka, 2003). For example, an increase in the components of the endocytic machinery, such as clathrin, dynamin and adaptor protein complex 2 (AP-2), were observed after morphine administration (Moron et al., 2006); and amphetamine-induced behavioral sensitization in the rat was prevented by systemic or intra-nucleus accumbens infusion of the membrane-permeable GluR2-derived peptide, which has also been used to block long-term depression (LTD) (Brebner et al., 2005).

In previous studies, AMPAR remodeling in hippocampal CA1 pyramidal neurons was investigated in response to flurazepam (FZP) withdrawal. AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) amplitude and AMPAR specific binding were increased in hippocampal CA1 pyramidal neurons after FZP withdrawal (Van Sickle and Tietz, 2002). Notably, upregulation of AMPAR function was associated with anxiety-like behavior (Van Sickle et al., 2004; Xiang and Tietz, 2007). The strengthening of CA1
neuron excitatory function and the expression of anxiety were mitigated by prior administration of pharmacological antagonist of AMPARs, underscoring the association of AMPAR potentiation and withdrawal symptoms (Van Sickle et al., 2004; Xiang and Tietz, 2007).

Knowledge of the neural mechanisms underlying the development of drug tolerance and dependence, in particular to the benzodiazepine remains incomplete. To investigate the gene profiles associated with chronic benzodiazepine treatment, microarray analysis followed by quantitative real-time PCR (qPCR) validation, were carried out in CA1 neuron minislices from 2 day FZP-withdrawn rats and their matched controls to identify the regulated transcripts at a time when both anticonvulsant tolerance and withdrawal-anxiety are present in vivo (Tietz et al., 1999b; Van Sickle et al., 2004). The analysis revealed 19 known up-regulated transcripts and 8 down-regulated gene products in FZP-withdrawn rats. Interestingly, within these, 4 upregulated transcripts, including amyloid precursor protein (APP), brain-derived neurotrophic factor (BDNF), Ca\(^{2+}\)/calmodulin dependent kinase II beta (CaMKII\(\beta\)) and microtubule-associated protein tau (MAPT), are thought to participate in activity-dependent plasticity (Shen et al., 1998; Barco et al., 2005; Mileusnic et al., 2005; Palmer et al., 2005).

To address the biological connections between the differentially regulated gene transcripts and seek their putative downstream targets, we explored Ingenuity pathway analysis (IPA) to build functional networks and pathways involving the regulated transcripts. This was accomplished with one network and several canonical pathways,
including long-term potentiation (LTP)-associated and neurotransmission pathways. Selected transcript alterations were further validated utilizing immunofluorescence labeling and confocal analysis. To test whether CaMKIIβ and hippocalcin may be involved in FZP-withdrawal-induced AMPAR regulation, the colocalization between GluR1-containing AMPARs and CaMKIIβ, as well as the colocalization between hippocalcin and GluR2/3 AMPARs were examined. The significantly elevated colocalization of each protein pair during FZP withdrawal indicated a possible increase in GluR1-containing AMPAR phosphorylation by CaMKII and facilitation of GluR2/3 AMPAR endocytosis via interaction with a hippocalcin-AP2 complex. These are the first gene profiling data to demonstrate that benzodiazepine dependence shares common mechanisms with those of neuronal plasticity.
Materials and methods

Animal model

All methods were approved by the University of Toledo College of Medicine (formerly the Medical University of Ohio), Institutional Animal Care and Use Committee (IACUC) and conformed to National Institutes of Health guidelines. One-week FZP treatment and withdrawal was carried out as previously established (Van Sickle et al., 2004; Xiang and Tietz, 2007). Male Sprague–Dawley rats (Harlan, Indianapolis, IN), P36-42 when evaluated, were first adapted 2-4 days to a 0.02% saccharin vehicle, then offered FZP (provided by the National Institute on Drug Abuse Drug Supply Program) in the vehicle for 1 week (100 mg/kg for 3 days, 150 mg/kg for 4 days) as their sole source of drinking water, appropriate to FZP’s relative potency and oral bioavailability (Gallager et al., 1985; Xie and Tietz, 1992; Izzo et al., 2001; Chouinard, 2004). Daily water consumption was monitored to adjust drug concentration. Rats that did not achieve a weekly average of 120 mg/kg/day were excluded. During drug withdrawal, rats received saccharin water for 2 days. Control rats received saccharin vehicle in parallel. This dosing regimen reliably induces manifestations of both benzodiazepine tolerance and dependence in 2-day FZP withdrawn rats (Poisbeau et al., 1997; Zeng and Tietz, 1999; Tietz et al., 1999b; Van Sickle et al., 2004; Xiang and Tietz, 2007).

RNA preparation

Total RNA was extracted from the hippocampal CA1 region of control (CON) and 2-day FZP-withdrawn (FZP) rats. Immediately after decapitation, hippocampi were removed
and the dorsal CA1 area was micro-dissected on ice from 2-3 mm slices. Total cellular RNA was isolated with TRIzol (Gibco-BRL, Grand Island, NY) from match pairs of CON and 2-day FZP-withdrawn rats, and further purified with the RNeasyMini Kit (Qiagen Inc, Germany). RNA yield and purity (A260/280) was quantified by spectrophotometer (Spectronic Genesys™ 8, Spectronic Instruments Inc., Rochester, NY). The integrity of the RNA was evaluated with a 1% agarose gel.

**Microarray expression analysis**

Microarray analysis was performed to identify candidate genes regulated during 2-day FZP withdrawal, using the manufacturer’s published techniques (Affymetrix, Santa Clara, CA). Briefly, 8.6 μg total RNA was converted to single-strand cDNAs with SuperScript II reverse transcriptase using T7-oligo (dT) as a primer. Second-strand cDNAs were synthesized with DNA polymerase I, DNA ligase and T4 DNA polymerase. Double-stranded cDNAs were purified and transcribed into biotin-labeled cRNAs. After fragmentation of cRNAs, the biotin-labeled cRNAs were first hybridized to 3 Affymetrix GeneChip Test arrays to determine the quality of the labeled cRNAs. After passing the quality control assay, the labeled cRNA fragments were hybridized to the Affymetrix GeneChip Rat Genome 230 2.0 Array (3 chips/group), and subsequently washed and stained, followed by signal amplification with anti-streptavidin antibody. All the washing and staining procedures were managed automatically by the Affymetrix Fluidics Station 400 (Affymetrix), according to the manufacturer’s instructions (http://www.affymetrix.com).
Microarray data processing

Initial processing of microarray data, including calculation of “average difference” expression intensity levels, was performed using GeneChip Operating Software (GCOS ver 1.4, Affymetrix) with default analysis parameters and global scaling to target signal = 150. Scaling factors for all arrays were between 1.5-1.9. Quality of array hybridizations was also assessed by ensuring that the housekeeping and spike controls were all present, and the ratio of 3’- to 5’-end probes for β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) did not exceed 3. Similar background, noise and the percentage of probe present were found among each sample, indicating good sample preparation. The files of 6 Affymetrix rat expression chips were then analyzed with a data mining tool (DMT, ver 3.0) to determine the intensities, the detection calls of present, marginal or absent (“P”, “M”or “A”) or increased or decreased (“I” or “D”), the fold-change [(FZP-CON)/CON] and the significance of each probe set. Based on the P value, the software generates a “P” call (P < 0.04), “M” call (P = 0.04–0.06) or “A” call (P ≥ 0.06) for each probe. All probe sets present in all chips were considered. In the absolute value analysis, a P value less than 0.05 were considered significant by Student’s t-test.

In the comparison analysis, each probe set on an experimental chip (FZP) was compared with each control chip (CON). Thus, 9 comparisons were performed among 3 pairs of animals. Comparison analysis was based on an algorithm that generates a qualitative difference call to identify if a transcript in the experimental array is increased
(I), marginally increased (MI), marginally decreased (MD), decreased (D) or equivalent to that on the control chip. Only the transcripts showing consistent “I” or “D” in more than 5 out of 9 comparisons were analyzed.

**Quantitative real-time PCR assay**

The changes in transcript levels detected by microarray expression profiling were validated by a SYBR green qPCR assay. In short, cellular RNAs from hippocampal CA1 areas were extracted following the same protocol as above. Prior to qPCR analysis, 5 μg RNAs from additional groups of Control and FZP withdrawn rats (CON, n = 6; FZP, n = 7) were reversed transcribed into cDNAs with Oligo (dT)20 primer (Invitrogen, Carlsbad, CA) and Superscript III reverse transcriptase (Invitrogen). Primers for qPCR were designed within the same sequences as for microarray probes, using an online tool, “Real-Time PCR Primer Design” (https://www.genscript.com/ssl-bin/app/primer, GenScript Corporation, Piscataway, NJ) or based on a previous publication (Guzman-Marin et al., 2006) for the CaMKIIα primer set. GAPDH expression, which did not significantly differ between CON and FZP groups and exhibited less variance than β-actin based on microarray analysis, was used to normalize the data. Quantitative real-time PCR was performed using a MyiQ Thermal Cycler (Bio-Rad, Hercules, CA) and carried out with iQ SYBR Green PCR Master Mix (Bio-Rad). The oligonucleotide sequences used as primers for APP, BDNF, CAMKIIA and CAMKIIB, HPCA, MAPT and GAPDH, are summarized in Table 1.
The qPCR reaction mixture contained 2 X Super SYBR Green PCR Master Mix, 2.5 μM primer pairs and 5 μl cDNA in a total volume of 25 μl. The PCR reaction was run with a two-step PCR program: initially the mixture was heated at 95°C for 3 min to activate iTaq DNA polymerase, followed by 40 cycles with denaturation at 95°C for 30 s, and annealing at 60°C for 30 s. Fluorescence was detected during the combined annealing/extension phase of each cycle. Melt-curve analysis was performed immediately following amplification by increasing the temperature in 0.5°C increments, starting at 55°C for 80 cycles of 10 s each. Standard curves for each PCR primer were created using serial dilutions of cDNA samples. A standard curve and negative controls (no cDNA samples) all in triplicate or quadruplicate wells were included in every assay for each specific gene in the same plate. RNA expression levels (Ct values, threshold cycle values) were calculated for each gene using the standard curves constructed for each PCR primer set and then normalized for GAPDH expression levels, as well as presented as fold-change (fold-change = $2^{\Delta\Delta Ct}$).

**Biological network and pathway analysis**

For Ingenuity network and pathway analysis, a data set containing only the significantly up- or down-regulated gene transcripts with their fold-changes were uploaded into Ingenuity software (http://www.ingenuity.com) to identify significantly regulated networks and pathways. This web-delivered application utilizes the Ingenuity System Pathways Knowledge Base (ISPKB) derived from the literature, containing large amounts of individually modeled relationships between genes, mRNAs and proteins to generate
significant biological networks and pathways. Networks were built upon the connections among all uploaded proteins based on ISPKB; while pathway clusters showed individual genes involved in created pathways based on ISPKB. A fold-change cutoff of at least 1.2 was set to further filter the genes whose expression was significantly differentially regulated. These genes, called “focus genes”, were then used as the starting point for generating biological networks. To start building networks, Ingenuity software inquires the ISPKB for interactions between focus genes and all the other genes stored in the database. Biological functions were calculated and assigned to each network. IPA then computed a score for each network/pathway according to the fit of the network to the set of focus genes. A cutoff of “2” was applied in network/pathway analysis. A score of “2” indicates that there is a 1 in 100 chance that the focus genes are together in a network due to chance. Therefore, scores of 2 or higher represent a 99% confidence level. Based on this criterion, 1 network and the top two pathways (LTP and neurotransmission pathways) which showed a score > 2 were used in this study.

**Double-labeling immunofluorescence and confocal analysis**

Immunofluorescence experiments were carried out in the presence of detergent to detect protein expression at the whole-cell level. Standard techniques were used for detection of CaMKIIβ, total GluR1 and their colocalization. Following blocking with donkey serum, sections were incubated with anti-CaMKIIβ antibody (1:100, Invitrogen, Carlsbad, CA) overnight and then labeled with Alexa 555-conjugated secondary (1:500, Invitrogen). After thorough washes, anti-carboxy-terminal GluR1 (anti-C-GluR1, 1:50, Chemicon,
Temecula, CA) was included during incubation and detected with an Alexa 488-conjugated secondary (1:500; Invitrogen). For hippocalcin and total GluR2/3 immunostaining and colocalization a modified methodology based on double-labeling techniques (http://www.jacksonimmuno.com/technical/techmain.asp) was used to detect two unlabeled primary antibodies from the same host species. In brief, immediately after dissection, 3 mm hippocampal blocks were post-fixed in 4% paraformaldehyde in 0.1 M PB at 4°C overnight. Free-floating sections (50 μm) were blocked with donkey serum, and then incubated with anti-hippocalcin antibody (1:1000, Abcam, Cambridge, MA) overnight at 4°C. After labeling with the secondary antibody (Alexa 488-conjugated donkey anti-rabbit antibody, 1:500, Invitrogen), excessive rabbit gamma globulin was used to saturate any open antigen binding sites on the first secondary antibody. Slices were then incubated with an excess amount (6X by weight of gamma globulin) of unconjugated donkey anti-rabbit IgG (H+L) F(ab’)2 fragment (Jackson Immuno Research Laboratories, Inc., West Grove, PA). Followed by thorough washes, slices were then incubated with anti-total GluR2/3 antibody (1:50, Upstate, Lake Placid, NY) overnight at 4°C, and antibody signal detected with an Alexa 555-conjugated donkey anti-rabbit secondary (1:500, Invitrogen). Experimental controls to verify appropriate background, antibody specificities and absence of cross-reactivity included omission of primary antibodies, administration with host-matched gamma globulin or incubation without F(ab’)2 fragments (data not shown). Signals were visualized and quantified using an Olympus BX51WI microscope (Olympus America Inc., Melville, NY), coupled to a
Radiance 2000 laser scanning confocal system (Bio-Rad, Hercules, CA) with constant laser power, gain, iris and offset settings between samples. To avoid cross-talk between channels, green and red signals were acquired separately within a given area of the same sections using different filters. All data collection and analysis were conducted with the observer unaware of experimental group.

Immunofluorescence labeling on confocal images was assessed using ImageJ software (National Institutes of Health, Bethesda, MD). Consistent and appropriate areas of interest were used to analyze the immunofluorescence signals between CON and FZP groups. For each individual signal, the intensity of immunofluorescence (mean density) was determined from the average grey scale value. Percentage of volume colocalized (% volume colocalized) was used to quantify colocalization signals.

**Statistical analysis**

Data were reported as mean ± SEM and significance level was set at $P < 0.05$. Error bars represented the SEM in all figures. Deviations from normality and homogeneity of variance were determined by Shapiro-Wilk test and F test, respectively. Statistical differences were determined by unpaired two-tailed Student’s $t$-test.
Results

Gene expression profiling during FZP withdrawal

To explore the molecular mechanisms underlying benzodiazepine withdrawal, the current study was focused on alterations in gene profiling in the CA1 area, in accordance to our previous findings of enhanced AMPAR-mediated function and decreased GABAR-mediated responses in the hippocampal CA1 area, rather than CA3 or dentate gyrus regions (Poisbeau et al., 1997; Zeng and Tietz, 1999; Van Sickle et al., 2004). We have previously shown that 2-day FZP-withdrawn rats possess the highest level of alterations in AMPARs (Van Sickle et al., 2004). This is also a time-point when specific GABAR subunit proteins are reduced after drug withdrawal and when reductions in GABAR mIPSCs first appear (Poisbeau et al., 1997; Zeng and Tietz, 1999).

Gene expression profiles of hippocampal CA1 neurons were obtained both from FZP and matched CON rats using the Affymetrix rat expression 230 3.0 arrays, which provide comprehensive coverage of rat genome transcripts, comprised of more than 31,000 probe sets. A “present” detection call filter was used to exclude the poorly performing probe sets and signals near background. A Students t-test analysis revealed 27 known gene transcripts defined as “differently expressed” in hippocampal CA1 neurons after 2-day FZP withdrawal, including 19 significantly up-regulated transcripts and 8 down-regulated mRNAs. The results could be distorted by individual values strongly deviating from the mean due to the variance between animals. Thus, we conducted a comparison analysis using the data mining tool, DMT, to confirm the findings. Nine
comparisons between each CON and FZP rat were carried out, and transcripts were considered to be “differentially regulated” when at least 5 out of 9 comparisons showed consistent “I” or “D” calls. Based on this additional criterion, the same 27 known gene transcripts were found to be differentially regulated, compatible with the absolute value analysis (Students t-test). The expression profiling of these genes, together with their molecular and biological functions were summarized in Table 2 and 3.

Within the cluster of upregulated transcripts, 4 genes [BDNF, CAMK2B (encoding CaMKIIβ), HPCA (encoding hippocalcin) and APP] are involved in neuronal plasticity (Shen et al., 1998; Barco et al., 2005; Mileusnic et al., 2005; Palmer et al., 2005); 7 genes [BDNF, NNAT (encoding neuronatin), NRN (CPG15, encoding neuritin), MAPT, SYN2 (encoding synapsin II), CAMK2B, and APP] are involved in the neurite formation or elongation and nervous system development (Ferreira et al., 1994; Brandt, 1996; Fink et al., 2003; Javaherian and Cline, 2005; Leyssen et al., 2005; Deumens et al., 2006; Park and Hong, 2006); 5 genes [BDNF, SYN2, STXBP1 (munc-18-1, encoding syntaxin binding protein I), SYT11 (encoding synaptotagmin XI) and APP] are involved in neurotransmitter secretion (Mikoshiba et al., 1999; Verhage et al., 2000; Atasoy and Kavalali, 2006; Hvalby et al., 2006; Priller et al., 2006); 6 genes [BDNF, STXBP1, CAMK2B, APP, MAPT (encoding microtubule-associated protein tau), SYN2] are involved in the assembly and organization of cytoskeletal proteins (Chilcote et al., 1994; Brandt, 1996; Shen et al., 1998; Trommsdorff et al., 1998; Gibney and Zheng, 2003; Bhaskar et al., 2004); 2 genes [KCNV1 (potassium channel subfamily V) and PMCA3
(plasma membrane Ca\(^{2+}\)-ATPase isoform 3]) are involved in ion homeostasis (Grover and Khan, 1992; Ebihara et al., 2004).

As shown in Table 3, in contrast to the upregulation of neuronal membrane calcium pump PMCA3, PMCA1, a non-tissue specific calcium pump (Stauffer et al., 1995), was significantly downregulated after FZP withdrawal, indicating differential regulation of calcium pumps in response to FZP withdrawal. The remaining downregulated transcripts include cytochrome P450, procollagen, inhibitor of DNA binding2, transcription factor 4, double-stranded RNA-binding protein p74, hemoglobin \(\alpha\)1 and \(\beta\) chain.

Meanwhile, microarray analysis revealed no alterations of the expression levels of the following transcripts: \(\alpha\)1, \(\gamma\)1, \(\gamma\)2, \(\theta\), \(\delta\), \(\epsilon\), \(\pi\) subunits of GABA\(_A\)Rs, GluR1-4 subunits of AMPARs, NR1, NR2A-D, NR3A subunits of N-methyl-D-aspartate receptors as well as GluR5, KA2-4 subunits of kainite receptors (data not shown).

Quantitative real time-PCR with SYBR green was used to validate the expression patterns of the transcripts identified by microarray analysis. Five up-regulated genes (APP, BDNF, CAMK2B, HPCA and MAPT) and 1 unchanged gene [CAMK2A (encoding CaMKII\(\alpha\))], which play important molecular signaling roles in neurons, were selected for real time-PCR analysis on additional groups of CON (n = 6) and FZP-withdrawn (n = 7) rats. For this validation, the primer sets of qPCR (Table 1) were designed within the same sequences used to develop microarray probes. Positive and negative transcript modulation was confirmed by qPCR in hippocampal CA1 neurons after FZP withdrawal, including significant upregulation of APP, BDNF, CaMKII\(\beta\) and
MAPT, as well as a lack of change in CaMKIIα level (Fig. 1). The mRNA expression levels obtained by qPCR were also comparable to those observed by microarray analysis. Meanwhile, in contrast to microarray analysis, no significant upregulation of hippocalcin was found by qPCR, though there was an increased trend in hippocalcin expression (1.7-fold increase) in the FZP-withdrawn group ($P = 0.40$).

**Pathway analysis**

To derive functional relationships between individual gene transcripts and identify signaling pathways associated with benzodiazepine withdrawal, we employed Ingenuity Pathway Analysis (IPA) of significantly regulated genes. IPA is a tool to construct relevant pathway information from the uploaded genes and integrates individual genes, biological subjects, and functional regulatory networks based on published literature.

Participation of genes in Ingenuity pathways clusters also provides information about protein properties, protein-protein interactions and the distribution of proteins. A detailed explanation of the edges and nodes in Ingenuity network and pathway clusters can be found in Fig. 2. As shown in the Ingenuity network (Fig. 2), our uploaded transcripts can be classified into various groups: protein transporter (ATP2B1, NNAT, STXBP1, HBB and HBA2), cytokine (BDNF), protein kinase (CAMK2B), transmembrane receptor (NPTXR), transcription regulator (TCF4) and others. Direct and indirect protein-protein interactions were exhibited between our uploaded proteins and their putative targets. Further, the extracellular and intracellular distributions of significantly regulated gene transcripts is illustrated by IPA: in the FZP group, 1 upregulated gene (BDNF) and 3
down-regulated genes (CHGB, COL3A1 and COL1A2) were found in extracellular space; 4 upregulated genes (SYN2, NNAT, APP and NPTXR) and 1 downregulated gene (ATP2B1) were found in membrane regions; 5 upregulated genes (CAMK2B, MAPT, NRN1, STXBP1 and BZW2) and 2 downregulated genes (HBA2 and HBB) were found in the cytoplasm; and 2 downregulated genes (ID2 and TCF4) were found in the nucleus.

To further address biological functions of individual transcripts, canonical pathway analysis was applied. The top two pathways established by IPA were the LTP-associated and neurotransmission pathways, as shown in Figs. 3 and 4, respectively. As for the LTP pathway, several genes encoding membrane receptors and protein kinases exhibit interactions with BDNF and APP, including GRIA1 (encoding GluR1), CHRNA7, CaMKIV, MAPK, PRKACA and NTRK2. The biological function among these proteins was also suggested, i.e. E (expression), A (activation/deactivation). On the other hand, a neurotransmission pathway (Fig. 4) showed BDNF interactions chiefly with membrane receptors [GRIA1, GRIK2 (encoding NR2C subunit)] and APP primarily related to vesicle docking and trafficking proteins (STBP1, STXBP5, DOC2B and UNC13B). The interaction of BDNF and APP points to a link between these membrane receptors and neurotransmitter release.

**Confirmation of gene expression profiles by immunofluorescence studies**

To investigate gene products of interest at the protein level, dual-immunofluorescence staining and confocal imaging was used. Hippocalcin and CaMKIIβ proteins were chosen for further examination, as both are involved in neuronal plasticity. As a calcium sensor,
hippocalcin binding to the AP2 adaptor complex, facilitates GluR2 endocytosis (Palmer et al., 2005); CaMKIIβ functions as a molecule to localize CaMKIIα/β hetero-oligomers, and promotes CaMKII translocation from the F-actin-bound to the PSD-bound state, which may result in protein phosphorylation at the synapse (Shen et al., 1998; Shen and Meyer, 1999).

AMPAR phosphorylation by CaMKII augments receptor surface/synapse trafficking and increases single-channel conductance (Derkach et al., 1999; Malinow and Malenka, 2002). In addition to acting as a protein kinase, the beta subunit of CaMKII is also an F-actin binding molecule, driving CaMKIIα/β heteromers from the cytosol to their membrane-associated targets (Shen et al., 1998) and facilitating phosphorylation reactions. Narrowing the distance between the GluR1 subunit and CaMKIIα driven by CaMKIIβ would increase the possibility of GluR1 phosphorylation by CaMKII and further AMPAR potentiation. To detect the expression level of total GluR1 and CaMKIIβ, as well as their colocalization, fixed hippocampal slices were incubated in anti-C-terminal GluR1 and anti-CaMKIIβ antibodies under permeabilizing conditions. A noteworthy increase in CaMKIIβ signal was observed in basal dendritic area (so: stratum oriens) of CA1 neurons in response to FZP withdrawal. In this area, neither total GluR1 nor GluR1/CaMKIIβ colocalization signals were noticeably differentially regulated (Fig. 5A). In contrast, in CA1 neuron somata (sp: stratum pyramidale) and apical dendrites (sr: stratum radiatum), FZP-withdrawn rats showed enhanced total GluR1 and CaMKIIβ immunolabelling and their colocalization (Fig. 5B, C). Quantification of confocal images
revealed that during FZP withdrawal CaMKIIβ expression was significantly upregulated in *so* (82.5% increase); and in the *sp* and *sr* regions the augmentation in total GluR1 (*sp*: 112.2%, *sr*: 137.2%) and CaMKIIβ (*sp*: 47.6%, *sr*: 110.0%) Immunofluorescence signal and their colocalization (*sp*: 248.9%, *sr*: 164.5%) were also statistically significant (Fig. 6). These findings suggest that during FZP withdrawal, CaMKIIβ may target CaMKIIα/β hetero-oligomers to GluR1-containing AMPARs and promote receptor phosphorylation.

Differential regulation of GluR1 and GluR2 AMPAR subunits were observed in CA1 pyramidal neurons in our previous study (Song and Tietz 2006 Soc. Neurosci Abstr.). An increase in membrane targeting of GluR1 subunits and increased GluR1/2 colocalization was observed, without alterations in GluR2 subunit incorporation. The results indicated that surface AMPAR subunit composition may switch from GluR2/3 to GluR1/2 heteromers in response to FZP withdrawal. One possible mechanism is that greater numbers of GluR2/3 heteromers are internalized and reassembled as GluR1/2 AMPARs. To address this question, anti-hippocalcin and GluR2/3 immunoreactivity was colocalized in the CA1 region of hippocampal slices. Hippocalcin is a calcium sensor protein, highly expressed in hippocampus, which plays important roles in membrane receptor endocytosis by acting with AP2 adaptor complex (Palmer et al., 2005). As the anti-hippocalcin and GluR2/3 antibodies are from the same host species, special gamma globulin blocking and F(ab′)2 fragment masking techniques were used between antibody incubations (see detailed methods). As shown in Figs. 7 and 8, there were no significant
differences in total GluR2/3 labeling in so, sp or sr regions between the two groups. In agreement with qPCR analysis, there were no differences in the levels of hippocalcin protein in CA1 neurons between FZP and CON groups. However, enhanced colocalization of signals was observed in the sp (118.9% increase) and sr (132.2% increase) regions, though the latter was not statistically significant ($P = 0.07$). This finding suggests that during FZP withdrawal hippocalcin may promote endocytosis of GluR2/3 heteromers.
Discussion

Previous studies from our laboratory and others suggest that changes in GABAergic system function are central to the development of benzodiazepine anticonvulsant tolerance (Gonsalves and Gallager, 1988; Tietz et al., 1999b; Bateson, 2002; Wafford, 2005); whereas withdrawal symptoms, such as anxiety which may underlie dependence, are at least in part mediated by increases in glutamatergic strength in hippocampal CA1 neurons (Izzo et al., 2001; Van Sickle et al., 2004; Xiang and Tietz, 2007). To provide further insights into the cellular and molecular mechanisms underlying benzodiazepine tolerance and dependence, microarray analysis followed by qPCR validation and Ingenuity pathway analysis was conducted to detect candidate gene transcripts that may be differentially regulated in hippocampal CA1 neurons. Using Affymetrix gene expression microarrays we identified a number of gene transcripts involved in neuronal plasticity that were upregulated in response to FZP withdrawal, including APP, BDNF, CAMKIIB and HPCA. There were no changes in other genes or pathways involved in modulation of inhibitory systems by hypoxia, ischemia or seizures, for example, glutamate decarboxylases, GABARs, voltage-gated calcium channels, metabotropic glutamate receptors, TrK receptors, Caspases, hypoxia-inducible factor (HIF), PKA (cAMP-dependent protein kinase A), stress-activated protein kinase, p42/44/MAPK and tyrosine kinase signaling pathway (Diverse-Pierluissi et al., 1997; Kotak et al., 2001; Seta et al., 2002; Telfeian et al., 2003; Gorter et al., 2006).
GABA\textsubscript{A}R subunit expression levels are regulated by chronic benzodiazepine exposure in a brain region-specific manner (Bateson, 2002). For example, Northern hybridization revealed \(\alpha_1\) mRNA level was significantly decreased in rat cerebral cortex but not in cerebellum after prolonged diazepam treatment (Heninger et al., 1990); in diazepam-tolerant rats, a reduction in GABA\textsubscript{A} receptor \(\alpha_1\) mRNA was observed in the frontoparietal motor cortex and hippocampus, but not in the frontoparietal cortex, striatum, olfactory bulb, and cerebellum (Impagnatiello et al., 1996). Meanwhile, regulation of \(\alpha_1\) subunit has been implicated in mediating both GABA/benzodiazepine uncoupling and decreased GABA receptor function during benzodiazepine tolerance and withdrawal (Bateson, 2002). In FZP-withdrawn rats, a significant downregulation of the GABA\textsubscript{A}R \(\alpha_1\) subunit mRNA and protein was observed in CA1 pyramidal neurons and Layer II-IV cortical neurons using \textit{in situ} hybridization and immunohistochemical techniques, respectively (Tietz et al., 1993; Chen et al., 1999). A decrease in \(\beta_3\) subunit mRNA and protein in CA1 and cortical neurons was accompanied by a concomitant upregulation of the \(\beta_2\) subunit in the CA1 region (Chen et al., 1999; Tietz et al., 1999a). \textit{In situ} hybridization and immunohistochemical studies did not reveal any changes in \(\alpha_2\), \(\alpha_4-6\), \(\beta_1\) and \(\gamma_2\) mRNA or protein levels (Chen et al., 1999; Tietz et al., 1999a). Consistent with the latter findings, the current microarray analysis also revealed no alterations in mRNA levels of the GABA\textsubscript{A}R \(\gamma_2\) subunit or other subunits (\(\gamma_1\), \(\theta\), \(\delta\), \(\epsilon\), \(\pi\)) in CA1 region from 2-day FZP-withdrawn rats. Surprisingly, a significant downregulation of GABA\textsubscript{A}R \(\alpha_1\) subunit mRNA levels was not detected in the present study. Given the
consistent finding of a reduction in $\alpha_1$ mRNA and protein levels in hippocampal tissue sections, the lack of change in $\alpha_1$ or $\beta_2/3$ mRNA levels with microarray analysis most likely reflects the very discreet nature of the regulation of these transcripts in CA1 pyramidal neurons (Tietz et al., 1993; Chen et al., 1999; Tietz et al., 1999a) and thus the differential sensitivity of these methodologies.

Microarray analysis following acute diazepam administration revealed a reduction in both BDNF (~1.3-fold) and CaMKII$\alpha$ (~1.4 fold) mRNA levels, also without alterations in GABA$_A$R subunit transcripts in wild-type mice (Huopaniemi et al., 2004). Interestingly, in mice with a point-mutation of the GABA$_A$R $\alpha_1$ subunit, which fail to be sedated by diazepam, there was no decrease in BDNF and CaMKII$\alpha$ mRNAs following acute diazepam (Huopaniemi et al., 2004), suggesting a possible role for GABA$_A$R $\alpha_1$ subunit in modulation in regulation of BDNF and CaMKII$\alpha$ expression. Thus in our model 2 days after FZP withdrawal, a decrease in GABA$_A$R $\alpha_1$ subunit protein expression (Chen et al., 1999; Tietz et al., 1999a) may rescue the reduction in BDNF and CaMKII$\alpha$ transcript levels.

On the contrary, in the present study, BDNF transcripts were increased in 2-day FZP-withdrawn rats. It is known that BDNF exhibits a series of actions on neurons ranging from acute enhancement of neurotransmission (presynaptic or postsynaptic) to protein synthesis, long-term promotion of neurite outgrowth and synaptogenesis associated with learning and memory (Narisawa-Saito et al., 1999; Lee et al., 2003b; Zakharenko et al., 2003; Ring et al., 2006). BDNF modulates LTP induction by binding
to TrkB receptors on the postsynaptic neuron (Manabe, 2002), followed by activation of mitogen-activated protein kinase (MAPK/ERK) (Patterson et al., 2001), which may regulate LTP-dependent gene expression via Elk-1/SRE or CREB/CRE pathways (Davis et al., 2000). In rodent neocortical neurons, BDNF application was demonstrated to enhance expression of AMPAR GluR1 and GluR2/3 subunits by activating Src-family protein tyrosine kinases (Narisawa-Saito et al., 1999). In addition, GluR1 phosphorylation in the PSD was enhanced after BDNF treatment, which is also TrkB receptor dependent (Wu et al., 2004). Therefore, the increased transcription level of BDNF in present study suggests that BDNF may regulate AMPAR receptor phosphorylation and gene expression during benzodiazepine withdrawal.

APP, involved in the pathogenesis of Alzheimer's disease, also has important effects on morphological differentiation of neurons, since it is highly expressed on proximal axon segments and in neuropil (Caporaso et al., 1994; Allinquant et al., 1995). The role of APP in physiological and pathological conditions is complex, with different levels of APP expression having neurotrophic or neurotoxic effects. Moderate over-expression of APP was accompanied by an increase in synapse formation; while with a several-fold higher expression of APP will cause a decrease in synaptotrophic effects in neuron-specific enolase and human APP in transgenic mice (Mucke et al., 1994). Further, APP also has been found to support survival and neurite extension of rat cortical neurons in a dose-dependent manner (Yamamoto et al., 1994). Since in our previous study there was no alteration in the frequency of mEPSCs after 2-day FZP withdrawal (Van Sickle
and Tietz, 2002), it appears that the moderate (~1.3 fold) augmentation of APP in hippocampal CA1 neurons during FZP withdrawal might have an *in vivo* neurotrophic effect possibly by preventing neuron damage from calcium influx through GluR2-lacking AMPARs, rather than by promoting new synapse formation.

One of the other upregulated proteins, CaMKIIβ was demonstrated to regulate AMPAR membrane distribution and function. As serine/threonine protein kinases, CaMKIIα and β isoforms exhibit different functions. CaMKIIβ, rather than CaMKIIα, selectively regulates neurite extension and synapse formation during development (Fink et al., 2003). CaMKIIβ bound to F-actin was also reported to localize CaMKIIα/β heteromers to dendritic spines (Shen et al., 1998). Coincident regulation of CaMKIIα and β was observed in some studies. For example, autophosphorylation of both alpha and beta isoforms was increased in the CA1 region after LTP induction (Fukunaga et al., 1996). However, inverse regulation of CaMKIIα and β isoforms was also reported. The alpha/beta ratio shifts toward alpha during increased activity (with bicuculline treatment) and beta during decreased activity (with TTX treatment) in cultured cells (Thiagarajan et al., 2002). In FZP-withdrawn rats, regulation of the CaMKIIα isoform was not observed either with microarray analysis or qPCR measurement, while mRNA and protein levels of CaMKIIβ were significantly upregulated, indicating differential regulation of alpha and beta isoforms during FZP withdrawal.

Although microarray analysis failed to detect an alteration in AMPAR GluR1 mRNA levels, an increase in total GluR1 protein expression in CA1 somata and apical, but not
basal dendrites was detected by immunofluorescence. These confocal data are also in agreement with our previous immunoblot and confocal immunofluorescence data respectively, which showed an increase GluR1 protein level in cytosolic, crude membrane and synaptic fractions, as well as the differential regulation of surface GluR1, but not GluR2 subunits in apical vs. basal dendrites during FZP withdrawal (Song and Tietz, 2006 Soc. Neurosci Abstr.). Post-transcriptional or post-translational modulation of GluR1 subunits might explain the discrepancy between findings of microarray and immunolabelling studies.

In contrast, colocalization of CaMKIIβ and total GluR1 transcripts was significantly augmented in somata and apical dendrites of CA1 pyramidal neurons in response to benzodiazepine withdrawal. Enhanced CaMKIIβ expression and colocalization of GluR1/CaMKIIβ in CA1 pyramidal neurons suggests that during benzodiazepine withdrawal, CaMKIIβ may act as a targeting molecule to drive CaMKIIα/β hetero-oligomers closer to GluR1-containing AMPARs and facilitate AMPAR phosphorylation, consistent with an increased AMPAR conductance in CA1 neurons from FZP-withdrawn rats (Derkach et al., 1999); Shen and Tietz, 2005 Soc. Neurosci Abstr.). Since autophosphorylation of CaMKIIβ is sufficient for F-actin dissociation into the cytosol promoting CaMKII translocation from the F-actin-bound to the PSD-bound state (Shen and Meyer, 1999), future studies investigating the autophosphorylation status of CaMKIIα and β isoforms, as well as the synaptic and extrasynaptic distribution of
these proteins will be helpful to clarify how CaMKII autophosphorylation is involved in benzodiazepine-withdrawal-induced AMPAR remodeling.

The increase in surface GluR1 subunits in FZP-withdrawn CA1 neurons without a change in GluR2 membrane insertion or increase in GluR1/2 colocalization, suggested that GluR2/3 AMPARs may be internalized and reassembled into GluR1/2-containing AMPARs during benzodiazepine withdrawal. An anti-GluR3 antibody is unavailable and the epitope of the commercially-available anti-GluR2/3 antibody is located intracellularly, thus membrane insertion of the GluR3 subunit could not be directly investigated. Nevertheless, the distribution of total GluR2 and GluR3 signals could be assessed with an anti-GluR2/3 antibody. Under permeabilizing conditions, total GluR2/3 labeling was also unaltered in CA1 pyramidal neurons, suggesting no differential regulation of GluR2/3 protein levels in response to FZP withdrawal. Together with previous findings, these data suggest that the redistribution of GluR3-containing AMPARs from membrane to cytosol, rather than a reduction in GluR3 protein level may contribute to a subunit switch of membrane-associated AMPAR from a composition of GluR2/3 to GluR1/2.

Hippocalcin as a member of the neuronal calcium sensor protein family, is highly expressed in hippocampal pyramidal neurons where it was reported to be required for learning and memory. Hippocalcin-deficient (−/−) mice exhibited both impaired cAMP-response element-binding protein (CREB) phosphorylation and discrimination learning (Kobayashi et al., 2005). In NMDA-induced chemical-LTD, hippocalcin was
found to be involved in GluR2-containing AMPAR endocytosis by binding to the beta2-adaptin subunit of the AP2 adaptor complex in a Ca\(^{2+}\)-sensitive manner (Palmer et al., 2005). Although microarray analysis detected a ~1.2-fold increase in hippocalcin mRNA levels, this finding was not validated by qPCR examination. Immunofluorescence studies further demonstrated that hippocalcin protein levels were not up-regulated during FZP withdrawal. However, hippocalcin and GluR2/3-containing AMPARs showed increased colocalization in CA1 somata, indicating that FZP withdrawal may promote the binding of hippocalcin and GluR2/3 subunits and facilitate GluR2/3 endocytosis, in agreement with our hypothesis that a subpopulation of AMPARs switched from GluR2/3 to GluR1/2 heteromers.

Five transcripts known to promote neurotransmitter secretion were observed by microarray analysis to be augmented during benzodiazepine withdrawal, including BDNF, APP, synapsin II, STXBPI (munc-18-1) and synaptotagmin XI, with the latter three belonging to a group of SNARE (soluble NSF attachment receptor) complex-interacting proteins. SNARE complex proteins have critical functions in regulating vesicular release of neurotransmitter. Synaptotagmins are a family of membrane-trafficking proteins also involved in neurotransmitter release. They are classified by their distribution as neuronal (synaptotagmin I-V, X, and XI) and ubiquitous (synaptotagmin VI-IX). Unlike synaptotagmin I, which triggers synaptic exocytosis by acting as a Ca\(^{2+}\)-sensor, synaptotagmin XI shows no Ca\(^{3+}\)-dependent phospholipid binding (von Poser et al., 1997) and moderates homo-oligomerization irrespective of the presence of Ca\(^{2+}\) (Fukuda and
Mikoshiba, 2000). To date, how synaptotagmin may be involved in Ca\textsuperscript{2+}-independent vesicle release remains uncertain. Syntaxin binding proteins (STXBPs) are cytosolic proteins. The function of STXBPs in membrane fusion is unclear. Their proposed functions include a role in vesicle docking or assisting SNARE complex formation. Additionally, in munc-18 knockout mice, syntaxin, a key element in SNARE complex, is reduced by 70%, though the residual syntaxin is still correctly targeted to synapses (Brunger, 2005). This finding indicates the possible roles of STXBPs in modulating neurotransmitter secretion via regulating syntaxin expression. Synapsins (I and II) are a family of neuron-specific phosphoproteins that play an important role in the regulation of synaptic vesicle trafficking and neurotransmitter release. Moreover, suppression of synapsin II inhibits the formation and maintenance of synapses (Ferreira et al., 1994). As a result, the upregulation of synapsin II, STXBPI and synaptotagmin XI after benzodiazepine withdrawal indicates the increased neurotransmitter release and/or synapse formation may occur in response to the FZP withdrawal. Nonetheless, an elevation of mEPSC amplitude did not accompany the increase in mEPSC frequency in 2 day FZP-withdrawn rats (Van Sickle et al., 2004), suggesting that glutamate release may be unchanged during this phase of drug withdrawal. However, modulation of transcripts involved in neurotransmitter release may signify recruitment of machinery required to modulate glutamate release related to postsynaptic remodeling of AMPARs. The involvement of similar machinery in normalization of GABA release in response to the
reduction in postsynaptic GABA function which also accompanies 1-week FZP treatment can also not be ruled out (Poisbeau et al., 1997; Zeng and Tietz, 1999).

Synapsins have also been demonstrated to function as mediators of BDNF-enhanced glutamate release in synapsomes (Jovanovic et al., 2000). BDNF potentiates synaptic transmission by elevating presynaptic intracellular Ca\(^{2+}\) release and inducing TrkB activation, which results in phosphorylation of synapsin and increases the number of synaptic vesicles available for release. Neurotrophins may also regulate synaptic transmission by increasing local release of neuregulin or other nerve-derived modulators (Zhan et al., 2003). Exposure of cortical neurons to BDNF also can enhance the expression of synapsin, synaptotagmin, and synaptophysin (Matsumoto et al., 2006), suggesting crosstalk between neurotrophins and SNARE-interacting proteins. The negative role for APP in neurotransmitter release has been found in cultured hippocampal neurons, as the size of the readily releasable synaptic vesicle pool was increased in the absence of APP (Priller et al., 2006); and it appears that APP expression can be stimulated by BDNF in a dose- and time-dependent fashion (Ruiz-Leon and Pascual, 2001). Therefore, upregulation of BDNF may result in enhanced APP mRNA levels in response to FZP withdrawal. Since these two proteins have opposite effects on neurotransmitter release, it’s possible that biphasic modulation of these neurotrophic factors may fine-tune neurotransmitter secretion in response to enhanced postsynaptic AMPA function.

Ingenuity Pathway Analysis was used to integrate the information derived from microarray analysis of benzodiazepine-withdrawal regulated genes. The network
generated from the uploaded genes suggests the extracellular and subcellular distribution of the respective proteins, as well as how these related genes may act in a coordinated fashion. Within the LTP-associated and neurotransmission pathways, BDNF and APP have the greatest number of interactions with other genes clusters, including AMPAR GluR1-4 subunits and various trafficking proteins, suggesting that modulation of BDNF and APP may be central to benzodiazepine withdrawal. In addition, regulation of these gene transcripts and others are related to cytoskeleton assembly and organization (BDNF, STXBP1, CaMKIIβ, APP, MAPT, synapsin II), and ion homeostasis (potassium channel subfamily V, PMCA1, PMCA3) were also observed to be differentially regulated in response to FZP withdrawal, indicating multiple signaling cascades may be engaged in during drug withdrawal and related to drug dependence.

In summary, the main findings of the present study show that a network of genes associated with activity-dependent plasticity (APP, BDNF, CAMKIIB, HPCA, etc.) are upregulated in hippocampal CA1 neurons during FZP withdrawal. Since such a high percentage of genes increased after FZP withdrawal were involved in neuronal plasticity and neurotransmitter secretion, it is likely that the increase in hippocampal CA1 neuron glutamatergic strength associated with benzodiazepine withdrawal shares similar signaling mechanisms to those associated with other models of activity-dependent plasticity such as LTP. To our knowledge, this is the first report investigating mRNA expression profiling after chronic benzodiazepine administration using microarray techniques coupled with Ingenuity pathway analysis. Our findings define key gene
transcripts and regulatory pathways in the hippocampus involved in benzodiazepine withdrawal, suggesting that manipulation of these genes may have therapeutic value in the management of drug dependence.
References


Guzman-Marin R., Ying Z., Suntova N., Methippara M., Bashir T., Szymusiak R.,
plasticity-related gene expression by sleep deprivation in rats. *J Physiol.* 575,
807-819.

Heninger C., Saito N., Tallman J. F., Garrett K. M., Vitek M. P., Duman R. S. and
Gallager D. W. (1990) Effects of continuous diazepam administration on GABA

Impagnatiello F., Pesold C., Longone P., Caruncho H., Fritschy J. M., Costa E. and
expression in rat neocortex during tolerance to diazepam. *Mol Pharmacol.* 49,
822-831.

Diazepam-induced adaptive plasticity revealed by alpha1 GABA receptor-specific

transmission from readily releasable synaptic vesicles in excitatory hippocampal
synapses in mice. *J Physiol.* 571, 75-82.

acid decarboxylase and glutamate receptor changes during tolerance and dependence


Tietz E. I., Huang X., Chen S. and Ferencak W.F. (1999a) Temporal and regional regulation of alpha1, beta2 and beta3, but not alpha2, alpha4, alpha5, alpha6, beta1 or gamma2 GABA(A) receptor subunit messenger RNAs following one-week oral flurazepam administration *Neuroscience*. **91**, 327-41.


**Figure 1.** Quantitative real-time PCR (qPCR) gene expression profiling in CA1 neurons. SYBR green qPCR was performed on hippocampal CA1 regions from individual CON (n = 6 rats) and 2 day FZP withdrawn (n = 7 rats) groups to validate microarray data. GAPDH RNA transcript was set as endogenous reference. Values representing the fluorescent product formed during the reaction are expressed as fold change between FZP and CON groups. The regulation of 5 out of 6 selected gene products (except hippocalcin) was confirmed with qPCR analysis. APP, BDNF, CaMKIIβ and MAPT were significantly upregulated during FZP withdrawal, compared with their matched controls (CON, P < 0.05, by unpaired Students t-test). CaMKIIα mRNA level, observed in the microarray study to be unaltered, was also unchanged when examined by qPCR. Black bars represent the average fold change obtained by microarray analysis, whereas the white bars denote fold change data gained by qPCR. Asterisks (*) represent statistical significance (P < 0.05).
**Figure 2.** Biological network of genes regulated during 2 day FZP withdrawn, constructed based on ISPKB database. The network is displayed graphically as nodes (gene or gene product) and edges (the biological relationships between nodes), as well as extracellular and subcellular distributions. The shape of the objects represents different types of gene products. The interactions between genes/gene products are represented as different letters. The greater the color intensity, the more upregulation or downregulation. Genes involved include: APP (amyloid beta precursor protein); BDNF (brain derived neurotrophic factor); CAMK2B (Ca$^{2+}$/calmodulin-dependent kinase II beta, CaMKIIβ); MAPT (microtubule-associated protein tau); ATP2B1 (PMCA1, plasma membrane Ca$^{2+}$-ATPase type 1); BCL2 (B-cell leukemia/lymphoma 2); BZW2 (basic leucine zipper and W2 domains 2); CHGB (chromogranin B); CMA1 (chymase 1); COL1A2 (collagen, type I, alpha 2); COL3A1 (collagen, type III, alpha 1); DOCK3 (dedicator of cytokinesis 3); HBA2 (hemoglobin, alpha 2); HBB (hemoglobin, beta); HIF1A (hypoxia-inducible factor 1, alpha subunit); ID2 (inhibitor of DNA binding 2); IL13 (interleukin 13); KLF7 (Kruppel-like factor 7); NNAT (neuronatin); NPR1 (natriuretic peptide receptor A/guanylate cyclase A); NPTXR (neuronal pentraxin receptor); NRN1 (neuritin 1); P4HA1 (procollagen-proline, alpha polypeptide I); P4HB (procollagen-proline, beta); SERPINH1 [serpin peptidase inhibitor, clade H (heat shock protein 47), member 1]; SP1 (Sp1 transcription factor); STXBP1 (syntaxin binding protein 1); SYN1/2/3 (synapsin I/II/III); TCF4 (transcription factor 4); TGFB (transforming growth factor, beta); TNF (tumor necrosis factor).
Figure 3. Connectivity map of LTP pathway constructed based on IPA canonical pathway databases. Explanations of symbols are shown in the Fig. 2 legend. BDNF and APP exhibit indirect interactions with AMPA and NMDA receptor subunits, the latter which play crucial roles in neuronal plasticity. Genes involved include: AKAP5 (AKAP79/150); AKAP11; APP; BDNF; CAMK4 (calcium/calmodulin-dependent protein kinase IV); CDH2 (neuronal-cadherin); CACNG2 (voltage-dependent calcium channel, gamma 2 subunit); CHRNA7 (nicotinic cholinergic receptor, alpha 7); CREB1 (cAMP responsive element binding protein 1); DLG4 [discs, large homolog 4 (Drosophila)]; FYN (FYN oncogene related to SRC, FGR, YES); GRIA1-4 (AMPA receptor GluR1-4); GRID2 (Glutamate receptor delta 2 subunit); GRIK2: Kainate receptor 2; GRIN1 (NMDA receptor subunit 1); HRAS (v-Ha-ras Harvey rat sarcoma viral oncogene homolog); IL10 (interleukin 10); KCNA4 (potassium voltage-gated channel, shaker-related subfamily, member 4); MAPK1 (mitogen-activated protein kinase 1); NRG1(neuregulin 1); NTRK2 (neurotrophic tyrosine kinase, receptor, type 2); PPP1R1B [protein phosphatase 1, regulatory subunit 1B (DARPP-32)]; PRKACA (PKA, catalytic subunit, alpha); PSEN1 (presenilin 1); PTK2B (protein tyrosine kinase 2 beta); PTPN5 (non-receptor protein tyrosine phosphatase, type 5); PTPRA (protein tyrosine phosphatase, receptor type A); PSEN1 (presenilin 1); PTPRD (protein tyrosine phosphatase, receptor type D); RYR3 (ryanodine receptor 3); S100B [S100 calcium binding protein, beta (neural)]. See Fig. 2 legend for details.
Figure 4. Connectivity map of neurotransmission pathway constructed based on IPA canonical pathway databases. Explanations of symbols are shown in the Fig. 2 legend. The pathway focus on BNDF, APP and STXBP1, which show direct and indirect interactions with other vesicle docking and trafficking proteins, i.e. STX3A, STXBP5, STX1A, DOC2B, UNC13B. Genes involved include: APBA1 (amyloid beta precursor protein-binding, family A, member 1); APBA2 (amyloid beta precursor protein-binding, family A, member 2); APP; BDNF; CACNG2; CASK [calcium/calmodulin-dependent serine protein kinase (MAGUK family)]; DLG4 (discs, large homolog 4, PSD95/SAP90); DOC2B (double C2-like domains, beta); EB-1 (E2a-Pbx1-associated protein); GRIA1-4; GRID2; GRIK2; GRIN1; GRIN2C (NMDA receptor, NR2C); GRIPAP1 (GRIP1 associated protein 1); HOMER1 (homer homolog 1); HTR2C (5-HT receptor 2C); LIN7C (lin-7 homolog C); MAP1A (microtubule-associated protein 1A); MYO6 (myosin VI); NAPA (NSF attachment protein, alpha); NARP (neuroactivity regulated petaxin); NSF (N-ethylmaleimide-sensitive factor); PRKCABP (protein kinase C, alpha binding protein, PICK1); STX1/3A (syntaxin 1/3A); STXBP1; STXBP5; UNC13B (unc-13 homolog B). See Fig. 2 legend for details.
Figure 5. Comparison of dual-immunofluorescence staining of total GluR1 and CaMKIIβ expression in CA1 neurons between CON and 2-day FZP-withdrawn groups. Quantification of GluR1 and CaMKIIβ IF data are shown in Figure 6. (A) Representative photomicrographs of GluR1 (left), CaMKIIβ (middle) and colocalization (right) in so (basal dendritic area) from the CON (upper panels) and FZP (lower panels) groups. Stronger CaMKIIβ labeling was observed in the FZP group. There was no apparent difference in GluR1 signal intensity or GluR1 and CaMKIIβ colocalization between two groups; (B) Representative photomicrographs of GluR1, CaMKIIβ and colocalization in sp (somatic area) from the CON and FZP groups. Stronger labelings of total-GluR1, CaMKIIβ and colocalization were found in FZP group; (C) Representative photomicrographs of GluR1, CaMKIIβ and colocalization in the first 100 μm of the apical dendritic area (sr) immediately adjacent to sp from two groups. Greater labeling intensity of total-GluR1, CaMKIIβ and colocalization were found in FZP group. Scale bar: 40 μm.
Figure 6. Quantification of total GluR1 and CaMKIIβ immunofluorescent signals in CA1 neurons from CON and FZP groups. Mean density, reflecting signal intensity/μm², was used to analyze total GluR1 and CaMKIIβ signals. Colocalization was defined as the percentage of the volume of overlapping signal. (A) In the so area, the FZP group showed a significant increase in CaMKIIβ signal compared with the CON group. However, no significant differences were found in total GluR1 labeling or GluR1 and CaMKIIβ colocalization between groups in this region; (B) and (C) In sp (B) and sr (C) regions, quantification of total GluR1 and CaMKIIβ IF signals showed significant increases in mean density, as well as colocalization volume in the FZP group. All data were analyzed by Students t-test and shown as mean ± SEM (n = 4 rats/group, *P < 0.05).
Figure 7. Compared dual-immunofluorescence staining of hippocalcin and total GluR2/3 expression in CA1 neurons between CON and 2-day FZP-withdrawn groups. Quantification of hippocalcin and total GluR2/3 IF data are shown in Figure 8. (A) Representative photomicrographs of hippocalcin (left), GluR2/3 (middle) and colocalization (right) in so from the CON (upper panels) and FZP (lower panels) groups. There was no obvious difference of hippocalcin or total GluR2/3 signal intensity and their colocalization between experimental groups; (B) Representative photomicrographs of hippocalcin, GluR2/3 and their colocalization in sp (somatic area) from the CON and FZP groups. A greater degree of colocalization of hippocalcin and GluR2/3 signal was found between the FZP and CON group; (C) Representative photomicrographs of hippocalcin, GluR2/3 and colocalization in the first 100 μm of the apical dendritic area (sr) immediately adjacent to sp from two groups. No obvious alterations in hippocalcin or GluR2/3 labeling intensity or their colocalization were observed in sr between groups. Scale bar: 40 μm.
Figure 8. Quantification of hippocalcin and total GluR2/3 immunofluorescent signals in CA1 neurons from CON and FZP groups. Mean density, reflecting signal intensity/μm², was used to analyze hippocalcin and GluR2/3 signals. Colocalization was defined as the percentage of the volume of overlapping signal. (A) and (C) There were no significant increases in hippocalcin, GluR2/3 or their colocalization in either the so or sr area between experimental groups; (B) In the sp region, though there was no significant increase in hippocalcin or GluR2/3 labeling between groups, quantification of IF signals showed a significant increase in their colocalization in the FZP, in comparison to the CON group. All data were analyzed by Students t-test (n = 4 rats/group, *P < 0.05).
<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank ID</th>
<th>Product</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>NM_012513</td>
<td>88</td>
<td>GGAGAAAGTCCCGGTATCAA</td>
<td>GCAGCCTCCTTCCGTGT</td>
</tr>
<tr>
<td>CAMK2B</td>
<td>NM_012920</td>
<td>76</td>
<td>AGCACCCTGGATCTCGC</td>
<td>TTCTTCAGGCAGTCCAC</td>
</tr>
<tr>
<td>CAMK2A</td>
<td>NM_021739</td>
<td>64</td>
<td>ACCAGCCAGTCCGAAGAG</td>
<td>AATGGACATTCTGCCAC</td>
</tr>
<tr>
<td>HPCA</td>
<td>NM_017122</td>
<td>76</td>
<td>GATTACTGGGCTGGGACT</td>
<td>AGAGCTGGGACAGGAA</td>
</tr>
<tr>
<td>MAPT</td>
<td>NM_017212</td>
<td>98</td>
<td>TCCCTTGCTCGCTTCTTG</td>
<td>TGATGCTGATGTCGCA</td>
</tr>
<tr>
<td>APP</td>
<td>NM_019288</td>
<td>79</td>
<td>GGTCGAGTTCTGTTGCTG</td>
<td>ATCGGAGGCTCTTCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>X02231</td>
<td>63</td>
<td>TATGACTCTACCCCGCAA</td>
<td>GATGACCCAGCCTTCC</td>
</tr>
</tbody>
</table>

**TABLE 1**
Summary of genes and primer sequences used for qPCR analysis.
<table>
<thead>
<tr>
<th>GenBank ID</th>
<th>Gene/Transcript</th>
<th>Fold Increase</th>
<th>Molecular Function</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_012513</td>
<td>BDNF</td>
<td>1.64 ± 0.18</td>
<td>neurotrophin TRKB receptor binding</td>
<td>nervous system development; neuron differentiation; regulation of long-term neuronal synaptic plasticity</td>
</tr>
<tr>
<td>NM_021739</td>
<td>Ca(^{2+})/CaM-dependent protein kinase II beta subunit</td>
<td>1.28 ± 0.04</td>
<td>Ca(^{2+})/CaM-dependent protein kinase activity</td>
<td>protein phosphorylation; synaptic plasticity</td>
</tr>
<tr>
<td>NM_017122</td>
<td>hippocalcin</td>
<td>1.23 ± 0.03</td>
<td>neuronal Ca(^{2+}) sensor; actin and tubulin binding; clathrin binding</td>
<td>neuronal plasticity and memory formation</td>
</tr>
<tr>
<td>NM_053601</td>
<td>neuronatin, variant 1</td>
<td>1.63 ± 0.11</td>
<td>auxiliary transport protein activity</td>
<td>brain development; neuron differentiation</td>
</tr>
<tr>
<td>NM_053346</td>
<td>neuritin</td>
<td>1.35 ± 0.05</td>
<td>Induced by neurotrophins</td>
<td>nervous system development</td>
</tr>
<tr>
<td>NM_019159</td>
<td>synapsin II</td>
<td>1.57 ± 0.12</td>
<td>cytoskeletal protein binding; protein dimerization activity</td>
<td>synaptic transmission</td>
</tr>
<tr>
<td>NM_013038</td>
<td>syntaxin binding protein 1</td>
<td>1.30 ± 0.04</td>
<td>syntaxin binding</td>
<td>vesicle docking and fusion during exocytosis;</td>
</tr>
<tr>
<td>NM_031667</td>
<td>synaptotagmin XI</td>
<td>1.38 ± 0.09</td>
<td>Ca(^{2+}) binding; Ca(^{2+})-dependent phospholipid binding; transporter activity</td>
<td>Ca(^{2+})-dependent exocytosis; transport</td>
</tr>
<tr>
<td>NM_021697</td>
<td>potassium channel, subfamily V, member 1</td>
<td>1.43 ± 0.05</td>
<td>voltage-gated potassium channel activity</td>
<td>potassium ion transport</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene Symbol</td>
<td>Description</td>
<td>Fold Change (Mean ± SE)</td>
<td>Function(s)</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>XM_343839</td>
<td>plasma membrane Ca(^{2+}) ATPase, isoform 3</td>
<td>1.60 ± 0.06</td>
<td>Ca(^{2+})-transporting ATPase activity, Ca(^{2+}) transport metabolism</td>
<td></td>
</tr>
<tr>
<td>NM_017212</td>
<td>microtubule-associated protein tau</td>
<td>1.35 ± 0.07</td>
<td>Structural constituent of cytoskeleton, protein binding, apoptosis, negative regulation of microtubule depolymerization, neuron differentiation</td>
<td></td>
</tr>
<tr>
<td>NM_019288</td>
<td>amyloid beta (A4) precursor protein</td>
<td>1.31 ± 0.04</td>
<td>Protein binding, heparin binding, metal ion binding, apoptosis, endocytosis, neuronal plasticity, extracellular matrix organization and biogenesis, Notch signaling pathway</td>
<td></td>
</tr>
<tr>
<td>NM_030841</td>
<td>neuronal pentraxin receptor</td>
<td>1.57 ± 0.08</td>
<td>Neuronal pentraxin binding activity</td>
<td></td>
</tr>
<tr>
<td>XM_574670</td>
<td>cartilage acidic protein 1</td>
<td>1.58 ± 0.16</td>
<td>Calcium ion binding, cell adhesion</td>
<td></td>
</tr>
<tr>
<td>NM_001001</td>
<td>spinal cord expression protein 4</td>
<td>1.32 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_053859</td>
<td>solute carrier family 17, member 7</td>
<td>1.31 ± 0.03</td>
<td>Transporter activity, sodium-dependent phosphate transporter activity, phosphate transport</td>
<td></td>
</tr>
<tr>
<td>NM_182668</td>
<td>nitrilase 1</td>
<td>1.47 ± 0.07</td>
<td>Nitrilase activity, hydrolase activity, nitrogen compound metabolism</td>
<td></td>
</tr>
<tr>
<td>NM_134402</td>
<td>basic leucine zipper and W2 domains 2</td>
<td>2.58 ± 0.16</td>
<td>Translation initiation factor activity, regulation of translational initiation</td>
<td></td>
</tr>
<tr>
<td>NM_012526</td>
<td>chromogranin B</td>
<td>1.35 ± 0.05</td>
<td>Regulation of IP3 receptor, extracellular space, secretory granule, facilitate calcium release from IP3 receptor, hormone activity</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 2

Mean fold change in RNA transcript levels in hippocampal CA1 neurons were assessed by Affymetrix microarray in response to 2-day FZP withdrawal. The 19 significantly upregulated transcripts and their biological function are shown. Analyzed by unpaired Student’s $t$-test, $P < 0.05$. 

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>GenBank ID</td>
<td>Gene Transcript</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NM_053311</td>
<td>plasma membrane Ca\textsuperscript{2+}-ATPase, isoform 1</td>
</tr>
<tr>
<td>NM_181087</td>
<td>cytochrome P450, family 26, subfamily b, polypeptide 1</td>
</tr>
<tr>
<td>NM_032085</td>
<td>procollagen, type III, alpha 1</td>
</tr>
<tr>
<td>NM_013060</td>
<td>Inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>NM_053369</td>
<td>transcription factor 4</td>
</tr>
<tr>
<td>NM_053416</td>
<td>double-stranded RNA-binding protein p74</td>
</tr>
<tr>
<td>NM_198776</td>
<td>hemoglobin beta chain</td>
</tr>
<tr>
<td>NM_0010138</td>
<td>hemoglobin alpha 53</td>
</tr>
</tbody>
</table>

**TABLE 3**

Mean fold change in RNA transcript levels in hippocampal CA1 neurons were assessed by Affymetrix microarray in response to 2-day FZP withdrawal. The 8 significantly downregulated transcripts are shown. Analyzed by unpaired Student’s \( t \)-test, \( P < 0.05 \).
CONCLUSIONS

1. Two days after 1-week FZP exposure, the amplitude of evoked AMPAR currents and mEPSCs was increased in hippocampal CA1 pyramidal neurons, in the absence of alterations in channel kinetics.

2. AMPAR potentiation was associated with a reduction in the rectification index and an increase in spermine analogue blockade, suggesting that more GluR1 homomers are anchored in the CA1 neuron membrane.

3. FZP withdrawal-induced increases membrane incorporation of AMPAR GluR1 subunits and GluR1/2 heteromers in CA1 neurons, without alterations in surface GluR2 labeling.

4. The levels of GluR1 subunit protein, rather than GluR2 levels, were augmented in all subcellular fractions examined in CA1 neurons after FZP withdrawal.

5. Increased GluR1 subunit phosphorylation by PKA proportionate to the upregulation of total GluR1, associated with enhanced expression of SAP97 and AKPA79/150 was observed in crude membrane and PSD-enriched compartments of CA1 neurons from 2-day FZP-withdrawn rats.

6. There were no alterations in the expression of PKA regulatory and catalytic subunits in the same subcellular fractions in response to FZP withdrawal.

7. Gene profiling analysis revealed 27 transcripts which were differentially regulated by FZP withdrawal, including BDNF, CAMKIIB, HPCA, MAPT and APP.
8. One network and two biological pathways were identified by Ingenuity Pathway Analysis, to be involved in the differential regulation of the altered gene transcripts.

9. Increased protein levels of CaMKIIβ and colocalization between CaMKIIβ and the GluR1 subunit protein suggested the possible enhancement of GluR1 subunit phosphorylation by CaMKII during FZP withdrawal.

10. Increased colocalization between hippocalcin and GluR2/3 indicated that hippocalcin-AP2 complex might promote GluR2/3 heteromers endocytosis and reassembly of GluR1/2 AMPARs in response to FZP withdrawal.
SUMMARY

The current study reveal several novel findings that further elucidate the mechanisms underlying BZ-withdrawal-induced AMPAR potentiation and support the idea that neuroadaptive glutamatergic remodeling is a common neurophysiological basis for drug dependence, as well as for the plastic changes which occur in hippocampus during learning and memory. First, the data provide direct evidence that AMPAR function is enhanced in response to FZP withdrawal, in conjunction with a subunit switch from AMPARs dominant in GluR2 subunit composition to an increased membrane/synapse incorporation of both GluR1 homomers and GluR1/2 heteromers. To our knowledge, this is the first study to demonstrate a critical role for an AMPAR subunit switch in BZ dependence. A switch in AMPAR composition has been observed in many forms of activity-dependent plasticity. For example, in cerebellar stellate cells, repetitive high-frequency synaptic activation of Ca\(^{2+}\)-permeable AMPARs caused a rapid reduction in Ca\(^{2+}\) permeability and a change in the amplitude of excitatory postsynaptic currents, resulting from the incorporation of GluR2-containing AMPARs (Liu and Cull-Candy, 2000); BDNF stimulation significantly promoted synaptic delivery of homomeric GluR1 AMPA receptors in cultured organotypic slices (Caldeira et al., 2007); in an *in vitro* model of brain ischemia, brief oxygen-glucose deprivation promoted GluR2-containing AMPARs endocytosis from synapses and delivery of GluR2-lacking AMPARs to synaptic sites (Liu et al., 2006). Moreover, increased GluR1 membrane/synapse delivery was also observed after brief dopamine D1 receptor stimulation (Sun et al., 2005); and Bellone et al.
found a significantly increased inward rectification in mouse VTA dopaminergic neurons after a single injection of cocaine (Bellone and Luscher, 2006), implicating an important role for increased expression of AMPAR GluR1 homomers associated with exposure to other drugs of abuse. Therefore, our findings coupled with the synaptic plasticity and drug addiction literature, support the idea that an AMPAR subtype switch may be a common and essential mechanism underlying neuronal adaptations to electrical and/or chemical stimulation.

Second, our observations suggest that mechanisms similar to those underlying AMPAR potentiation in different forms of activity-dependent plasticity may also occur in hippocampal CA1 pyramidal neurons in our BZ-withdrawal model. We found an elevated AMPAR-mediated EPSCs amplitude and increased GluR1 subunit expression in hippocampal CA1 neurons after FZP withdrawal. The augmentation in GluR1 subunit levels was associated with a proportionate increase in the amount of AMPAR GluR1 subunit phosphorylated at Ser\textsuperscript{845}, coupled with enhanced expression of membrane/synapse-associated SAP97 and AKAP79/150. In an analogous fashion in synaptic plasticity, PKA phosphorylation of GluR1 Ser\textsuperscript{845} increased AMPAR channel open probability (Banke et al., 2000), promoted AMPAR protein synthesis (Nayak et al., 1998) and facilitated receptor synaptic trafficking (Esteban et al., 2003). Thus, interactions among SAP97-AKAP-PKA complexes and GluR1 subunits may be centrally involved in regulating AMPAR remodeling after exposure to BZs as well as other forms of activity-dependent plasticity. In excitatory synapses, glutamate receptors
and PKA are recruited into a macromolecular signaling complex through direct interaction between the MAGUK proteins, PSD95 and SAP97, and AKAP79/150 (Colledge et al., 2000), favoring GluR1 substrate phosphorylation. On the other hand, disruption of PKA anchoring onto AKAP79/150 will cause a reduction in surface GluR1 (Snyder et al., 2005) and decreased AMPAR currents (Tavalin et al., 2002), which shares similarities with the mechanisms underlying LTD. Similarly, the concomitant augmentation of SAP97 and AKAP79/150 observed in our study suggests that the latter complex may recruit more PKA to GluR1 subunits and promote receptor phosphorylation and potentiation. To date, PKA signaling pathways have been reported to be involved in cannabinoid withdrawal (Tzavara et al., 2000), ethanol and opioid tolerance and dependence (Liu and Anand, 2001; Wand et al., 2001), as well as chronic cocaine exposure (Freeman et al., 2001). However, no direct evidence demonstrates whether SAP97-AKAP-PKA interactions are critical to AMPAR remodeling induced by exposure to drugs of abuse. Thus, our study also provides the first evidence that these interactions may occur in a manner similar to activity-dependent plasticity and that SAP97-AKAP-PKA complexes may be crucial elements in regulating GluR1-containing AMPAR phosphorylation, surface receptor delivery and channel function during BZ withdrawal. Future co-immunoprecipitation studies with anti-AKAP79/150 and anti-PKA regulatory subunit antibodies will clarify whether a direct interaction between them is enhanced in BZ-withdrawn rats. In addition, interruption of the AKAP/PKA complex with st-Ht31, a cell-permeable stearated form of AKAP, will further confirm the
importance of AKAP/PKA interactions to AMPAR phosphorylation and potentiation of AMPAR function.

Finally, the third manuscript in this dissertation represents the first comprehensive study investigating gene profiling in hippocampal CA1 neurons after chronic BZ withdrawal, accompanied by analysis of plausible functional biological pathways. The Affymetrix rat expression 230 3.0 arrays used in this study, comprised of more than 31,000 probe sets, provide thorough coverage of rat genome transcripts. Twenty-seven gene transcripts were found to be differentially regulated in CA1 minislices from 2-day FZP-withdrawn rats. They were separated into different clusters by their distinct biological functions, such as neurotransmitter secretion, neuronal plasticity, as well as the assembly and organization of cytoskeletal proteins. However, the functional relationships among individual gene transcripts are still unsolved. Therefore, we employed Ingenuity Pathway Analysis to integrate the information derived from microarray analysis of BZ-withdrawal regulated genes as a tool to construct relevant pathway information from the uploaded genes. The analysis integrates individual genes, biological pathways, and functional regulatory networks based on published literature. Participation of genes in Ingenuity pathways clusters also provides information about protein properties, protein-protein interactions and the cellular distribution of proteins. One network and two canonical pathways were constructed based on the significantly regulated genes in response to BZ withdrawal, providing important information about the possible interactions among these regulated gene products.
The selective modification of AMPARs during FZP withdrawal is also coincident with GABA/BZ site uncoupling, downregulation of GABA_A receptor expression, reduction in GABA_A receptor function and BZ anticonvulsive tolerance (Tietz et al., 1989; Zeng et al., 1995; Tietz et al., 1999a; Tietz et al., 1999b; Zeng and Tietz, 1999). Meanwhile, GABA_A receptor function is also controlled by PKA-mediated phosphorylation. For example, PKA phosphorylation of GABA_A receptors has been demonstrated to protect the GABA_A receptor from endocytosis and subsequently enhance mIPSC amplitude and GABA_A receptor-mediated whole-cell currents (Kittler et al., 2005). Although it was demonstrated that AKAP79/150 is associated with excitatory rather than inhibitory synapses in CA1 pyramidal neurons (Lilly et al., 2005), it remains unknown how PKA spatially regulates excitatory and inhibitory synaptic functions in hippocampal CA1 pyramidal neurons during BZ withdrawal. Excitatory and inhibitory synapses can be distinguished from their distribution and structure: in brain most excitatory glutamatergic synapses locate on dendritic spines, which are small actin-rich protrusions (Harris and Kater, 1994); while GABAergic inhibitory synapses primarily occur on cell body and dendritic shafts with GABA_A receptor attached to microtubule cytoskeleton via gephyrin and/or GABA_A receptor-associated protein (Essrich et al., 1998; Wang et al., 1999). In addition, excitatory and inhibitory synapses can also be separated by distinct biomarkers. As in cultured hippocampal neurons, excitatory and inhibitory synapses were distinguished with presynaptic markers, vesicular glutamate transporter VGLUT1 and glutamate decarboxylase GAD65, respectively (Liu, 2004).
PSD95 and spinophilin have also been used as excitatory postsynaptic markers (Li et al., 2004). Future studies focusing on the distribution and function of PKA subunits in excitatory or inhibitory synapses in CA1 pyramidal neurons in our FZP-withdrawal model could be conducted based on the synaptic distribution of these different biomarkers, will be helpful to explain how PKA may spatially regulate the glutamatergic and GABAergic systems. Biomarker pull-down assay can also be used to examine the spatial expression and activity of other protein kinases (CaMKII) and phosphotases (PP1 and PP2B), at excitatory/inhibitory synapses.
BIBLIOGRAPHY


Carlezon WA, Jr., Rasmussen K, Nestler EJ (1999) AMPA antagonist LY293558 blocks the development, without blocking the expression, of behavioral sensitization to morphine. Synapse 31:256-262.


Gonsalves SF, Gallager DW (1988) Persistent reversal of tolerance to anticonvulsant effects and GABAergic subsensitivity by a single exposure to benzodiazepine antagonist during chronic benzodiazepine administration. J Pharmacol Exp Ther 244:79-83.


Otten AD, Parenteau LA, Doskeland S, McKnight GS (1991) Hormonal activation of
gene transcription in ras-transformed NIH3T3 cells overexpressing RII alpha and
RII beta subunits of the cAMP-dependent protein kinase. J Biol Chem
266:23074-23082.

Palmer CL, Lim W, Hastie PG, Tward M, Korolchuk VI, Burbidge SA, Banting G,
Collingridge GL, Isaac JT, Henley JM (2005) Hippocalcin functions as a calcium
sensor in hippocampal LTD. Neuron 47:487-494.

Park S, Hong YW (2006) Transcriptional regulation of artemin is related to neurite
outgrowth and actin polymerization in mature DRG neurons. Neurosci Lett
404:61-66.

Partin KM, Mayer ML (1996) Negative allosteric modulation of wild-type and mutant

Passafaro M, Piech V, Sheng M (2001) Subunit-specific temporal and spatial patterns of
AMPA receptor exocytosis in hippocampal neurons. Nat Neurosci 4:917-926.

Patneau DK, Mayer ML (1991) Kinetic analysis of interactions between kainate and
AMPA: evidence for activation of a single receptor in mouse hippocampal

Patterson SL, Pittenger C, Morozov A, Martin KC, Scanlin H, Drake C, Kandel ER
(2001) Some forms of cAMP-mediated long-lasting potentiation are associated
with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron
32:123-140.


Schiffer HH, Swanson GT, Heinemann SF (1997) Rat GluR7 and a carboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate. Neuron 19:1141-1146.


Tietz EI, Huang X, Chen S, Ferencak WF, 3rd (1999a) Temporal and regional regulation of alpha1, beta2 and beta3, but not alpha2, alpha4, alpha5, alpha6, beta1 or gamma2 GABA(A) receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience 91:327-341.


ABSTRACT

Glutamatergic system adaptive remodeling has newly been identified as an important neural substrate of drug dependence. As extensively used anticonvulsant and hypnotic drugs, benzodiazepines act primarily as allosteric modulators of $\text{GABA}_A$ receptors; while the withdrawal symptoms are associated with enhanced AMPAR function. To investigate AMPAR remodeling during benzodiazepine withdrawal, whole-cell recordings were conducted in rat hippocampal CA1 neurons. The amplitude of AMPAR-mediated currents was enhanced 30-50% after 2-day FZP withdrawal, without changes in channel kinetic properties. Agonist-elicited AMPA currents were significantly blocked by spermine, suggesting augmented membrane incorporation of GluR2-lacking AMPARs. As GluR1-containing AMPARs are centrally involved in neuronal plasticity, we sought to determine whether changes in GluR1 subunit expression and distribution occurred during benzodiazepine withdrawal. Confocal imaging analysis revealed that FZP withdrawal promoted GluR1 subunit membrane incorporation, in the absence of GluR2 subunit alterations. Immunoblotting assays showed that GluR1, but not GluR2, subunit protein levels were enhanced in all subcellular fractions from CA1 minislices. As with LTP, FZP-withdrawal-induced GluR1 incorporation into membrane/synapse may require the GluR1-trafficking protein SAP97 and AMPAR phosphorylation by PKA may be mediated via a SAP97-AKAP interaction. Therefore, the expression and membrane/synapse redistribution of these proteins were investigated using subcellular fractionation and immunoblotting techniques. Consistent with increases in SAP97
immunoreactivity, protein levels of phospho-Ser$^{845}$ GluR1 and AKAP79/150 were concomitantly elevated in membrane/synapse-associated compartments from FZP-withdrawn rats. There were no alterations in the immunoreactivity of PKA regulatory and catalytic subunits. These findings indicate that increased interaction between SAP97-AKAP-PKA, rather than enhanced PKA expression, resulted in increases in phosphorylated AMPARs and thus may be involved in AMPAR potentiation. The molecular mechanisms underlying benzodiazepine withdrawal were further explored by microarray analysis and Ingenuity Pathway Analysis. Twenty-seven known gene transcripts were differentially expressed during FZP withdrawal, including 19 significantly upregulated and 8 downregulated transcripts. Based on the transcripts identified, one network and two biological (LTP-associated and neurotransmission) pathways were constructed by IPA, illustrating the interactions among these genes products. Collectively, our findings provide evidence that during FZP withdrawal, increased expression and phosphorylation of GluR1-containing AMPARs and associated upregulation of AMPAR function in hippocampal CA1 pyramidal neurons share fundamental similarities with the mechanisms underlying activity-dependent synaptic plasticity.