Bidirectional regulation of AMPA and NMDA receptors during benzodiazepine withdrawal

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Bidirectional Regulation of AMPA and NMDA Receptors during Benzodiazepine Withdrawal

Submitted by:
Guofu Shen

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

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Date of Defense: April 29, 2009
Bidirectional Regulation of AMPA and NMDA Receptors during Benzodiazepine Withdrawal

Guofu Shen

University of Toledo
Health Science Campus
2009
DEDICATION

This work is dedicated to my incoming daughter, Zoey Lee Shen, and to my beloved wife, Yupin Li, for her endless support, love and encouragement.
ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my major advisor, Dr. Elizabeth I. Tietz, for her guidance, patience, understanding and encouragement for my dissertation study in the past five years. I have been lucky enough to have her all around support to explore in the biomedical science.

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Nobody has been more important to me in the pursuit of my PhD degree than the members of my family, my parents and my brother. Their love and support were the source of my strength to pursue my goals and happiness.

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INTRODUCTION:

Gamma-aminobutyric acid type-A receptors (GABA_ARs) are the major inhibitory receptor in the central nervous system (CNS) whose activation will open chloride (Cl^-) channels and hyperpolarize postsynaptic neurons. Benzodiazepines (BZs) are allosteric modulators of GABA_ARs, which can increase the affinity of GABA binding and increase the frequency of Cl^- channel opening (Macdonald and Olsen, 1994; Olsen and Leeb-Lundberg, 1981). This potentiation of inhibition makes them widely prescribed as sedatives, hypnotics and anticonvulsants. Despite the relatively high therapeutic index of BZs, their long-term use can lead to tolerance and dependence which limit their clinical utility (Griffiths and Johnson, 2005).

Tolerance is defined as the decreased responsiveness to a drug following repeated exposure. Development of BZ tolerance has been linked to dysfunction of the GABAergic system. Structural and functional changes in GABA_ARs following chronic BZ administration include decreased number of BZ binding sites, uncoupling of the allosteric linkage between GABA and BZ binding sites, plus modulation of transcription, expression and turnover of GABA_AR subunits (Bateson, 2002).

GABA_ARs belong to the cys-loop superfamily of ligand-gated channels characterized by a cysteine loop in their N-terminus, and include the GABA_C receptor, nicotinic acetylcholine receptor, serotonin receptor and glycine receptor. GABA_ARs are pentameric assemblies of 19 subunits (α1-6, β1-3, γ1-3, ρ1-3, σ, π, ε and θ) with different alternatively spliced variants (Costa et al, 2002; Macdonald et al, 1994). The
presence of a γ subunit is required for BZ anticonvulsant and hypnotic actions. BZs bind to the interface of α and γ subunits, while α1 subunit containing GABA\textsubscript{A}Rs confer the hypnotic effect and α2 subunit containing GABA\textsubscript{A}Rs mediate their anxiolytic actions (Mohler et al, 2002).

Unlike BZ tolerance, in which the proposed mechanism is related to changes in the GABAergic system, the molecular mechanisms underlying BZs dependence may involve not only changes at GABAergic synapses, but also modulation of the glutamatergic system (Allison and Pratt, 2003; Wafford, 2005). In fact, glutamate receptors have been broadly implicated in the withdrawal syndromes associated with a variety of drugs of abuse, including the BZs (Jackson et al, 2000). Pharmacological experiments with antagonists of specific glutamate receptor subtypes suggested that both alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR)-dependent mechanisms might underlie the expression of BZ dependence (Dunworth and Stephens, 1998; Koff et al, 1997; Steppuhn and Turski, 1993; Tsuda et al, 1998; Van Sickle et al, 2004; Xiang and Tietz, 2007). Transcription and expression studies of glutamate receptors in rat hippocampus and frontal cortex suggest that they are also regulated following BZ withdrawal (Allison et al, 2003; Izzo et al, 2001; Song et al, 2007; Van Sickle et al, 2002a; Van Sickle and Tietz, 2002b).

AMPAR and NMDAR are the two major subtypes of ionotropic glutamate receptors mediating excitatory neurotransmission in CNS. Both are ligand-gated cation channels composed of four subunits. All these subunits share a similar structure: four hydrophobic
transmembrane domains between the extracellular N-terminus and the intracellular C-terminus. The transmembrane domain II is a re-entrant loop and forms the wall of ion permeable pore of the receptor (Dingledine et al, 1999).

AMPARs mediate fast excitatory synaptic neurotransmission in the mammalian brain, and the strength of the transmission is regulated by multiple signaling pathways based on different internal and external stimuli (Derkach et al, 2007). Four genes encode GluR1-GluR4 subunits (Hollmann and Heinemann, 1994). In rat hippocampus, especially in the CA1/CA2 region, two major heterodimeric AMPAR are dominant: GluR1/GluR2 and GluR2/GluR3., yet about 8% percent of AMPAR composed of GluR1 homomers (Wenthold et al, 1996), which have a relatively higher Ca$^{2+}$ permeability (Thiagarajan et al, 2005) and higher channel conductance (Oh and Derkach, 2005). These homomeric GluR1 AMPARs have a critical role in activity-dependent plasticity, which refers to the long-lasting change of glutamatergic synaptic strength induced by previous neuronal activity (Derkach et al, 2007), through increased homomer insertion (Hayashi et al, 2000) and CaMKII-mediated phosphorylation of a specific amino acid, Ser$^{831}$ (Barria et al, 1997b).

In contrast, seven NMDAR subunits have been identified from three different families: NR1, NR2A-D and NR3A-B. The majority of functional NMDARs are composed of two NR1 and two NR2 subunits (Cull-Candy et al, 2001). NR2A and NR2B are the two major NR2 subunits present in hippocampus. Unlike the NR1 subunit, which has a relatively short intracellular C-terminus (about 100 amino acid residues), the NR2 subunit has a
much longer intracellular C-terminus (more than 600 amino acid residues) (Wenthold et al, 2003) which interacts with scaffold and signaling proteins in the postsynaptic area. Specifically, NR2B subunits with their long intracellular tail can bind to Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and further recruit PSD95, SynGAP, nNOS, etc, to form a signaling complex at the postsynapse (Kennedy, 2000). The functional presence of NR2B-containing NMDAR is also important for acquisition of LTP in different brain regions, including hippocampus and amygdala (Barria and Malinow, 2005; Bauer et al, 2002; Rodrigues et al, 2001).

In a well-established rat model of BZ physical dependence in our laboratory (Song et al, 2007; Van Sickle et al, 2002b; Van Sickle et al, 2004; Zeng and Tietz, 1999), withdrawal induced anxiety-like behavior, measured in the elevated-plus maze, was observed after 1-day withdrawal from 1-week oral treatment with the water soluble BZ, flurazepam (FZP). AMPAR function undergoes a progressive upregulation characterized by an increased miniature excitatory postsynaptic currents (mESPCs) in 1-day and 2-day FZP-withdrawn rats and this change was correlated with anxiety-like behavior, though only one day after ending FZP-treatment (Van Sickle et al. 2004). In contrast to the potentiation of AMPARs, NMDAR currents, both evoked EPSCs and NMDA-induced whole-cell currents were found to decrease only after 2-day FZP withdrawal. The time-course of the bidirectional functional changes in AMPAR and NMDAR currents in hippocampal CA1 neurons revealed a possible causal relationship between AMPAR upregulation and NMDAR downregulation. If the downregulation of NMDARs in 2-day FZP withdrawn
rats was prevented on day-2, anxiety could be expressed (Xiang et al, 2007). Additional experiments had revealed that systemic injection of GYKI-52466, an AMPAR antagonist, would not only prevent the potentiation of AMPARs in 1-day and 2-day FZP-withdrawn rats, but also prevent the depression of NMDAR function after 2-day withdrawal. Moreover, blockade of Ca$^{2+}$ influx through L-type voltage-gated Ca$^{2+}$ channels, but not NMDARs, could prevent anxiety (Xiang et al, 2007).

The bidirectional modulation of AMPARs and NMDARs after BZ withdrawal will be studied using our well-established animal model of BZ physical dependence. Since the enhancement of AMPAR function in CA1 neurons from FZP-withdrawn rats resembled what occurred during LTP, the levels of expression of CaMKII and its activated form Thr$^{286}$CaMKII, was hypothesized to be required for AMPAR potentiation. Increased synaptic insertion of GluR1 homomeric AMPARs and subsequent Ser$^{831}$GluR1 phosphorylation were hypothesized to be possible consequences of CaMKII activation and to lead to increased AMPAR single-channel conductance. Similar to the occlusion of LTP observed by the infusion of active CaMKII (Lledo et al, 1995), impairment of LTP was anticipated in CA1 neurons from FZP-withdrawn rats associated with an impairment of memory. Since NR2B subunits are more mobile and experienced intensive internalization (Groc et al. 2006), it was predicted that NR2B subunits would be likely to mediate the depression of NMDAR currents during FZP withdrawal. Therefore, NR2B-mediated synaptic currents and NR2B subunit expression levels were hypothesized to decrease in FZP withdrawn rats. The depression of NMDAR currents was further
hypothesized to counteract AMPAR potentiation observed in neurons from 2-day FZP withdrawn rats.

Electrophysiological recording of miniature and evoked EPSCs in the presence of a variety of pharmacological tools was used to dissect AMPAR- and NMDAR-mediated current responses to drug withdrawal and their modulation by Ca$^{2+}$-mediated signaling pathways. Immunoblot analyses were also employed to detect changes in the expression of AMPAR and NMDAR-related subunits, including GluR1, GluR2, NR1, NR2A and NR2B, in the postsynaptic density (PSD)-enriched fraction of CA1 minislices obtained by ultracentrifugation. The time course and regulation of these two subtypes of glutamate receptors was assessed, and a possible negative feedback loop between AMPAR and NMDAR was also proposed as the mechanism to maintain homeostasis of glutamatergic transmission.

Analogous to other models of activity-dependent plasticity, the first manuscript focused on the study of CaMKII-related mechanisms of increased AMPAR function during BZ withdrawal and the possible consequences for synaptic plasticity and memory. First, AMPAR-mediated miniature EPSCs (mEPSCs) were recorded and analyzed by nonstationary noise analysis (NSNA). The increase in AMPAR-mediated mEPSC amplitude and estimated single-channel conductance showed a strong similarity with mechanisms proposed to underlie activity-dependent plasticity, in such models as long-term potentiation (LTP) and fear conditioning. These findings were further supported by the elevated slope of the evoked AMPAR EPSCs input-output curve which confirmed
that AMPAR-mediated synaptic transmission was potentiated. An increase in GluR1-homomeric AMPAR-mediated synaptic currents was suggested in hippocampal slices from FZP-withdrawn rats by the increased blockade with the GluR1-specific antagonist, 1-naphthylacetyl spermine (NAS). Second, the importance of the contribution of postsynaptic CaMKII to AMPAR potentiation was shown through the reversal of AMPAR potentiation by the CaMKII inhibitors, KN-93 and AIP applied extracellularly or intracellularly, respectively. The possible mechanism by which CaMKII regulated AMPAR potentiation upon BZ withdrawal was also studied. A two-step process which involves enhanced GluR1 subunit insertion and further phosphorylation of a specific site on the GluR1 subunit might occur upon BZ withdrawal similar to that which occurs with LTP induction. Immunoblot studies in subfractionated tissues of CA1 minislices revealed the expression of GluR1 was increased in 1-day and 2-day FZP-withdrawn rats but phospho-Ser$^{831}$ GluR1, the substrate of active CaMKII shown to mediate the increased AMPAR single-channel conductance (Derkach et al, 1999), was increased only in 2-day, not 1-day FZP-withdrawn rats. Finally, the consequences of AMPAR potentiation were evaluated using additional electrophysiological approaches and behavioral methods. Namely, the expression of LTP induced with theta burst protocol was occluded, although memory tests based on a novelty preference paradigm indicated no observable deficit in FZP withdrawn rats.

The second manuscript concentrated on the mechanisms underlying the decrease in NMDAR function and its possible physiological significance. First, consistent with
previous work from our laboratory (Van Sickle et al, 2002a; Van Sickle et al, 2004) downregulation of NMDARs was shown to be mediated by NR2B-containing receptors through recordings of evoked EPSC in the presence or absence of the NR2B-specific antagonist, ifenprodil. The synaptic expression levels of different NMDAR subunits were also evaluated by immunoblot analyses of PSD-enriched fractions collected from CA1 minislices and confirmed the electrophysiological findings indicating a decline of NR2B-containing NMDAR. The reduction of NR1 and NR2B subunits and the lack of anxiety-like behavior in 2-day FZP withdrawn rats (Van Sickle et al, 2004), resembles the loss of fear conditioning by blocking NR2B-containing NMDAR (Rodrigues et al, 2001). In fact, the synaptic loss of the NR2B subunit itself might hamper NMDAR-mediated synaptic transmission, and/or the loss of the link to downstream signaling molecules, might contribute to the disappearance of anxiety in 2-day FZP withdrawn rats. Since previous findings indicated that a decrease in NMDAR currents only occurred in 2-day FZP withdrawn rats (Van Sickle et al, 2002a) and could be disrupted by pre-injection of AMPAR antagonist GYKI-52466 (Van Sickle et al, 2004; Xiang et al, 2007), the findings implied that NMDAR downregulation was secondary to AMPAR potentiation (Van Sickle et al, 2004). Preincubation of hippocampal slices from 1-day FZP withdrawn rats with AMPA was used to explore the relationship between AMPAR potentiation and depression of NMDAR function. The decreased amplitude of NMDAR-mediated evoked EPSCs, which typically required 2 days of withdrawal (Van Sickle et al, 2004; Xiang et al, 2007), emerged after 0.5 hr AMPA preincubation of slices from 1-day FZP withdrawn
rats. The synaptic transmission mediated by glutamate receptors was measured by the charge transferred during eEPSCs in slices from 2-day FZP withdrawn rats. The result revealed that a decrease in NMDAR-mediated current could counteract the enhancement of AMPAR-mediated currents during FZP withdrawal which might serve as a marker to explain the disappearance of anxiety in the 2-day FZP withdrawn rats. Based on the collective findings, Ca$^{2+}$ influx likely mediated by L-type voltage gated Ca$^{2+}$ channels (Xiang et al, 2008; Xiang et al, 2007) was proposed as a possible feedback mechanism to maintain homeostasis at CA1 neuron glutamatergic synapses.

The possible mechanisms underlying the bidirectional modulation of two types of ionotropic glutamate receptors were examined in hippocampal CA1 neurons of BZ-withdrawn rats. The findings in this dissertation study revealed an important role of glutamatergic synaptic transmission in the appearance and disappearance of anxiety-like behavior upon withdrawal from chronic BZ administration. Similar to the mechanisms underlying activity-dependent plasticity, such as LTP and fear conditioning, the modification of synaptic AMPAR and NMDAR function may serve as the common final output during drug-induced withdrawal via similar signal transduction pathways and thus may underlie BZ physical dependence.
LITERATURE REVIEW:

BZ tolerance and dependence:

Benzodiazepine and its action on GABA$_\lambda$ receptors: The GABA$_\lambda$ receptor is one member of the cys-loop superfamily of ligand-gated ion channels, which also includes the nicotinic acetylcholine receptor, the 5-hydroxytryptamine type-3 receptor, and the glycine receptor. It’s a pentameric membrane protein assembled with 7 main subunit families ($\alpha_{1-6}, \beta_{1-3}, \gamma_{1-3}, \delta, \varepsilon, \pi, \theta$) encoded by 16 different genes (Costa et al, 2002; Macdonald et al, 1994; Rudolph et al, 1999). There are three $\rho$ subunits, which constitute GABA$_C$ receptors (Olsen and Sieghart, 2008). The most abundant combination of subunits is $\alpha_1\beta_2\gamma_2$, which can be found in most brain areas, including hippocampal and cortical interneurons and cerebellar Purkinje cells (McKernan and Whiting, 1996). Each subunit has four conserved transmembrane domains with a long extracellular N-terminal domain, a long residue chain between TM3 and TM4 that forms an intracellular loop and a small extracellular C-terminal tail (Costa et al, 2002). Studies of recombinant GABA$_\lambda$ receptors indicate that the expression of the $\gamma_2$ subunit is critical for BZ sensitivity (Pritchett et al, 1989) and is required for BZ binding together with an $\alpha$ subunit (Olsen and Tobin, 1990). Different subtypes of $\alpha$ subunits mediate the diverse pharmacology of BZs. It was reported that heterooligomeric GABA$_\lambda$ receptors containing $\alpha_1$ subunits mediate the sedative, amnestic, and anticonvulsant activity of the BZs (Rudolph et al, 1999); while the anxiolytic action of BZs is mediated via $\alpha_2$-containing GABA$_\lambda$ receptors (Low et al, 2000; Rudolph et al, 1999).
The GABA<sub>A</sub> receptor is the major inhibitory receptor in the CNS. BZs can bind to a high-affinity BZ recognition site on the GABA<sub>A</sub> receptor (Mohler and Okada, 1977), facilitate GABA binding and increase the frequency of Cl⁻ channel opening in response to GABA without altering the mean duration of channel open time (Macdonald et al, 1994; Polc, 1988). Thus, BZs prolong inhibitory postsynaptic current decay and increase phasic inhibitory tone of GABAergic synapses (Nusser and Mody, 2002). Since BZs can promote GABA<sub>A</sub> receptor’s inhibition, they are widely used in clinical practice for their hypnotic, anxiolytic and anti-seizure effects.

**Tolerance and dependence of BZ:** Although BZs are quite safe in clinical use, BZs have been associated with the susceptibility to develop tolerance and physical dependence with prolonged use. Tolerance is defined as the reduction in the pharmacological effects of a drug on repeated exposure to a given dose or the need to increase the amount of drug intake to obtain the same effect. Chronic exposure to BZs, which leads to changes in GABA<sub>A</sub> receptor allosteric properties, turnover and expression, has been related to BZ tolerance (Bateson, 2002).

Since BZs function is manifested by a cooperative increase in the binding of GABA to the GABA<sub>A</sub> receptor facilitating inhibition, the uncoupling of this allosteric modulation has been proposed as a mechanism for tolerance. A number of early studies provided evidence to suggest that following chronic BZ administration to experimental animals, the ability of BZ agonist to potentiate the action of GABA was diminished in parallel to a decrease in the ability of GABA to potentiate the binding of BZ-site ligands (Bateson,
2002). Allosteric coupling between the benzodiazepine and GABA recognition sites was compromised immediately after the 4-week treatment period, but returned to normal 2 days later. There was also no effect of acute treatment on allosteric modulation as measured in rats given diazepam 30 minutes prior to sacrifice (Tietz et al, 1989). In the stably transfected mouse Ltk cell line, chronic FZP or muscimol treatments caused downregulation of BZ potentiation of muscimol-stimulated $[^{36}\text{Cl}]$ uptake (Klein et al, 1995). It’s still not clear whether the uncoupling of benzodiazepine and GABA was an acute effect (Holt et al, 1999). Still, these various findings of in vivo and in vitro studies of $\text{GABA}_A$ receptor uncoupling are highly suggestive of a role for the GABAergic system as a primary mediator of BZ tolerance.

Substance dependence is a maladaptive pattern of substance use leading to clinical problems. As a sign of physical dependence, a withdrawal syndrome is a series of physical, psychological and behavioral changes experienced when chronic drug treatment is discontinued or ceased abruptly. The symptoms of the BZ withdrawal syndrome mirror their therapeutic effects, including anxiety, insomnia, decreased threshold of seizure induction etc. For example, in a now well-established BZ dependence model in our laboratory, a withdrawal syndrome manifested as anxiety-like behavior was measured in the elevated plus-maze after withdrawal from 1-week chronic FZP administration (Van Sickle et al, 2004; Xiang et al, 2007). While the involvement of $\text{GABA}_A$Rs in BZ dependence has been proposed (Wafford, 2005), a role for changes in excitatory receptor systems mediated by AMPARs (Allison et al, 2003) and a role for L-type voltage-gated
calcium channels (VGCCs) (Dolin et al, 1990; Xiang et al, 2008) have also been suggested, but the detailed mechanisms still remain unclear.

**Glutamate receptor involvement in BZ tolerance and dependence:** There is growing evidence of neuroadaptive changes in the glutamatergic system associated with BZ withdrawal (Allison et al, 2003; Stephens, 1995; Van Sickle et al, 2004). Administration of GYKI-52466 prior to the appearance of withdrawal symptoms could prevent the subsequent development of withdrawal signs (Steppuhn et al, 1993; Van Sickle et al, 2004). Changes in expression of particular AMPA receptor subunits and regionally specific alterations in AMPA receptor subunit mRNAs was found following withdrawal from BZ associated with withdrawal anxiety in rats (Allison et al, 2003; Izzo et al, 2001). Withdrawal from 1-week oral administration of FZP, increased AMPAR-mediated mEPSCs amplitude in slices (Van Sickle et al, 2002b) and increased glutamate-induced currents in dissociated CA1 neurons (Song et al, 2007). Increased GluR1, but not GluR2 immunogold reactivity was observed at CA1 synapses using electron microscopy (EM) (Das et al, 2008). A shift in GluR1-mediated rectification index (Song et al, 2007) offered further evidence of an involvement of AMPAR regulation in BZ withdrawal behaviors. Thus, AMPAR-mediated neuronal hyperexcitability may contribute an important part to a functional anatomic circuit to mediate anxiety (Millan, 2003), which is a necessary component of the neurophysiological mechanisms underlying benzodiazepine dependence (Van Sickle et al, 2004).

Extensive studies have also demonstrated a role for NMDA receptors in tolerance and
dependence to drugs of abuse (Trujillo, 1995). It has been shown that co-administration of the competitive NMDA antagonist CPP, but not the competitive AMPA receptor antagonist GYKI-52466, to mice undergoing chronic diazepam treatment prevents the development of sedative tolerance, indicating that NMDA receptors have a role in benzodiazepine tolerance development. Administration of CPP also could prevent withdrawal symptoms (Steppuhn et al, 1993). MK-801, a noncompetitive NMDA antagonist, prevented the development of tolerance to the sedative effects, but not the anxiolytic effects of diazepam in rats (Fernandes and File, 1999; File and Fernandes, 1994). Tolerance to the anticonvulsant affects of lorazepam in mice was prevented by the NMDA receptor antagonist CPP (Koff et al, 1997). Taken together, although there are a number of pieces of evidence to suggest the involvement of NMDA receptors in expression of BZ tolerance and physical dependence following chronic BZ administration, the precise mechanism through which the NMDA receptor is involved in BZ dependence remain to be clarified.

**Glutamate receptors and anxiety:** Limbic structures, including the septo-hippocampal system, amygdala, hypothalamus and the periaqueductal gray matter are the anatomic core of the genesis of fear and anxiety (Charney and Deutch, 1996). The hippocampus and prefrontal cortex also play roles in expression of anxiety in response to contextual signals (Bergink et al, 2004; Simon and Gorman, 2006). It has been shown that GABA, 5-hydroxytryptamine (5-HT) and noradrenaline (NE) systems were involved in anxiety disorders (Bergink et al, 2004). Recently, glutamate receptors have also been implicated
in anxiety and blockade of glutamate synaptic transmission in the amygdala can alleviate anxiety (Bergink et al, 2004). Using a fear conditioning model, intra-amygdala injection of an NMDAR antagonist (Kim and McGaugh, 1992; Miserendino et al, 1990), or more specifically NR2A and NR2B selective antagonist (Walker and Davis, 2008), could reduce the anxiety response. One common pathophysiological feature of several anxiety conditions is excessive excitatory amino acid neurotransmission in response to stress. Loss of balance between GABAR-mediated inhibition and glutamate receptor-mediated excitation is one critical factor for the behavioral and physiological response associated with anxiety (Sajdyk and Shekhar, 1997a, b). The modification of glutamatergic neurotransmission in the CNS has been used as an approach to finding more effective and better tolerated treatments (Krystal et al, 2002; Mathew et al, 2001; Paul and Skolnick, 2003; Skolnick et al, 1996).

**Glutamate receptor:**

L-glutamate is the major excitatory neurotransmitter in the mammalian CNS, acting through both ligand-gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. Upon activation, these receptors mediated both basal excitatory synaptic transmission and many forms of synaptic plasticity, such as long-term potentiation (LTP) /long-term depression (LTD) and fear conditioning (Dingledine et al, 1999; Maren, 1996, 2005).

The ionotropic glutamate receptors can be further divided into three subtypes by their selectivity to agonists other than glutamate, i.e. AMPA, NMDA and KA receptors. Each
subtype of ionotropic receptor is a tetramer composed of a set of different subunits defined according to its subtype. These subunits share a similar structure: four hydrophobic transmembrane domains between the extracellular N-terminus and the intracellular C-terminus. The transmembrane domain II is a re-entry loop and forms the wall of ion permeable pore of the receptor (Dingledine et al, 1999).

AMPA receptors mediate fast excitatory synaptic transmission in CNS, whereas NMDA receptors, whose response to the ligand binding is much slower compared with AMPAR, are crucial for the induction of specific forms of synaptic plasticity and play important roles in several neuropsychiatric disorders (Song and Huganir, 2002). KA receptors are less studied, compared with the other two subtypes of glutamate receptor.

**AMPA receptor structure:** AMPA receptors are composed of four types of homologous subunits GluR1–4, products of four separate genes (Hollmann et al, 1994). The subunit composition of AMPA receptors varies based on differences in cell type, developmental stage and brain region. Two dominant heterodimers of AMPAR were found in adult hippocampus: GluR1/GluR2 heterodimer and Glu2/GluR3 heterodimer (Wenthold et al, 1996). Immature hippocampus, as well as mature brain regions, express the GluR4 subunit, which could form the functional heteromeric AMPAR with GluR2 (Zhu et al, 2000). Although both the extracellular domain and transmembrane domain of AMPAR subunits are of high homology, their intracellular cytoplasmic tails are quite different. Two categories of subunit can be distinguished based on the length and homology of their intracellular domain. GluR1, GluR4, and an alternatively spliced form of GluR2 (GluR2L)
have longer cytoplasmic tails. In contrast, the predominant splice form of GluR2, GluR3, and an alternatively spliced form of GluR4, primarily expressed in cerebellum (GluR4c) have shorter, homologous cytoplasmic tails. Through their C-terminal tails, each subunit interacts with specific sets of cytoplasmic proteins. Characterized by protein-protein interaction, most of these AMPAR-interacting proteins have single or multiple PDZ (PSD/DiscLarge/Zonula occludens) domains, which often interact with the extreme C-terminal tails of target proteins (Sheng and Sala, 2001). GluR1 is a group I PDZ ligand, whereas GluR2, GluR3, and GluR4c are categorized as group II PDZ ligands. GluR4 and GluR2L have variant C-terminal tails, whether they interact with classical PDZ-domain proteins is not clear. In a variety of cell types, proteins containing PDZ domains have been implicated in playing an important role in the targeting and clustering of membrane proteins to specific subcellular domains (Sheng et al, 2001). AMPARs can also interact with the actin cytoskeleton via band 4.1N through its homologous region in GluR1 subunit (Shen et al, 2000). These AMPAR-interacting proteins, either contain PDZ domains or interact with the actin cytoskeleton, are implicated in membrane fusion or in controlling the trafficking of AMPARs and/or their stabilization at synapses.

**Q/R editing and calcium permeability of AMPARs:** RNA editing describes a variety of RNA transcript modification phenomena. The nucleotide of messenger RNA is changed through several different enzymatic mechanisms (Simpson and Emeson, 1996). Mammals have several adenosine deaminases that can hydrolyze one base from adenosine and turn it to inosine. For example, upon A-to-I editing the CAG codon for
glutamine (Q) becomes CIG, now coding for arginine (R). Q/R editing from uncharged glutamine (Q607) to positively charged arginine (R) at TM2, a posttranscriptional modification of GluR2 subunit mRNA, turns off the calcium permeability of the GluR2 subunit (Sommer et al, 1991). Since the Q/R site of the GluR2 subunit is edited virtually 100% (Seeburg et al, 2001), the presence of a GluR2 subunit will significantly reduce the calcium permeability of GluR2-containing AMPARs. So the GluR2 is a dominant subunit in determining the biophysical properties of the AMPAR channel (Dingledine et al, 1999). Channels containing GluR2 subunits have a linear current–voltage relationship (Boulter et al, 1990) and are impermeable to Ca$^{2+}$, whereas those lacking GluR2 are Ca$^{2+}$ permeable and show inward rectification due to a voltage-dependent block by endogenous polyamines (Bowie and Mayer, 1995; Geiger et al, 1995; Kamboj et al, 1995). In rat hippocampus, especially in the CA1/CA2 region, two major heterodimeric AMPARs are dominant: GluR1/GluR2 and GluR2/GluR3. Only about 8% percent of AMPARs are composed of GluR1 homomers (Wenthold et al, 1996). These AMPARs composed of homomers of GluR1 show calcium permeability and inward rectification (Thiagarajan et al, 2005). Interestingly, a group of compounds, polyamine and its derivatives, either endogenous intracellular spermine or spermidine or exogenous spider or wasp toxins, polyamine toxins, can bind to AMPAR intracellularly or extracellularly. The inward rectification property of GluR2-lacking AMPARs is related to the presence of a certain concentration of intracellular polyamine, which will block the channel pore in a voltage-dependent manner (Stromgaard and Mellor, 2004). Since Q/R editing renders
AMPAR a positive charged R in its channel pore, it will hamper the access of polyamine. That’s why GluR2-containing AMPAR don’t show inward rectification (Stromgaard et al, 2004). As with polyamine, the polyamine toxins are non-competitive channel blockers which manifest use- and voltage-dependent blockade (Stromgaard et al, 2004). They can selectively block calcium permeable AMPARs or KARs, which make them very useful pharmacological tools. The derivatives of spermine toxin, such as 1-naphthylacetyl spermine (NAS), are also useful pharmacological tools for distinguishing GluR1 homomeric AMPAR (Koike et al, 1997). Another important thing to consider is that NAS also shows some effect on NMDAR blockade (Stromgaard et al, 2004).

**AMPAR phosphorylation:** The phosphorylation of AMPARs is crucial for the regulation of AMPAR function. Early conclusions regarding the role of phosphorylation were drawn by manipulating the activity of protein kinases in neurons and analyzing the resulting changes in AMPAR function or in excitatory synaptic transmission. Intracellular application of peptides that inhibit PKA (cAMP-dependent protein kinase) was shown to inhibit AMPAR-mediated currents in cultured hippocampal neurons (Greengard et al, 1991; Rosenmund et al, 1994), intracellular perfusion of constitutively active CaMKII (Ca$^{2+}$/calmodulin-dependent protein kinase II) or PKM (a constitutively active form of protein kinase C) was shown to increase AMPAR-mediated synaptic responses (McGlade-McCulloh et al, 1993; Wang et al, 1994). We now know that all 4 subunits of AMPARs have several identified phosphorylation sites on their intracellular C-termini that regulate their function, and many more are currently being characterized.
and are involved in mediating various forms of synaptic plasticity (Song et al, 2002). For example, PKC can phosphorylate GluR1 at Ser\textsuperscript{818} and mediate the synaptic incorporation of the GluR1 subunit (Boehm et al, 2006); PKC can also phosphorylate Ser\textsuperscript{880} of GluR2, and this phosphorylation can interfere with the binding of GluR2 to PDZ-domain–containing proteins such as GRIP (glutamate receptor interacting protein) and PICK-1 (protein interacting with C kinase) (Chung et al, 2000; Matsuda et al, 1999). Interestingly, the binding of GluR2 with PICK-1 will lead to its rapid internalization (Perez et al, 2001).

Among all the phosphorylation sites of AMPAR subunits, Ser\textsuperscript{831} and Ser\textsuperscript{845} of the GluR1 subunit are the most intensively studied and have been well-documented to modulate AMPAR function. Ser\textsuperscript{845} of GluR1 was shown to be a substrate of PKA, since the C-terminus of the GluR1 (S845A) mutant could not be phosphorylated after incubation with PKA (Roche et al, 1996). Moreover, increased phosphorylation of Ser\textsuperscript{845} was detected by immunoblotting in hippocampal slices after forskolin treatment (Mammen et al, 1997; Oh et al, 2006); and PKA activation appears to increase the open probability of recombinant GluR1 receptors but not of GluR1 (S845A) mutant receptors (Banke et al, 2000). PKA can increase channel open probability (Banke et al, 2000) or open time (Greengard et al, 1991) in cultured neurons. Changes in the phosphorylation status of Ser\textsuperscript{845} is specifically associated with synaptic plasticity, which may be mediated by increased surface trafficking of AMPAR (Ehlers, 2000; Esteban et al, 2003; Oh et al, 2006). Ser\textsuperscript{831} can be phosphorylated by both PKC and CaMKII. Purified PKC can directly phosphorylate
Ser\textsuperscript{831} of GluR1 in transfected HEK cells (Roche et al, 1996). But the physiological significance of this phosphorylation was not well defined. Another calcium dependent protein kinase, CaMKII, also can phosphorylate Ser\textsuperscript{831} of GluR1 (Barria et al, 1997b). This phosphorylation was correlated with the activation and autophosphorylation of CaMKII, and was blocked by the CaMKII inhibitor KN-62. \textsuperscript{32}P-Isotope-labeled phosphopeptide mapping also showed the direct phosphorylation of Ser\textsuperscript{831} (Barria et al, 1997b). This property of CaMKII makes it the perfect molecule to mediate LTP which will be discussed later.

**NMDA receptor structure and properties:** NMDARs exhibit remarkable properties that distinguish them from other types of ligand-gated ion channels: 1) The ion channel is subject to a voltage-dependent block by physiological levels of extracellular Mg\textsuperscript{2+}. Relief from Mg\textsuperscript{2+} blockade at depolarized potentials provides NMDAR the capacity to be a molecular coincidence detector, permitting cations to flow only when pre- and postsynaptic cells are excited simultaneously. 2) Activation of NMDARs requires not only the presence of glutamate, but also the co-agonist glycine which binds at the interface between the NR1 and NR2 subunits. 3) Upon activation, its high calcium permeability and slow kinetic properties may lead to a large calcium influx to the postsynaptic region and trigger a series of downstream signaling cascades, leading to LTP/LTD, excitotoxicity, etc. (Cull-Candy and Leszkiewicz, 2004). Seven NMDAR subunits have been identified from three different families: NR1, NR2A-D and NR3A-B. Similar to the AMPAR, the functional NMDAR is a tetramer composed of two NR1
subunits and two NR2 subunits. There are eight isoforms of NR1 arising from one single gene. These are generated by the insertion of exon 5 in the N terminus (N1 cassette), the deletion of exon 21 (encode C1 cassette) in the C terminus, and use of an alternative splice acceptor site of exon 22 (encode C2 cassette) in the C terminus. Exon 22 (C2) contains an alternate acceptor splice site that can generate one different reading frames for C2’ cassette (Dingledine et al, 1999). Three exons (C0, C1, and C2) encode the NR1 intracellular C terminus. All NR1 subunits contain the invariant C0; the C1 and C2 cassettes are alternatively spliced in a developmentally regulated manner (Zukin and Bennett, 1995). The C0 cassette, which is next to TM4 of NR1 subunit, was the target of binding with either calcium/calmodulin or α-actinin, which plays an important role of calcium-dependent inactivation of NMDAR (Wyszynski et al, 1997; Zhang et al, 1998). The type of NR1 splice variant in the receptor assembly is critical in influencing certain key features, such as inhibition by protons, potentiation by polyamines, and inhibition by Zn$^{2+}$. The C1 cassette is involved in receptor clustering, for example, it binds to neurofilaments and the intracellular protein yotioa (Ehlers et al, 1998; Lin et al, 1998). Furthermore, the C1 cassette contains protein kinase C (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). After kindling a transient reduction of C1-containing splice variants was found in rats (Vezzani et al, 1995), but whether the expected functional receptor alterations contribute to the kindled state is unknown.
On the other hand, the identity of the NR2 subunits is critical in determining many biophysical and pharmacological properties of the receptor—including its high affinity for glutamate, modulation by glycine, sensitivity to Mg$^{2+}$, fractional Ca$^{2+}$ current, and channel kinetics (Cull-Candy et al, 2004). Both recombinant (Vicini et al, 1998; Wyllie et al, 1998) and native NMDARs (Cathala et al, 2000; Misra et al, 2000) showed different deactivation times to a brief glutamate application, from fastest to slowest: NR2A < 2C = 2B << 2D. Patch-clamp studies of native and recombinant NMDARs have demonstrated that two other functionally important properties—single-channel conductance and blockade by Mg$^{2+}$ ions—also depend on subunit composition (Brimecombe et al, 1997; Cull-Candy et al, 2001; Farrant et al, 1994; Momiyama et al, 1996; Stern et al, 1994; Takahashi et al, 1996). The high conductive 50 pS openings that display a high sensitivity to Mg$^{2+}$ block are associated with NR2A- or NR2B-containing receptors, whereas low-conductance (~35 and 18 pS) openings, with a low sensitivity to Mg$^{2+}$, arise from NR2C- and NR2D-containing receptors (Farrant et al, 1994; Wyllie et al, 1998). The various responses of NR2 subunits to pharmacological agents provide useful tools for the study of NMDAR. The best characterized of these compounds is ifenprodil, a phenylethanolamine, whose IC$_{50}$ is about 400-fold lower for NR2B- than for NR2A-, NR2C- or NR2D-containing receptors (Williams, 1993). Ifenprodil suppresses the activation of NMDARs containing the NR2B subunit by enhancing their sensitivity to inhibition by protons (Mott et al, 1998). NR2A-containing receptors can also be identified with the Zn$^{2+}$ chelator N,N,N$'$,N$''$-tetakis-[2-pyridylmethyl]-ethylenediamine
(TPEN), which enhances the NMDAR response by removing the tonic inhibition caused by low levels of Zn$^{2+}$ present in experimental solutions (Paoletti et al, 1997). By selectively potentiating responses from NR2A-containing receptors, TPEN provides a convenient distinction between NR2A- and NR2B-containing NMDARs (Misra et al, 2000; Tovar et al, 2000).

**NMDAR distribution and trafficking:** More and more evidence shows that extrasynaptic and synaptic NMDARs have different subunits composition in some cells. For example, NR2A and NR2B subunits target preferentially to synaptic and extrasynaptic locations, respectively, in a variety of cells (Rumbaugh and Vicini, 1999; Stocca and Vicini, 1998). The origin and functional significance of these differences in subunit targeting remain unclear. But some studies may offer clues about the cause of this difference. Lavezzari et al. showed that NR2A and NR2B used different endocytosis motifs in their distal C-termini and with different affinity to bind with its adaptor protein. And in the 12 days in vitro (DIV) neuronal culture, NR2B had an increased internalization compared with NR2A (Lavezzari et al, 2004). With single molecular imaging, Groc showed the surface mobility of NMDARs depends on the NR2 subunit subtype, with NR2A-containing NMDARs being more stable than NR2B-containing ones. NR2A subunit overexpression stabilized surface NR2B-containing NMDARs (Groc et al, 2006). Studies on organotypic slices and dissociated neurons have shown that NMDAR trafficking and integration at synaptic sites is dictated by neuronal activity, and the level of NMDAR activation itself plays a crucial role (Barria and Malinow, 2002;
Chen and Bear, 2007; Rao and Craig, 1997). Synaptic NMDARs are the key molecules in the postsynaptic area, where they are structurally organized into a large macromolecular signaling complex with synaptic scaffolding and adaptor proteins which are physically adjacent or link the receptors to kinases, phosphatases and other downstream signaling molecules (Lau and Zukin, 2007). The NMDAR stay relatively stable in the synapse, in contrast to the rapid turnover of a synaptic AMPARs (Bredt and Nicoll, 2003). For example, the half-life of NMDARs measured in cerebellar granule cells and cortical neurons in culture by pulse-chase receptor labeling and surface biotinylation was estimated as about 20h for NR1, NR2A and NR2B subunits (Ehlers, 2003; Huh and Wenthold, 1999). Disruption of endocytosis and exocytosis have no effect on NMDAR-mediated EPSCs suggesting that NMDAR are only slowly mobilized at synapses (Luscher et al, 1999), characterized by both less lateral motility (Groc et al, 2004) and modest activity-dependent internalization (Ehlers, 2000; Lin et al, 2000). However, they can still undergo constant movement in cell-specific and synapse-specific manner during development and in response to neuronal activity or sensory experience (Lau and Zukin, 2007). NMDAR-mediated EPSCs showed an anomalous recovery following non-competitive MK-801 block. The recovery from blockade could not be attributed to MK-801 unbinding or insertion of new receptors, suggesting that membrane receptors move laterally into the synapse (Tovar and Westbrook, 2002). The targeting of the NMDAR to the synapse and its localization at the synapse depend on a series of interactions with other proteins, many of these interactions involve the NMDAR’s long carboxyl terminal
domain. NR2 subunits have much longer C-termini compared with NR1 subunits. For example, the NR2A subunit has 627 amino acids while the NR1-1 subunit has 105 amino acids (Wenthold et al, 2003), NR2 subunits may play a more important role in anchoring NMDAR at the postsynapse. In fact, synaptic expression of NMDAR was affected by its assembly, trafficking, targeting and anchoring. NR1 and NR2 play distinct roles in the processes of intracellular trafficking and surface expression of NMDAR. The NR1/NR2 complex can assemble before leaving the endoplasmic reticulum (ER), although specific individual subunits can be exported from the ER on their own. The exocytosis signal of NR1 subunit is critical in the release of the NMDAR from the ER. And the conditional knockout of NR1 subunit will lead to retention of NR2 within the ER and dramatically decrease the dendritic expression of NR2A and NR2B subunits (Fukaya et al, 2003). The overall activity of a neuron can change the surface expression pattern of NMDARs by modifying their export from the ER (Mu et al, 2003). It should be kept in mind that although NR2A and NR2B subunits are not able to be transported to the cell surface unless it assembles into a functional NMDAR with NR1, most splice variants of NR1 can reach the cell surface in the absence of NR2 (Scott et al, 2001; Xia et al, 2001). In contrast, NR2 appears to be more important in later steps of NMDAR delivery to the cell membrane (including the synaptic site). Distinct interactions between different dendritic and postsynaptic proteins with the NR2 subunit are critical for dendritic transport, synaptic localization, and trafficking of receptors, and finally will affect its distribution and function (Wenthold et al, 2003). The surface expression is not only determined by
delivery, but also by internalization. Roche et al found internalization of NR2B subunits can be initiated by the interaction of a YEKL motif in its C terminus with components of the endocytic machinery (Roche et al, 2001). Phosphorylation of this Tyr1472 in NR2B C-terminal by Src family kinase Fyn (Nakazawa et al, 2001) can block the binding of AP-2 (Bonifacino and Traub, 2003; Owen and Evans, 1998) and consequently increase the number of synaptic NMDAR (Prybylowski et al, 2005). Glycine binding to the NMDARs, without receptor activation, will also initiate the internalization of the receptor, decreasing NMDAR-mediated whole-cell responses (Nong et al, 2003). The membrane-associated guanylate kinases family (MAGUKs, which include PSD-93, PSD-95, SAP-97, SAP-102, and chapsyn-110), contains PDZ domains responsible for binding and stabilizing NMDARs in the plasma membrane (Sheng et al, 2001).

**Calcium-dependent inactivation of NMDAR:** The activity of NMDAR channels is negatively modulated by intracellular Ca\(^{2+}\). Three main modulatory effects of calcium on NMDAR channels have been described: 1) irreversible inhibition or run-down; 2) irreversible acceleration of glycine-independent desensitization (Tong and Jahr, 1994; Vyklicky, 1993); and 3) reversible (transient) inactivation, a phenomenon which is independent of the activation of NMDA receptor (Legendre et al, 1993; Medina et al, 1994; Vyklicky, 1993). Postsynaptic depolarizing pulses transiently inactivated NMDAR-mediated EPSCs and could be suppressed by removal of extracellular Ca\(^{2+}\) or cadmium, a voltage-gated calcium channel blocker (Medina et al, 1999). Ca\(^{2+}\) regulation of NMDARs has been postulated to involve a linkage between the receptor and the
cytoskeleton since prevention of actin depolymerization could block the Ca$^{2+}$-induced NMDAR rundown and vice-versa (Rosenmund and Westbrook, 1993). This regulation may be mediated through activation of various Ca$^{2+}$-dependent proteins, including calmodulin (Zhang et al, 1998), calcineurin, PKC, and α-actinin-2 (Rycroft and Gibb, 2002). The inactivation was proposed to occur after C0 cassette dissociates from α-actinin by two distinct but converging calcium dependent processes: competitive displacement of α-actinin by calmodulin and reduction in the affinity of α-actinin for C0 after Ca$^{2+}$ binding to α-actinin (Krupp et al, 1999). α-Actinin-2, a member of the spectrin/dystrophin family of actin-binding proteins, is identified as a brain postsynaptic density protein that colocalizes in dendritic spines with NMDA receptors and the NMDA receptor-clustering molecule PSD-95. α-Actinin-2 can bind by its central rod domain to the C-termini of both NR1 and NR2B subunits of the NMDA receptor, and can be immunoprecipitated with NMDA receptors and PSD-95 from rat brain. Intriguingly, NR1-α-actinin binding is directly antagonized by Ca$^{2+}$/calmodulin (Wyszynski et al, 1997). Zhang et al. demonstrated that the binding of Ca$^{2+}$/calmodulin to NR1 mediates CDI of the NMDA receptor and suggest that inactivation occurs via Ca$^{2+}$/calmodulin-dependent release of the receptor complex from the neuronal cytoskeleton (Zhang et al, 1998).

**Synaptic plasticity: from molecules, cells to behavior:** Although Hebb proposed his famous hypothesis about use-dependent synaptic modification 60 years ago, the phenomena of long-term potentiation was only discovered in the early 70’s (Bliss and...
Lomo, 1973). As one form of synaptic plasticity most extensively studied, LTP and long-term depression (LTD) are characterized by a long-lasting modification of synaptic strength that is elicited by a brief period of coordinated neuronal activity. It was widely accepted that postsynaptic changes of AMPAR function act as common output pathway for the expression of LTP/LTD, and its function is under tight control by serial upstream pathways, from membrane proteins (NMDARs (Bear and Malenka, 1994), VGCCs (Ito et al, 1995), mGluRs (Riedel and Reymann, 1996), etc), scaffold and anchoring proteins (AKAP (Genin et al, 2003), MAGUK (Dell'Acqua et al, 2006), etc) to many kinds of kinases and phosphotases (PKA (Esteban et al, 2003), CaMKII (Lisman et al, 2002), and calcineurin (Xia and Storm, 2005), etc). Simply speaking, LTP could involve the modulation of AMPARs that are already expressed at the synapse and/or the rapid new recruitment of AMPARs to the synapse to alter the number present (Benke et al, 1998). The increased function of AMPARs includes increased AMPA binding with autoradiography (Maren et al, 1993), increased AMPAR-mediated EPSC amplitude (Isaac et al, 1998), and increased single-channel conductance (Benke et al, 1998). The dual ligand and depolarization-dependent activation of NMDARs makes the receptor a good co-incidence detector for Ca^{2+} influx (Yuste et al, 1999) during the induction of LTP. LTP is regarded as an important mechanism for learning and memory. A recent study also showed a solid link between this form of cellular plasticity and memory behavior. Whitlock et al. showed that inhibitory avoidance learning in rats produced the same changes in hippocampal glutamate receptors as high frequency induction of LTP.
And this avoidance learning procedure caused enhanced synaptic transmission in CA1 in vivo (Whitlock et al, 2006). In fact, another well-studied activity-dependent plasticity model, fear-conditioning, also showed a link between glutamate facilitation within the amygdala (Maren, 1996, 2005; McKernan and Shinnick-Gallagher, 1997). Fear conditioning is established by combining an unconditioned stimulus (i.e. electric shock), which can generate a certain degree of unconditional response such as startle, with a conditioned stimulus (i.e. a certain mild sound or light). After fear conditioning is established, the conditioned stimulus can also generate an unconditioned response. Two pathways may be involved in the development of fear conditioning: afferent fibers through thalamo-amygdala synapses undergo postsynaptic modification mediated by activation of NMDAR (Rodrigues et al, 2001) and L-type VGCC (Weisskopf et al, 1999) pathways. Cortico-amygdala synapses also show a change in presynaptic neurotransmitter release (Maren, 2005; McKernan et al, 1997). Recent studies showed that fear conditioning can lead to a rise of GluR1 homomeric AMPAR incorporation in lateral amygdala (Rumpel et al, 2005), similar to mechanisms underlying LTP (Hayashi et al, 2000).

**CaMKII and synaptic plasticity:** Calcium/calmodulin dependent protein kinase II (CaMKII) is the most abundant protein (about 1-2% of total brain protein) present in the postsynapse (Merrill et al, 2005). The CaMKII holoenzyme is a large complex formed by 12 subunits. There are two main isoforms in the mammalian brain, CaMKIIα and CaMKIIβ, which can form mixed α-β heterododecameric assembly (Lisman et al, 2002).
Each subunit consists of (from N to C terminal) a catalytic domain, an autoinhibitory domain, a variable segment and a self-association domain. The catalytic domain has ATP and substrate binding sites (s site) for a phosphotransfer reaction. The autoinhibitory domain has the pseudosubstrate site with similar amino acid sequence of CaMKII’s substrate. In the inactive state, the autoinhibitory domain binds with catalytic domain turning off the catalytic effect. Calcium/calmodulin can bind to the autoinhibitory domain at the pseudosubstrate region and dissociate binding between autoinhibitory domain and catalytic domain. It will not only turn on catalytic activity, but also expose Thr\(^{286}/287\) (α/β isoform, respectively) which can be phosphorylated by adjacent subunits. The self-association domain is located on the C-terminus. It helps to assemble hexameric ring and stack two rings into a dodecameric homoenzyme (Lisman et al, 2002). CaMKII can be activated to different degrees dependent on the magnitude of the Ca\(^{2+}\) signal. A weak Ca\(^{2+}\) signal can activate CaMKII without autophosphorylation but will be inactivated in less than 1 second. When the Ca\(^{2+}\) signal is great enough to induce autophosphorylation of Thr\(^{286}/287\), CaMKII will remain active even when Ca\(^{2+}\) returns to basal levels until the Thr\(^{286}\) is dephosphorylated by phosphatases. Persistent activation of CaMKII can occur when the rate of autophosphorylation exceeds the rate of dephosphorylation of Thr\(^{286}/287\) (Lisman et al, 2002). The ability to turn a short pulse of Ca\(^{2+}\) into long lasting enzymatic activity makes CaMKII an excellent candidate to mediate LTP. At many excitatory synapses, LTP is triggered by Ca\(^{2+}\) entry into the postsynaptic cell. CaMKII is believed to be the molecule to detect this Ca\(^{2+}\) elevation turning the short-term Ca\(^{2+}\) signal into long
lasting potentiation of synaptic transmission (Lisman et al, 2002). Interestingly, NR2B subunit of NMDAR can bind with autophosphorylated CaMKII and keep it in active form, even in the absence of Ca\(^{2+}\) and promote synaptic translocation of CaMKII (Bayer et al, 2001; Bayer et al, 2006; Strack and Colbran, 1998; Tsui and Malenka, 2006). Among all the substrates of CaMKII, GluR1 can be phosphorylated by endogenous kinases in the presence of Ca\(^{2+}\) and calmodulin (Hayashi et al, 1997) or by exogenous, constitutively active CaMKII (Barria et al, 1997a; Barria et al, 1997b). The phosphorylation site of GluR1 by CaMKII was identified as Ser\(^{831}\) and plays an important role in synaptic plasticity: only mutation affecting this amino acid altered the \(^{32}\)P phosphopeptide map of GluR1 from HEK293 co-expressed with active CaMKII; this phosphorylation site is specific to GluR1, as CaMKII did not phosphorylate or potentiate currents in cells expressing GluR2 subunits; once Ser\(^{831}\) was mutated to Ala, the potentiation also disappeared (Barria et al, 1997a). Co-expression 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 revealed CaMKII can increase the single channel conductance of 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). Indirect evidence also showed that CaMKII application or transfection could reduce the probability of synaptic failures (Lledo et al, 1995) and “unsilence” silent synapse (Wu et
al, 1996). Direct evidence showed that CaMKII, also acts in parallel with PKA, to enhance GluR1 synaptic incorporation (Esteban et al, 2003). This membrane insertion was believed to be mediated by Ras small GTPase relayed by the NMDAR-CaMKII pathway (Zhu et al, 2002). On the contrary, lack of CaMKII can lead to loss of LTP and spatial memory (Silva et al, 1992a; Silva et al, 1992b), which lends strong support for CaMKII’s contribution to LTP. The most eloquent experiment about CaMKII’s mediation of LTP was conducted by Pettit et al. Their results indicated that active CaMKII not only can potentiate synaptic transmission, but also can occlude further potentiation (Lledo et al, 1995; Pettit et al, 1994).

**Drug abuse and synaptic plasticity:** The central nervous system exhibits a high adaptive ability in response to internal and external stimuli. It makes the brain not only perfect for learning and memory, but also vulnerable to drug abuse. The development of drug addiction may share common features with traditional learning models. In fact, synaptic plasticity has been shown to be involved in neural reward circuitry and the acquisition of addictive behavior related to dopaminergic and glutamatergic systems (Jones and Bonci, 2005; Kelley, 2004). Excitatory synaptic function within the limbic system, especially the ventral tegmental area (VTA) and nucleus accumbens (NAc) dopamine circuits, are the two major targets of the neural adaptations that underlie addiction (Kauer and Malenka, 2007). Drugs of abuse have profound effects on dopamine and glutamate signal transduction. Dopamine was regarded as the key player for drug addiction, but a recent study showed glutamate was also involved either in the addiction
or in learning and other adaptive processes (Jones et al, 2005). A lot of evidence demonstrated that the exposure to abusive drugs could induce quick and persistent synaptic plasticity in both VTA and NAc (Jones et al, 2005; Kalivas et al, 2009; Kalivas and O'Brien, 2008; Kauer et al, 2007; Kelley, 2004). For example, a single dose of cocaine could cause a long lasting increase in the AMPAR/NMDAR ratio of eEPSC amplitude, which was used as an indicator of synaptic strength (Ungless et al, 2001). Nicotine, amphetamine, alcohol and morphine could induce similar long lasting potentiation in VTA dopaminergic neurons (Kauer et al, 2007). The potentiation of excitatory synapse in dopaminergic neurons may initiate the incentive effects and lead to more important downstream circuitry outputs (Kauer et al, 2007). In NAc, synaptic depressions are more prevalent in excitatory synapses. For instance, chronic cocaine administration could cause the decrease of AMPAR/NMDAR ratio 10-14 days after the withdrawal. Drug-induced depression may contribute to the long lasting sensitized behavior responses generated by previous drug exposure (Kauer et al, 2007).

The long lasting change of excitatory glutamate synapses in dopamine neurons has physiological impact on drug addiction. The glutamatergic synaptic inputs to the VTA dopaminergic neuron may further affect the dopaminergic neurons output. The VTA dopaminergic cells exhibit two major firing patterns in vivo: the irregular single spike firing, and burst firing. The burst firing of VTA dopaminergic neurons encodes the occurrence of salient reward-associated stimuli. The fine tuning of dopaminergic fire pattern and increased dopamine release would enhance the reward related circuitry and
promote addiction (Jones et al, 2005). And a therapeutic role of regulating glutamate mediated synaptic plasticity was proposed to alleviate the development and expression of addictive behavior (Kalivas et al, 2009).
Calcium/calmodulin dependent protein kinase II activity contributes to enhancement of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor currents mediated by GluR1 homomers during benzodiazepine withdrawal

Abbreviated Title: **CaMKII involvement in benzodiazepine-induced plasticity**

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Abstract:

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) currents are enhanced in hippocampal CA1 pyramidal neurons during withdrawal from 1-week oral benzodiazepine, flurazepam (FZP) administration, related to withdrawal-anxiety and attributable to increased GluR1 subunit-containing AMPARs. As with models of activity-dependent plasticity, the contribution of CaMKII to enhance glutamatergic synaptic strength during drug withdrawal was examined in hippocampal slices from FZP-withdrawn rats. AMPAR-mediated miniature excitatory postsynaptic current (mEPSC) amplitude was increased 30% in CA1 neurons with a 2-fold increase in single-channel conductance. Pre-incubation of slices with the CaMKII inhibitor, KN-93, or intracellular inclusion of autocamtide-2 related inhibitory peptide (AIP) prevented both phenomena. Increased 1-naphthylacetyl spermine inhibition was consistent with synaptic incorporation of homomeric GluR1 AMPARs. In 1-day withdrawn rats, only GluR1 levels were increased in immunoblots of the postsynaptic density (PSD)-enriched subfraction from CA1 minislices consistent with increased mEPSC amplitude, but not conductance. In 2-day withdrawn rats, total, but not relative phospho-Thr286 CaMKII levels increased in the PSD-enriched subfraction in parallel with increased GluR1 and phospho-Ser831 GluR1 expression levels implying that CaMKII mediates AMPAR phosphorylation and increased channel conductance in FZP-withdrawn CA1 neurons. Whole-cell input-output curves revealed a parallel enhancement of synaptic efficacy. Whole-cell and field (f)EPSP recordings revealed that LTP expression, induced by low-
intensity theta burst stimulation was impaired in CA1 neurons from FZP-withdrawn rats though no memory deficits were detectable using a novelty preference paradigm. Collectively, the findings suggest that synaptic insertion and subsequent CaMKII-mediated phosphorylation of homomeric GluR1 AMPARs might contribute to BZ withdrawal-induced potentiation of AMPARs analogous to mechanisms underlying activity-dependent plasticity.

Keywords: benzodiazepine withdrawal, CaMKII, AMPA receptor, long-term potentiation, drug dependence
Introduction:

Benzodiazepines, allosteric modulators of the GABA_A receptor, are safe, well-tolerated and effective therapies for anxiety, insomnia and seizures, yet carry some abuse liability and signs of physical dependence may emerge after long-term use (Griffiths and Johnston, 2005).

While BZs tolerance has been associated primarily with GABA_A receptor regulation (Bateson, 2002), mechanisms involving both GABAergic and glutamatergic systems have been proposed to underlie manifestations of BZs dependence (Allison et al, 2003; Wafford, 2005). Moreover, pharmacological approaches using specific glutamate receptor antagonists to modify withdrawal phenomena suggested that activation of excitatory amino acid receptors might be central to the expression of BZs physical dependence (Dunworth et al, 1998; Koff et al, 1997; Van Sickle et al, 2004; Xiang et al, 2007).

Abrupt withdrawal from prolonged benzodiazepine administration results in potentiation of AMPAR-mediated synaptic currents associated with withdrawal anxiety (Van Sickle et al, 2002b; Xiang et al, 2007). The amplitude of AMPAR-mediated miniature (m)EPSCs is progressively increased in hippocampal CA1 pyramidal neurons from 1-day and 2-day flurazepam (FZP)-withdrawn rats associated with a localized increase in AMPAR binding (Van Sickle et al, 2002b; Van Sickle et al, 2004). Immunofluorescence and immunoblot studies revealed that GluR1 subunit expression is increased in CA1 neurons from 2-day FZP-withdrawn rats consistent with an increase in
macroscopic glutamate-elicited currents (Song et al, 2007). Postembedding immunogold electron microscopy confirmed an increased incorporation of GluR1-containing AMPARs into CA1 neuron synapses, without an increase in GluR2 immunoreactivity (Das et al., 2008).

The GluR1 subunit, one of two major hippocampal AMPAR subunits, has a long cytoplasmic carboxyl terminus, which undergoes phosphorylation. Phosphorylation-dependent GluR1 trafficking contributes to AMPAR-mediated activity-dependent synaptic plasticity (Derkach et al., 2007). As with long-term potentiation (LTP), increased mEPSC amplitude may stem from increased AMPAR numbers. In particular a transient increase homomeric GluR1 AMPARs (Plant et al, 2006) can be detected as both a shift in the current rectification index in the presence of spermine analogues (Washburn and Dingledine, 1996) and by an alteration in AMPAR single-channel properties (Benke et al, 1998; Luthi et al, 2004). Calcium/calmodulin dependent protein kinase II (CaMKII), one of the most abundant proteins in the postsynaptic density (PSD), is required for membrane incorporation of GluR1 subunit-containing AMPARs (Esteban et al, 2003), and subsequent phosphorylation of GluR1-subunits at Ser$^{831}$ enhancing single-channel conductance of GluR2-lacking AMPARs (Derkach et al, 1999; Oh et al, 2005).

To further explore the contribution of CaMKII activation in mediating AMPAR potentiation during benzodiazepine withdrawal, mEPSCs were recorded in CA1 pyramidal neurons in hippocampal slices from FZP-withdrawn rats to evaluate mEPSC amplitude and kinetic parameters and to estimate AMPAR single-channel conductance in
the presence and absence of extracellular and intracellular CaMKII inhibitors. AMPAR-mediated synaptic current rectification was evaluated by extracellular application of a potent spermine analogue. Total and phosphorylated CaMKII and GluR1 protein levels were detected and quantitated by immunoblot analysis in a PSD-enriched subfraction of the CA1 region. Since constitutive activation of CaMKII underlies LTP induction and maintenance (Barria et al., 1997b; Sanhueza et al., 2007) and LTP occlusion was observed after infusion of active CaMKII (Lledo et al., 1995), LTP and an associated memory task were also evaluated in CA1 neurons from FZP-withdrawn rats.
Materials and Methods:

**FZP withdrawal model:** An FZP dosing regimen was used which reliably induces manifestations of both benzodiazepine tolerance and dependence (Song et al, 2007; Van Sickle et al, 2002b; Van Sickle et al, 2004; Zeng et al, 1999). Male Sprague-Dawley rats (Harlan, Indianapolis, IN), P36-42 at the time of study were handled in accordance with institutional and NIH guidelines and approved by the University of Toledo Institutional Animal Care and Use Committee, Rats were first adapted to the animal facility and to the 0.02% saccharin vehicle for 2-4 days, then offered FZP (provided by the National Institute of Drug Abuse Supply Program) in vehicle for 1 week (100 mg/kg X 3 days; 150 mg/kg X 4 days) as their sole source of drinking water, followed by 1 or 2 days of drug withdrawal. Daily water consumption was monitored to adjust the drug concentration to offer the desired dose. Rats that did not reach a weekly average of 120 mg/kg/day were excluded. FZP-treated rats received saccharin water during the withdrawal period. Control rats received saccharin vehicle in parallel throughout the course of the study.

**Hippocampal slice preparation:** Following decapitation, the hippocampus was rapidly dissected. Transverse dorsal hippocampal slices (400 μm) were cut on vibratome (Ted Pella, Redding CA) in ice-cold, pre-gassed, low-calcium artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 120; KCl 2.5; CaCl₂ 0.5; MgCl₂ 7.0; NaH₂PO₄ 1.2; NaHCO₃ 2; D-glucose 20; ascorbate 1.3; pH 7.4. Slices were maintained at room temperature (22°C) for 15 min in gassed low-calcium ACSF, and then transferred to
normal ACSF containing (in mM): NaCl 119; KCl 2.5; CaCl₂ 1.8; MgSO₄ 1.3; NaH₂PO₄ 1.25; NaHCO₃ 26; D-glucose 10; pH 7.4. Slices recovered at room temperature for ≥ 2 hr in ACSF prior to electrophysiological recording. During recording, slices were perfused at a rate of 2.5 ml/min with gassed ACSF at room temperature. In some experiments, an active (KN-93, 10 μM), or inactive (KN-92, 10 μM) (EMD Chemicals, Inc, Gibbstown, NJ) CaMKII inhibitor was added to the ACSF 2 hr prior to mEPSC recording. Alternately, either the CaMKII inhibitor autcamtide-2, a CaMKII substrate (H-KKALRRQETVDAL-OH, 5 μM, EMD Chemicals) or the more potent, autcamtide-2 inhibitory peptide (H-KKKLRRQEAFDAL-OH, AIP, 5 μM, EMD Chemicals) were added to the micropipette prior to mEPSC recordings. A control scrambled peptide (H-ELRKFQADLKRKA-OH) used at the same micropipette concentration was designed to disrupt both the basic C-terminal and hydrophobic N-terminal groups of AIP. The scrambled sequence was analyzed online using Blastp, and no similar sequence was found in the current database.

Whole-cell electrophysiological recording: For whole-cell AMPAR-mediated mEPSC recordings, hippocampal slices were continuously perfused with oxygenated ACSF and visualized on an upright Zeiss Axioskop. Blind whole-cell patch-clamp recordings from CA1 pyramidal neurons were made using borosilicate micropipettes (4-7 MΩ, WPI, Sarasota, FL) containing (in mM): Cs methanesulfonate 132.5; CsCl 17.5; HEPES 10; EGTA 0.2; NaCl 8; Mg-ATP 2; Na₃-GTP 0.3; QX-314 2; pH 7.2. mEPSCs were recorded
under voltage clamp ($V_H = -80$ mV) in the presence of 1 μM tetrodotoxin, 50 μM picROTOXIN and 25 μM CGP-35348 in ACSF. Signals were amplified with an Axoclamp2A amplifier coupled to a 4-pole Bessel filter/amplifier (1 kHz, 100X, Cornerstone) and digitized online (20 kHz, Digidata 1200A, Axon) then stored on computer disk for later analysis.

Non-stationary noise analysis: AMPAR single-channel conductance was estimated by offline non-stationary noise analysis (NSNA) of mEPSCs using MiniAnalysis 6.0 (Synaptosoft, Inc, Decatur, GA). Miniature events were detected over the 5 (1-day) or 20 min (2-day) recording. Only fast events (10-90% rise time ≤ 3 ms) were used. Since records from 1-day FZP-withdrawn neurons and matched control neurons (Van Sickle et al., 2002) were 5 min in duration all previously detected events were used for NSNA analysis. For 2-day FZP-withdrawn neurons and their controls, events with an amplitude ≥ 8 pA were selected from the 20 min record. All selected events were baseline-adjusted, peak-scaled, superimposed and compared to the peak-scaled mean. The variance of amplitudes during the decay phase was plotted against mean amplitude of mEPSC currents and fitted with the equation: $\sigma^2 = i^* (I^2 - I^2/N)$ where $\sigma^2$ is the variance of the mEPSC, $i$ indicates the unitary current, $I$ represents the mean of whole-cell current, and $N$ is the total number of channels. The single-channel conductance was calculated by Ohm’s law: $\gamma = i/(V_H - V_{rev})$, where $V_H$ is –80 mV and $V_{rev}$ of AMPAR currents was 0 mV.
Spermine analogue inhibition: Three AMPAR subpopulations are primarily expressed in rat hippocampus, GluR1 homomers and GluR1/2 and GluR2/3 heteromers (Wenthold et al., 1996). Heteromeric GluR2-containing AMPARs exhibit low permeability for divalent cations such as Ca\(^{2+}\), whereas GluR2-lacking receptors have high divalent cation permeability (Hollmann et al, 1991). Furthermore, GluR2-containing and GluR2-lacking AMPARs also differ in their pharmacological properties, in particular blockade by extracellularly applied polyamine-containing spider and wasp toxins (Washburn et al, 1996). To evaluate whether GluR2-lacking receptors were present at CA1 neurons synapses during FZP-withdrawal, 100 μM 1-naphthylacetyl spermine (NAS) was added to the perfusate during AMPAR-mediated (\(V_H=-80\) mV) mEPSC recordings before (10 min) and after (7 min) NAS application. Mean mEPSCs amplitude was measured before and after NAS application and the percentage decrease after NAS was calculated.

Whole-cell LTP: Potentiation of whole-cell evoked EPSCs (eEPSCs, \(V_H=-80\) mV) was induced in individual CA1 neurons with a stimulating electrode placed in the Schaffer collateral pathway. Input-output curves were generated by increasing stimulus intensity, until maximal eEPSC amplitude was achieved or until an action potential was elicited. Stimulus intensity was then adjusted to induce 100-200 pA responses. Baseline eEPSCs were recorded 5 min before theta burst stimulation (TBS; 10 bursts of 4 pulses at 100 Hz; 200 ms interburst interval) was delivered at the same intensity. LTP was induced by switching the amplifier to current clamp mode and delivering TBS. eEPSCs were
recorded at 15 sec intervals for 60 min after TBS. LTP was defined as the percentage increase in eEPSC amplitude after TBS compared with the baseline amplitude averaged from 5 determinations.

Systemic AMPAR antagonist administration: Both AMPAR upregulation 1-day and 2-day FZP-withdrawn rats and N-methyl-D-aspartate receptor (NMDAR) downregulation in 2-day FZP-withdrawn rats can be prevented by an acute systemic injection of the AMPAR antagonist, GYKI-52466 24 hr prior to slice preparation and mEPSC recording (Van Sickle et al, 2002a; Van Sickle et al, 2002b; Van Sickle et al, 2004). To assess whether regulation of these receptors could affect the lack of LTP maintenance noted in FZP-withdrawn rats, an additional group of 2-day FZP-withdrawn and matched control rats were given a single injection of GYKI-52466 (0.5 mg/kg, i.p.) or the TWEEN 20 vehicle (1 ml/kg) the day prior to LTP recording.

Extracellular LTP: For extracellular recordings of field evoked potentials (fEPSPs and population spikes) transverse hippocampal slices were cut on a tissue chopper (Stoelting, Wood Dale, IL) from 2-day and 7-day FZP-withdrawn adult rats (PN55-60) or their matched control groups. AMPAR upregulation in this age group is similar to that in juvenile rats (Van Sickle et al., 2002). Slices were transferred to ice-cold pregassed (95% O₂/5% CO₂) buffer [(mM) 120 NaCl, 5.0 KCl, 1.3 MgSO₄, 1.2 NaH₂PO₄, 2.4 CaCl₂, 26 NaH₂CO₃, 10 D-glucose], then to an interface chamber and allowed to equilibrate for 2 hr at 33-35°C prior to electrophysiological recording of field potentials. Extracellular recordings were with 2M NaCl-filled glass microelectrodes (2-5 mΩ), in stratum
pyramidale of CA1 to record the population spike or in stratum radiatum, 250-300 µm from stratum pyramidale, to record the field excitatory postsynaptic potentials (fEPSPs). Evoked potentials were elicited with a bipolar, tungsten stimulating electrode, similarly placed in the Schaffer collateral pathway, using a S48 stimulator (Grass Instr. Co., Quincy, MA) coupled with isolator unit (SIU5, Grass). Evoked potentials were filtered (10KHz and 3KHz), then amplified 1000-fold using an AC differential amplifier (DAM 80, World Precision Instruments, Inc.; WPI, Sarasota, FL). Signals were acquired with a MacLab analog-to-digital converter (WPI) interfaced with a MACII computer, monitored using MacLab Scope v. 3.1 software and stored for later analysis. Input-output curves were generated by Schaffer collateral pathway stimulation until a maximal fEPSP or population spike amplitude was achieved, then stimulus intensity was adjusted to 50% of maximum. Average baseline fEPSP amplitude was the mean of 3 determinations at 5 min intervals. Each determination was the average of 4 sweeps at 30 sec intervals. Slices were excluded if the fEPSP response was unstable, i.e. varied greater than 10% across the 15 min baseline period. LTP was elicited at the half-maximal stimulus intensity by TBS as described above. fEPSP amplitude (mV) was defined for each response as the difference between the baseline recording immediately prior to stimulation to the peak negative point of the response. The percent change in fEPSP amplitude was calculated as the change from the average baseline fEPSP amplitude at several time points (0.5-60 min) after TBS. Initial fEPSP slope (mV/ms) measured from a 0.5-ms segment during the
initial negative change in voltage was calculated as percent change from the mean baseline fEPSP slope at the same post-TBS interval.

**Immunoblot analyses of CA1 minislices:**

Subcellular fractionation: For immunoblotting, hippocampi were isolated from matched pairs of control and FZP-withdrawn rats. The CA1 region was microdissected, homogenized and centrifuged to obtain a cytosolic fraction (S2), crude membrane pellet (P2) and a PSD-enriched fraction (P3) as previously described (Song et al, 2007). All procedures were conducted at 0-4°C.

Immunoblotting: Protein (15 μg/well) was separated by 10% SDS-PAGE and wet-transferred to nitrocellulose. Primary antibodies anti-CaMKII (1:5,000, Chemicon, Temecula, CA), anti-phospho-Thr286-CaMKII (1:1,000, Promega Corporation, Madison, WI), anti-GluR1 (1:2,000, Chemicon), anti-phospho-Ser831-GluR1 (1:1,000, Chemicon), anti-actin (1:50,000, Chemicon) or anti-GAPDH (1:20,000, Abcam Inc, Cambridge, MA) were incubated with membranes overnight at 4°C. Antibody signals were detected with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch, West Grove, PA), followed by enhanced chemiluminescence (ECL) (Denville Scientific, Metuchen, NJ). Images of immunoblots were scanned and immunoreactivity quantified with ImageJ. CaMKII or GluR1 signals were normalized to the corresponding GAPDH or actin signal.
Novelty Preference Paradigm:

Since LTP maintenance was impaired an additional group of control (n=11) and 2-day FZP-withdrawn (n=12) rats was evaluated behaviorally in a novelty preference paradigm for novel object recognition, place preference and contextual memory using methods modified from Mumby et al. (2002), a procedure which required little prior training. Briefly, during saccharin water administration rats were acclimation for 20 min to an open-field arena (70 cm X 70 cm X 25 cm, length X width X height) with two identical objects not used in the later test session. For object trials, rats were allowed to familiarize themselves with two identical objects at symmetric locations for 5 min. After a 5 min retention interval, one of the two objects was replaced with a novel object and the rat was returned for another 3 min exploration. For the spatial trial, one of two familiar objects was moved to a new location and the rat was returned for another 3 min exploration. For the context trial, the arena was placed in one room with one pair of objects for 5 min familiarization and then moved to another room with another pair of objects for an additional 5 min of familiarization. After the 5 min retention interval, the arena was returned to the first room for the 3 min object recognition test. A rat was considered to be engaged in object exploration when its head was oriented at 45° and within 4 cm of an object. Rearing with the head oriented upward was also included if at least one of forepaw was on the object. Trials were excluded if a rat spend less than 1 sec exploring both objects during the retention test. The exploration ratio during each 3 min test period was defined as $t_{novel}/(t_{novel} + t_{sample})$ and compared by Student’s t-test.
Results:

Potentiation of AMPAR mEPSCs via postsynaptic CaMKII activation

As previously reported (Van Sickle et al, 2002b; Xiang et al, 2007), the amplitude of AMPAR-mediated mEPSCs was increased in CA1 neurons from 2-day FZP-withdrawn rats (Fig. 1A-C) without an effect on mEPSC kinetics (Table 1). To investigate CaMKII’s contribution to this potentiation, hippocampal slices were incubated for 2 hr in ACSF in the presence of 10 μM KN-93, a membrane permeant CaMKII inhibitor, which competes with CaM for CaMKII activation (Sumi et al, 1991). KN-93 effectively abolished the potentiation of AMPAR-mediated mEPSCs in the FZP-withdrawn group. The inactive analog, KN-92 (10 μM), had no effect, i.e. AMPAR-mediated mEPSC amplitude remained elevated (Fig 1A and C, Table 1).

An enhancement in AMPAR current amplitude could be due to an increase in AMPAR numbers or an increase in channel conductance. As with LTP (Derkach et al, 1999; Esteban et al, 2003; Hayashi et al, 2000), the number of AMPAR binding sites and of GluR1-containing AMPARs are increased at CA1 neuron synapses in FZP-withdrawn rats (Van Sickle and Tietz, 2002; Song et al., 2007; Das et al., 2008), an effect that may be mediated by several kinases including CaMKII. In studies of recombinant receptors, CaMKII activation leads to Ser831 phosphorylation of the GluR1 subunit accompanied by an increase in AMPAR single-channel conductance (Derkach et al, 1999). Single-channel conductance is also enhanced up to 4-fold following LTP induction (Benke et al., 1998). To evaluate whether this CaMKII-mediated effect occurs at the CA1 neurons of FZP-
withdrawn rats, single-channel conductance of AMPAR mEPSCs was estimated by NSNA. As shown in Table 1, the estimated AMPAR single-channel conductance in control neurons ($\gamma = 8.8 \pm 0.7$ pS) was comparable to a previous report using similar techniques (Benke et al., 1998). In comparison to controls, AMPAR unitary conductance was increased ~2-fold in the FZP-withdrawn group (FZP: 14.6 ± 2.1 pS; p< 0.01). Preincubation in KN-93 also eliminated the enhanced single-channel conductance in the FZP-withdrawn group (CON: 9.8 ± 1.6 pS; FZP: 9.1 ± 0.7 pS; p< 0.05, Fig 1D), similar to its effect on mEPSC amplitude. Single-channel conductance did not return to control levels in FZP-withdrawn neuron recordings following KN-92 preincubation.

CaMKII is highly expressed in the pre- and post-synapse and contributes to short-term facilitation and long-term potentiation of synaptic transmission. The finding that AMPAR mEPSCs amplitude, but not event frequency was enhanced in FZP-withdrawn neurons (Van Sickle and Tietz, 2002) suggests that a postsynaptic mechanism mediates AMPAR potentiation. To confirm that cellular mechanisms downstream of CaMKII activation may contribute to this postsynaptic effect, in some groups of cells 5μM AIP or autocamtide-2 (ACM), were included in the micropipette to inhibit CaMKII activity intracellularly. Scrambled AIP (sAIP) at the same concentration was used as a control. The representative traces (Fig 2A) show that AIP reversed the increased amplitude of AMPAR mEPSCs to control levels in FZP-withdrawn rats. Postsynaptic inclusion of AIP also blocked the increase in AMPAR conductance (Fig 2B). As shown in Table 2, AIP prevented the enhancement of AMPAR mEPSC amplitude induced by FZP withdrawal.
(Fig. 2C). The single-channel conductance derived from the same sets of recordings showed a parallel return to control levels (Fig. 2D). ACM, a relatively less-potent CaMKII, substrate also showed an effect to prevent AMPAR potentiation in FZP-withdrawn rat which did not reach statistical significance (p=.06) (Table 2).

**Synaptic incorporation of GluR1 subunits: Further evidence for the involvement of CaMKII in FZP withdrawal-induced AMPAR plasticity**

An increase in single-channel conductance reflects an increase in synaptic incorporation of homomeric GluR1 AMPARs, whereas the presence of a GluR2 subunit maintains GluR1/GluR2 heteromers in a low-conductance state regardless of GluR1 Ser{sup 831} phosphorylation by CaMKII (Oh et al, 2005). Inward rectification in the presence of spermine or its analogs can be used as an electrophysiological tag for the detection of GluR1 homomers (Derkach et al, 1999). mEPSCs recorded from CA1 neurons in hippocampal slices from FZP-withdrawn rats showed a decreased +40/-60 rectification index with 0.1 mM spermine in the micropipette (Song et al, 2007). As illustrated by its effect on representative average mEPSCs (Fig. 3A), extracellular application of NAS, another selective inhibitor of AMPAR currents mediated by GluR1 homomeric receptors, had a significant greater effect to inhibit AMPAR-mediated mEPSC amplitude in CA1 neurons from FZP-withdrawn vs. control rats (Fig. 3B). Removing the synaptic current component mediated by GluR1 homomers with NAS also abolished the potentiation of mEPSC amplitude in neurons from FZP-withdrawn rats (Fig. 3C). This finding lends
further support to the hypothesis that upregulation of AMPARs during drug withdrawal is mediated by GluR1 homomer incorporation into CA1 neuron synapses.

**Time-course of increased GluR1 and CaMKII levels in the PSD-enriched fraction**

In models of activity-dependent plasticity, such as LTP, CaMKII activated by Ca\(^{2+}\) influx through NMDARs potentiates synaptic efficacy by inducing synaptic insertion of AMPARs, as well as increasing AMPAR single-channel conductance via Ser\(^{831}\) GluR1 subunit phosphorylation (Lisman et al, 2002). Accordingly, phospho- and total GluR1 subunit levels were examined by immuoblot analysis of subfractionated proteins in cytosolic (S2), crude membrane (P2) and PSD-enriched fractions (P3) of CA1 minislices from 2-day FZP withdrawn rats. Consistent with an increase in single-channel conductance of GluR1-containing AMPARs, both the absolute and relative phospho-Ser\(^{831}\) GluR1 subunit level was significantly increased in the P3 fraction of the FZP-withdrawn group. Total GluR1 expression levels in the PSD-enriched fraction (P3) were also elevated (Fig. 4A) as reported previously (Song et al, 2007).

To probe the possible underlying mechanism for GluR1 Ser\(^{831}\) phosphorylation, the levels of CaMKII and its constitutively active, Thr\(^{286}\) phosphorylated form were also evaluated. Total CaMKII protein levels were significantly increased in all fractions derived from FZP-withdrawn CA1 minislices (Fig. 4B) providing a mechanism for GluR1 Ser\(^{831}\) phosphorylation. Neither the total nor the relative amounts of phospho-Thr\(^{286}\) CaMKII protein was increased.
Since AMPAR current amplitude and conductance were both increased in neurons from 2-day FZP-withdrawn rats (30-50%) and AMPAR mEPSC amplitude was previously reported to be significantly increased (~15%) in 1-day FZP-withdrawn rats (Van Sickle and Tietz, 2002) total and phospho GluR1 and CaMKII protein levels, as well as GluR2 subunit levels were similarly examined in the P3 fraction at this time-point after withdrawal. Despite a significant increase in total GluR1 level consistent with an increase in mEPSC amplitude there was no parallel increase in phopho-Ser\textsuperscript{831} GluR1 expression in the PSD-enriched fraction (Fig. 5A). Phospho- and total CaMKII protein levels (Fig. 5B) were also unchanged in comparison to control CA1 subfractions. Consistent with previous immunohistochemical findings in 2-day FZP-withdrawn tissues (Song et al., 2007; Das et al., 2008), GluR2 subunit expression level was likewise unchanged in the PSD-enriched fraction from 1-day FZP withdrawn rats (Fig 5D).

Since phopho-Ser\textsuperscript{831} GluR1 expression levels were not increased in 1-day FZP-withdrawn rats, previous mEPSC recordings were reexamined using NSNA. Estimated single-channel conductance derived from these data (Van Sickle and Tietz, 2002) revealed a similar conductance level (Fig 5C, CON: 7.7 ± 0.8 pS; FZP: 7.7 ± 0.3 pS, p = .92) in control and 1-day FZP-withdrawn rats despite a significant increase in both rise-time-aligned (see Van Sickle et al., 2002) or peak aligned mEPSC amplitude (CON: -11.4 ± 0.3 pA; and FZP: -12.6 ± 0.3 pA, p=.01). The increased expression of GluR1, but not GluR2 subunits in CA1 minislices from 1-day FZP-withdrawn rats provides substantial support for the proposal that homomeric GluR1 AMPARs were incorporated into CA1
synapses prior to Ser\(^{831}\) phosphorylation by CaMKII resulting in a subsequent functional increase in AMPAR single channel conductance (Oh and Derkach, 2005).

**Impaired LTP expression in CA1 neurons from FZP-withdrawn rats**

Activation of CaMKII is essential for LTP. Transfection of CaMKII (Pettit et al, 1994), intracellular infusion of the active form of CaMKII (Lledo et al, 1995) or of Ca\(^{2+}\)/CaM (Wang and Kelly, 1995) can all potentiate synaptic transmission and occlude tetanus-induced LTP. As expression of CaMKII was increased in all fractions of CA1 minislices and AMPAR-mediated synaptic transmission is enhanced in FZP-withdrawn rats, LTP was predicted to be occluded.

Prior to the study of LTP, an input-output (I/O) curve (Fig. 6A) was generated to evaluate this stimulus-response relationship and to select the optimal stimulus intensity for inducing whole-cell LTP. AMPAR-mediated evoked EPSC amplitude was plotted against stimulus intensity delivered to the Schaffer collateral pathway and fitted by linear regression. A 2.4-fold enhancement in the slope of the I/O curve (CON, 205.3 ± 44.5 pA/V vs. FZP, 440.0 ± 79.4 pA/V, p<0.05, Fig 6B) was consistent with both an enhancement in AMPAR-mediated synaptic efficacy and a role for \(\alpha\)-CaMKII in synaptic transmission as revealed in CaMKII mutant mice (Hinds et al, 1998).

LTP induced by TBS lasted up to the 60 min recorded in control slices, measured using either whole-cell or extracellular techniques (Fig. 7). For whole-cell LTP studies, stimulus intensities were adjusted to obtain 100-200 pA basal responses. Neither the
minimal LTP stimulus intensity (CON, 0.50 ± 0.04 V; FZP, 0.59 ± 0.06 V, n=7, p = 0.26) used to elicit whole-cell LTP, nor baseline eEPSC responses were different between groups (CON, 159 ± 28 pA; FZP, 175 ± 47 pA, n=7, p = 0.77, Fig. 5A, Inset). In whole-cell EPSC studies, LTP induction was evident in both groups immediately after TBS. The enhancement of AMPAR-mediated eEPSCs amplitude persisted for the 60 min recording period in CA1 neurons from control rats (Fig 7A). A significant difference between FZP-withdrawn and control neurons emerged within 15 min after TBS was delivered.

To evaluate whether enhanced AMPAR glutamatergic strength could result in ‘depotentiation’ of LTP, the ability of a systemic injection of GYKI-52466, previously shown to reverse AMPAR potentiation (Van Sickle et al., 2004, Xiang et al., 2007) to restore LTP was examined after FZP withdrawal (supplemental Fig 1). All groups injected with either Tween 20 or with GYKI-52466 showed a similar downward shift in LTP expression compared to the preinjection state suggesting that perhaps stress due to handling or the injection may have had similar effects to negatively modulated LTP expression (Alfarez et al., 2003) in both the control and experimental groups. As expected, inclusion of AIP in the micropipette blocked LTP induction in neurons from both experimental groups (CON, n = 4; FZP n = 3 neurons/group, data not shown).

In CA1 neuron responses recorded extracellularly from adult FZP-withdrawn rats (11 slices/6 rats), potentiation was similarly induced shortly after TBS, yet as with whole-cell LTP in neurons from juvenile rats, was not maintained. Importantly there were also no significant differences in the mean stimulus intensity (CON, 3.6 ± 0.2 V; FZP, 4.0 ±
0.3 V, p = 0.32) used to evoke half-maximal, baseline fEPSPs, also not different between groups (CON, 0.81 ± 0.4 mV; FZP, 0.81± 0.5 mV, p = 0.95). Though there were no differences in baseline fEPSP slope (CON, 0.53 ± 0.12 mV/ms; FZP, 0.48 ± 0.09 mV/ms, p = 0.65), the slope of the fEPSP showed a steady decline toward baseline after TBS (Fig. 6D). Within 25 min there was significant difference in the degree of potentiation remaining between control and FZP-withdrawn rats. The amplitude of both fEPSPs and population spikes showed a similar trend towards baseline across the 60 min recording period (data not shown). Though baseline fEPSP slope and amplitude were unchanged between groups, the population spike amplitude increase ~1.7 fold (CON, 4.5 ± 0.5 mV; FZP, 7.6 ± 0.07 mV, p = 0.001) consistent with the enhancement of the slope of the whole cell I/O curve (Fig. 5) and an increase in synaptic efficacy.

CA1 neurons derived from 7-day withdrawn rats are neither tolerant nor physically dependent (Van Sickle et al, 2004; Zeng et al, 1999). Field potentials in CA1 neurons from both 7-day FZP withdrawn slices and slices from match control rats showed a similar degree of potentiation of the population spike, fEPSP amplitude and fEPSP slope (supplementary Fig. 2) across the entire 60 min recording period.

Though FZP-withdrawn rats showed a tendency toward differential behavioral effects in the novelty preference paradigm vs. control rats across each 3 min test periods no deficits were observed between the control and the 2-day FZP-withdrawn group in object recognition, or spatial or contextual memory (Fig. 8 and Supplementary Fig. 3), in contrast to the impairment of LTP expression.
Discussion:

A significant link has been established between the increased efficacy of hippocampal CA1 pyramidal neuron AMPAR-mediated synaptic transmission and CA1 neuron hyperexcitability (Van Sickle et al., 2004; Xiang and Tietz, 2007), suggesting increased excitability within the neural circuits mediating expression of withdrawal-induced anxiety-like behavior. The enhancement of macroscopic glutamate currents can be attributed to an increase in GluR1-, but not GluR2-, containing AMPARs at CA1 neuron synapses (Song et al., 2007; Das et al., 2008). This postsynaptic change is reminiscent of CaMKII-mediated, activity-dependent remodeling of glutamatergic transmission, which occurs at CA3-CA1 neuronal synapses during early LTP (Malinow and Malenka, 2002; Nicoll, 2003; Derkach et al., 2007). A large body of evidence suggests that CaMKII activation is necessary and sufficient to enhance AMPAR-mediated synaptic strength and plays a central role in LTP induction (Lledo et al., 1995; Mammen et al., 1997; Benke et al., 1998; Derkach et al., 1999; Poncer et al., 2002), as well as maintenance (Lisman et al., 2002; Sanhueza et al., 2007). CaMKII can be persistently activated for at least an hour through autophosphorylation of Thr\textsuperscript{286}CaMKII by NMDAR-mediated Ca\textsuperscript{2+} entry during LTP induction (Fukunaga et al., 1993). Persistently active CaMKII is translocated to the PSD (Otmakhov et al., 2004) where it phosphorylates homomeric GluR1-containing AMPARs on Ser\textsuperscript{831}, enhancing channel conductance at least two-fold (Barria et al., 1997a; Barria et al., 1997b; Derkach et al., 1999) an effect mimicked by intracellular perfusion or viral expression of active CaMKII
(Pettit et al., 1994), or by tetanic stimulation (Benke et al., 1998). Conversely, blocking the kinase by pharmacological (Malinow et al., 1989) or genetic (Silva et al., 1992; Giese et al., 1998) approaches, blocks or reduces LTP induction and maintenance. Both protein kinase A and CaMKII are necessary for activity-dependent incorporation of recombinant GluR1-containing AMPARs into CA1 neuron synapses, independent of Ser\(^{831}\) GluR1 phosphorylation (Esteban et al., 2003). Once incorporated, GluR2-lacking AMPARs are readily phosphorylated by CaMKII resulting in an increase in channel conductance (Oh and Derkach, 2005).

As reported previously (Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Song et al., 2007) AMPAR-mediated synaptic transmission was potentiated in FZP-withdrawn rats (Fig. 1A and C, Fig 2A and C, Fig. 6) consistent with an increase in trafficking and/or phosphorylation of GluR1-containing AMPARs (Barria et al, 1997b; Derkach et al, 1999; Esteban et al, 2003; Hayashi et al, 2000). Homomeric GluR1 channels expressed in HEK-293 cells exhibit conductance states of 9, 14, 20 and 28 pS and co-expression of active CaMKII and GluR1 increases the frequency of transitions to the two highest conductance states (Barria et al, 1997a; Barria et al, 1997b; Derkach et al, 1999). Mutation of Ser\(^{831}\) GluR1 to Asp further enhanced transitions such that the highest conductance states comprise 80% of total activity (Derkach et al., 1999). Non-stationary noise analysis (NSNA) was used to estimate average unitary synaptic current from whole-cell recordings of mEPSCs. Though the method can be affected by changes in membrane capacitance, membrane resistance and access resistance, it can still provide an
accurate estimate of changes in single-channel conductance at intact synapses (Benke et al., 1998). The ~2-fold increase in AMPAR single-channel conductance detected (Fig 1D) in CA1 neuron synapses (Fig 1B) is consistent with a role for CaMKII-mediated phosphorylation of GluR1-containing (i.e. GluR-2 lacking) AMPARs on Ser^831 (Oh et al., 2005) during drug withdrawal. These findings coupled with previous results also suggest that, as with other models of activity-dependent plasticity (Esteban et al., 2003, Oh and Derkach, 2005), CaMKII may have dual mechanisms to modulate AMPAR potentiation, affecting both the numbers of GluR1-containing AMPARs at CA1 synapses (Das et al., 2008), as well their phosphorylation state.

Alterations in the downstream effectors of CaMKII activation provided further evidence that enhanced glutamatergic strength at CA1 neuron synapses is ‘LTP-like’ in benzodiazepine withdrawn rats. A potential role for CaMKII in mediating increased numbers of GluR1-containing AMPARs and a shift in their conductance state was first evaluated by preincubation of hippocampal slices with KN-93 (10 μM). The CaMKII inhibitor restored the increased AMPAR current amplitude and conductance in neurons from FZP-withdrawn rats to control levels, while the inactive analog KN-92, had no effect (Fig 1C and D). Though numerous experiments fail to support the presynaptic mechanisms for LTP (Nicoll, 2003) and mEPSC frequency is unaltered in FZP-withdrawn neurons (Van Sickle and Tietz, 2002), CaMKII can also affect neurotransmitter vesicle release (Llinas et al., 1985; Margrie, 1998). Therefore in additional experiments, CaMKII inhibitors with alternate mechanisms of action were also
included in the micropipette to rule out effects on presynaptic release. Autocamtide-2 related inhibitory peptide (AIP), but not the scrambled peptide had similar effects to prevent potentiation of AMPAR synaptic current (Fig 2C and D, Table 2), providing additional evidence that CaMKII activation is involved in the CA1 neuron hyperexcitability that contributes to drug-induced withdrawal anxiety.

Since CaMKII also leads to synaptic delivery of GluR1 subunits during LTP (Poncer et al, 2002; Zhu et al, 2002) NAS, a potent spermine analogue, was used as an electrophysiological tool to detect increased functional incorporation of GluR1 subunits into CA1 synapses of FZP-withdrawn rats. AMPARs in hippocampus are predominantly composed of GluR1/2 and GluR2/3 subunits (Wenthold et al., 1996). Q/R editing renders GluR2-containing AMPAR calcium impermeable and insensitive to spermine blockade while GluR2-lacking AMPARs show inward rectification (Washburn and Dingledine, 1996). Thus, the shift in the rectification index in FZP-withdrawn neurons (Song et al., 2007) was supportive evidence for synaptic incorporation of GluR1 homomers (Esteban et al, 2003; Shi et al, 1999). Intracellular and extracellular spermine application, either to intact or acutely dissociated FZP-withdrawn CA1 neurons, also inhibited endogenous and exogenous glutamate-elicited currents (Song et al, 2007). As with spermine application, NAS inhibition of mEPSCs was significantly increased in FZP-withdrawn neurons suggesting that an increased proportion of GluR1 homomers, rather than GluR1/2 heteromers, was incorporated into CA1 synapses (Fig. 3C) corroborating electron microscopic studies in 2-day FZP-withdrawn tissues (Das et al., 2008).
AMPAR mEPSC amplitude progressively increased in 1-day and 2-day FZP-withdrawn rats, yet an increase in single-channel conductance was only evident in parallel with enhanced Ser\textsuperscript{831}GluR1 phosphorylation on day 2 (Fig 4 and 5). Notably, since phosphorylation of GluR1 at Ser\textsuperscript{831} does not result in an increase in conductance in presence of the GluR2 subunit (Oh et al, 2005) these findings lend further support to the interpretation that GluR1 homomers are driven into CA1 synapses in 1-day FZP-withdrawn rats, then subsequently phosphorylated by CaMKII on day 2 leading to an increase in channel conductance. Taken together, these findings may represent the first example of a drug withdrawal-induced functional expression of Ser\textsuperscript{831} phosphorylation of GluR1 homomers in native hippocampal neurons reflected in an enhancement of AMPAR single-channel conductance.

To assess whether a similar sequence of events might mediate the downstream effects of CaMKII activation as with other models of plasticity, the levels of total CaMKII protein and the active, phospho-Thr\textsuperscript{286} form were measured in fractionated CA1 minislices (Fig 4). In contrast to LTP (Barria et al, 1997b), CaMKII levels were elevated 50% above control levels, whereas the absolute amount of the phospho-protein was unchanged in the FZP-withdrawn group. This may not be surprising since with LTP only 10% of synapses are potentiated (Buchs and Muller, 1996), whereas during withdrawal from chronic drug treatment a relatively larger portion of the synapses might be expected to be affected during the course of chronic drug treatment and upon withdrawal. Alternately, this difference may arise from the source of elevated intracellular Ca\textsuperscript{2+}
during FZP withdrawal, which may be predominantly mediated by an increase L-type voltage-gated Ca\(^{2+}\) channels (Xiang et al., 2007, Xiang et al., 2008). Moreover, levels of CaMKII inactive, active and constitutively active forms, depend on the status of calcium/calmodulin binding and further autophosphorylation of Thr\(^{286}\), respectively. Even if there were no differences in the constitutively active form of CaMKII, there still may be sufficient amounts of active CaMKII available to affect AMPAR potentiation (Lisman et al., 2002). Indeed since phospho-Ser\(^{831}\) GluR1 levels were significantly elevated in the PSD-enriched fraction concomitant with an increase in AMPAR channel conductance, such a scenario is quite conceivable. The findings of immunoblot studies lend additional support to the possibility that a post-translational modification involving a sequence of events similar to the other forms of activity-dependent plasticity and downstream of CaMKII activation may mediate enhanced glutamatergic strength at CA1 neuron asymmetric synapses during drug withdrawal.

CaMKII activation has been shown to be sufficient to augment excitatory synaptic strength and this amplification shares similar underlying mechanisms to the enhancement observed with LTP (Lledo et al, 1995). AMPAR potentiation can be mutually occluded as a function of CaMKII activation or by tetanic stimulation (Lledo et al., 1995; Wang and Kelly, 1995). Since the upregulation of AMPAR currents was linked to CaMKII activation the possibility that LTP induction or maintenance was occluded was also evaluated in FZP-withdrawn neurons. Theta burst stimulation induced LTP to an equivalent degree in both groups. Initial attempts to uncover a mechanism for the lack of
LTP maintenance and its behavioral consequences did not provide any additional insights into the nature of this occlusion or its functional consequences and the basis for this phenomenon remains under investigation.

While LTP expression was impaired in FZP-withdrawn neurons (Fig. 6), there were no effects on object recognition or spatial or contextual memory (Fig 8). Several reasons may account for this discrepancy. First, LTP was inducible in FZP-withdrawn rats and the impairment of LTP expression may not have been sufficient to result in memory impairment. Alternatively, the novelty preference paradigm may have been insensitive to detect a minor hippocampal memory deficit. On the other hand, more recent reports in GluR1 knockout mice and other gene deletion experiments suggest that LTP may not be closely correlated with hippocampal-related memory tasks. (Hölscher, 1999; Reisel et al., 2002).

Collectively, the findings suggest a major contribution of CaMKII activation to the potentiation of CA1 neuron AMPAR-mediated currents in 2-day FZP-withdrawn rats. The evidence suggests that CaMKII activation contributes to a shift in AMPAR subunit composition toward one dominated by homomeric GluR1-containing receptors and subsequent phosphorylation of GluR1 at Ser\textsuperscript{831}, further enhancing the conductance of synaptically incorporated GluR1-containing AMPARs. Moreover as with models of activity-dependent plasticity such as LTP, CaMKII may play a similar, dual role in mediating enhanced AMPAR glutamatergic strength in the hippocampus, an important locus within the neural circuits mediating withdrawal-anxiety. Activation of the same
CaMKII-mediated pathways may be highly conserved promoting both activity-dependent plasticity and drug-induced adaptations at CA1 pyramidal neuron synapses associated with benzodiazepine physical dependence and may contribute to dependence on other drugs of abuse (Fan et al, 1999; Tang et al, 2006).
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Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1


Ca(2+)/calmodulin-dependent protein kinase II. J Pharmacol Exp Ther 317:901-909.


Figure 1. CaMKII activity is required for AMPAR potentiation in 2-day FZP-withdrawn rats.

AMPAR-mediated mEPSCs obtained from control (CON), 2-day FZP-withdrawn rats (FZP), with or without 10 μM KN-93, or KN-92 pre-incubation. (A) Mean amplitude of AMPAR-mediated mEPSCs is increased in 2-day FZP-withdrawn rats. Two hr KN-93 incubation prevented the increase in mEPSC amplitude in the FZP, but not CON group. (B) Non-stationary noise analysis (NSNA) was used to extract single-channel information from whole-cell recordings as described in the methods. mEPSC variance was plotted vs. mean amplitude and fitted with a parabola. The slope of the parabolic curves at I=0 was used to estimate single-channel current. The mean conductance in the FZP-withdrawn group was ~2-fold greater (14.6 ± 2.1 pS, n = 9 neurons; open circles/dotted line) compared with the CON group (8.5 ± 0.7 pS, n = 10 neurons, close circles/solid line). Average mEPSC (C) amplitude or (D) conductance in CA1 neurons from CON (closed bars) and FZP-withdrawn (open bars) rats following 2 hr incubation in artificial cerebrospinal fluid (ACSF) plus KN93 or KN92, an inactive analog of KN-93. The significant increase in mEPSC amplitude and conductance (**, p<0.01) in FZP-withdrawn vs. CON neurons recorded in ACSF was blocked by preincubation with KN-93 (*, p<0.05, ** p<0.01), but not KN-92.
**Figure 2.** Intracellular CaMKII inhibitors block FZP-withdrawal induced AMPAR potentiation. AMPAR-mediated mEPSCs were recorded from control or FZP-withdrawn rats with either 5 μM scrambled autocamtide inhibitory peptide-2 (+sAIP, used as control for AIP), Autocamtide inhibitory peptide-2 (+AIP) or autocamtide-2 (+ACM) inside the micropipette. (A) The mean amplitude of AMPAR-mediated mEPSCs was increased in FZP-withdrawn neurons with sAIP in the micropipette. Inclusion of 5 μM AIP restored the amplitude to control levels. Autocamtide-2 had a more modest effect compared with AIP. (B) A representative NSNA plot shows that both mEPSC amplitude and conductance were increased in FZP-withdrawn rats (black dotted line) with sAIP in micropipette vs. control neurons with sAIP (black solid line). AIP could restore both amplitude and conductance to control levels (grey line). Mean mEPSC (C) amplitude or (D) conductance from CON (closed bars) and FZP-withdrawn (open bars) neurons with 5 μM sAIP, AIP or ACM. The significant increase in mEPSC amplitude and conductance (*, p<0.05; **, p<0.01) in FZP-withdrawn vs. CON neurons recorded in ACSF was blocked by preincubation with AIP (*, p<0.05). ACM, a less potent substrate of CaMKII, was unable to significantly (p=.06) restore the mEPSC amplitude levels amplitude and conductance to those recorded with scrambled peptide.
Figure 3. Greater NAS inhibition in neurons from FZP-withdrawn rats indicative of synaptic incorporation of GluR1 homomers. (A) AMPAR-mediated mEPSCs were recorded before (solid line) and after (dotted line) external application of the potent spermine analogue NAS (100μM). (B) NAS inhibition of mEPSC current amplitude was increased ~2-fold in CA1 neurons from FZP-withdrawn rats (p<0.05). (C) External application of NAS abolished the increased AMPAR-mediated mEPSC in FZP-withdrawn neurons (FZP, 10.6 ± 0.3 pA vs. FZP + NAS, 8.4 ± 0.4 pA, n=12) and was without effect in control neurons (CON, 9.0 ± 0.3 pA vs. CON + NAS, 8.1 ± 0.4 pA, n=7), supporting the hypothesis that AMPAR current potentiation is mediated by synaptic GluR1 homomer incorporation.
Figure 4. Total and phospho-GluR1 and CaMKII protein levels in fractionated CA1 minislices. CA1 minislices were pooled from the hippocampus of 3 control (CON) or 3 2-day FZP-withdrawn (FZP) rats were fractionated to obtain S2 (cytosolic), P2 (crude membrane) and P3 (PSD-enriched) fractions. Phospho-Ser$^{831}$ GluR1 was detected with ECL and normalized to the respective actin signal. The nitrocellulose membrane was striped and re-probed for total GluR1. Phospho-Thr$^{286}$ CaMKII protein levels were detected and normalized to the respective GAPDH signal. The nitrocellulose membrane was then striped and re-probed for total CaMKII. (A) Immunoblots of Phospho-Ser$^{831}$ GluR1 and total GluR1 are in the left column with actin blots underneath. Histograms representing integrated signal density in the S2, P2 and P3 fractions are shown (CON: closed bars, FZP: open bars, n = 7 pooled subfraction samples/21 rats/group). (B) Immunoblots of phospho-Thr$^{286}$ CaMKII and total CaMKII relative to GAPDH and the respective histograms are similarly displayed in the right column (single asterisk: p<0.05; double asterisk: p<0.01).
Figure 5 GluR1, CaMKII and GluR2 expression in the PSD-enriched fraction of 1-day FZP-withdrawn CA1 minislices consistent with lack of change in AMPAR conductance. (A) Representative immunoblots and respective histograms illustrating no change in phospho-Ser<sup>831</sup> GluR1 levels (upper panel) despite an enhanced GluR1 subunit expression levels (lower panel) in the PSD-enriched fraction from 1-day FZP-withdrawn CA1 minislices. (B) No differences in either phospho-Thr<sup>286</sup> (upper panel) or total CaMKII levels (lower panel) were observed. (C) NSNA was conducted on miniature EPSCs previously recorded in 1-day FZP withdrawn rats and no increase in estimated single-channel conductance was detected, though mEPSC amplitude aligned by peak (p=.01) or rise-time (Van Sickle et al., 2002) was significantly increased. (D) As in 2-day FZP-withdrawn rats GluR2 subunit levels were unchanged in 1-day FZP-withdrawn minislices.
Figure 6. Increased slope of input–output (I/O) relationships, measuring evoked EPSC amplitude (pA) as a function of Schaffer collateral pathway stimulus intensity (V) in CA1 neurons from control and FZP-withdrawn rats. (A) Linear regression of pooled input-output relationships showed a ~2.5 fold increase in the stimulus response relationship in CA1 neurons from FZP-withdrawn (open circles, n = 9) compared to control rats (closed circles, n = 7). Inset: There were no significant differences in the stimulus intensity used (CON: 0.50 ± 0.04V vs FZP: 0.59 ± 0.06V) to elicit 100-200 pA eEPSC responses or in the amplitude of the responses between control and FZP-withdrawn groups. (B) The mean slope of the input-output curve derived from the fits of individual I/O curves generated in neurons from FZP-withdrawn rats (440.0 ± 79.4 pA/V, n = 9) was significantly higher from control rats (205.3 ± 44.5 pA/V, n = 7).
Figure 7. Lack of LTP maintenance in CA1 neurons from 2-day FZP-withdrawn rats. LTP was induced by TBS and measured using both whole-cell (A, C eEPSC) and extracellular (B, D, fEPSP) techniques. Representative traces of eEPSCs (A) and fEPSPs (B) elicited 1, 30 and 60 min after theta burst stimulation (TBS; 10 bursts of 4 pulses at 100 Hz; 200 ms interburst interval) are superimposed over the baseline response, shown in grey. (C) Whole-cell evoked EPSCs were recorded for 60 min. The degree of stimulus-induced potentiation was normalized to the baseline response. LTP was induced in CA1 neurons derived from both control rats (close circles, n = 7) and 2-day FZP-withdrawn rats (open circles, n = 7) though could only be maintained for <15 min in the FZP-withdrawn group. (D) In control hippocampal slices fEPSP slope remained significantly elevated for the 60 min observation period (close circles, n = 10). In slices from 2-day FZP-withdrawn rats (open circles, n = 10), fEPSP slope steadily declined 1 min after TBS and thereafter was no longer significantly different from baseline by 45 min (p = 0.96).
**Figure 8.** Novelty paradigm designed to measure object recognition, spatial and contextual memory in 2-day FZP-withdrawn rats. Mean exploration ratios, as the proportion of object exploration time spent on novel objects $t_{\text{novel}}/(t_{\text{novel}} + t_{\text{sample}})$, are shown for object, place and context trials (CON: closed bars, $n = 11$ vs. FZP: open bars, $n=12$). Both groups showed a similar recognition of novel objects, as well as their place and context suggesting no observable memory deficit despite an impairment of LTP expression at this time of drug withdrawal.
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<th>Frequency (Hz)</th>
<th>Rise time (ms)</th>
<th>Amplitude (pA)</th>
<th>Decay tau (ms)</th>
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Values are listed as means ± S.E.M.
Table 2. mEPSC characteristics after postsynaptic application of CaMKII inhibitors

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Values are listed as means ± S.E.M.
Supplementary Figure 1. Effect of GYKI-52466 injection on LTP expression. Injection of GYKI-52466 (grey closed circles) or vehicle (1% Tween 20, grey open circles) in (A) control (GYKI, n = 7 vs. TWEEN20, n = 7) or in (B) 2-day FZP withdrawn rats (GYKI, n = 9 vs. TWEEN20, n = 9). Control (black closed circles, n = 7) and FZP-withdrawn rats (black open circles, n = 7) as shown in Fig. 7 were also shown for comparison. In control rats a significant downward shift of LTP expression was observed in after both GYKI and vehicle injection. The post-injection pattern was similar in FZP-withdrawn rats.
**Supplementary Figure 2.** No difference was found in the slope of \( f \)EPSPs recorded from 7-day FZP-withdrawn rats. The slope of \( f \)EPSPs was normalized to baseline before TBS was delivered. At this time point, neither glutamatergic nor GABAergic synaptic transmission was different from matched control.
Supplementary Figure 3. No significant memory deficits were found in 2-day FZP withdrawn rats. During the (A) trial and (B) familiarization phases for each memory test, there were no significant differences in the time engaged in object exploration between control and FZP-withdrawn rats. For the context trial, data are shown separately for the first (Ctx1) and second (Ctx2) familiarization phases. Mean exploration ratios, which represent the proportion of time spent on exploring novel objects during the test phase, are shown for (C) object recognition, (D) place (E) and context. The dashed line (0.5) represents no discrimination between sample and target objects. Data were shown separately for the first, second and third min of the trial and for all 3 min of the trial.
MANUSCRIPT 2

Downregulation of synaptic NR2B subunit-containing NMDA receptors: A natural brake for over-excitation of glutamatergic transmission during benzodiazepine withdrawal

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Abstract:

Excitatory glutamatergic neurotransmission is modified in rats withdrawn from 1 week oral administration of the benzodiazepine, flurazepam (FZP). NMDAR-mediated evoked EPSCs were decreased 2-fold in 2-day FZP withdrawn rats (CON, 491 ± 87 pA vs. FZP, 247 ± 43 pA, p< 0.05). The current mediated by NR2B subunit-containing NMDAR, measured by the ifenprodil-sensitive component, showed a significant reduction in the 2-day FZP withdrawn group (CON, 47.9 ± 8.5% vs. FZP, 9.5 ± 8.9%, p<0.05). NR2B subunit-containing NMDAR mediated the depression of function since blockade of ifenprodil also eliminated the difference between currents in neurons from control and 2-day FZP withdrawn rats. Decreased synaptic expression of the NR2B subunit (CON, 100 ± 7.3% vs. FZP, 69 ± 6.6%, p<0.05), along with the NR1 subunit (CON, 100 ± 2.4% vs. FZP, 85 ± 3.9%, p<0.05), may serve as the molecular basis of the depression of NMDAR currents. A lack of change in NMDAR expression levels in subfractions from 1-day FZP withdrawn rats corresponded with the absence of electrophysiological findings at this time point. Though the total charge transfer was unchanged between control and 2-day FZP-withdrawn neurons there was an increase in charge transfer mediated by AMPAR and decrease in charge transfer mediated by NMDAR. The depression of NMDAR currents was secondary to the potentiation of AMPAR since preincubation of slices with AMPA (1μM) could accelerate the depression of NR2B subunit-mediated NMDAR downregulation currents in slices from 1-day FZP- withdrawn rats, a time-point at which no NMDAR change had been observed. Collectively, these findings suggest that a
reduction of NR2B-containing NMDAR may serve as a compensatory mechanism to modulate glutamatergic synaptic strength during FZP withdrawal to alleviate benzodiazepine withdrawal symptoms.
Introduction:

N-methyl-D-aspartate receptors (NMDARs) are one of the major ionotropic glutamate receptors that consist of heteromeric complexes composed primarily of NR1, NR2 (NR2A-NR2D), and NR3 (NR3A-NR3B) subunits (Cull-Candy et al. 2001). NMDARs have been implicated in neuronal development, synaptic plasticity (Dingledine et al. 1999), excitotoxicity (Auzmendi et al. 2009) and addiction (Lau and Zukin 2007) related to their high calcium permeability. The functional NMDAR is a tetramer of two NR1 and two NR2 subunits (Cull-Candy et al. 2001; Wenthold et al. 2003). NR2A and NR2B are the two major subunits expressed in mammalian forebrain (Cull-Candy et al. 2001; Wenthold et al. 2003) but have different temporal and spatial distribution. While hippocampal NR2B immunoreactivity parallels NR1 staining, there is also considerable overlap with NR2A immunoreactivity. Immunoprecipitation studies of adult mouse forebrain indicated that only 6% of total NMDAR contained all three subunits (NR1/NR2A/NR2B) suggesting that this assembly may constitute a minor subtype. Ternary complexes containing NR1 and NR2A or NR2B subunits with NR2D subunits are rare and localized to the CA3 region (Chazot and Stephenson 1997; Thompson et al. 2002). NR2B subunits are expressed prenatally (Kutsuwada et al. 1996) and gradually increased NR2A subunit expression (Monyer et al. 1994; Sheng et al. 1994) and synaptic incorporation (Stocca and Vicini 1998; Tovar and Westbrook 1999) during development. NR2A subunits are most likely present in postsynaptic region while NR2B subunits are
majorly extrasynaptic in cortical and hippocampal pyramidal neurons (Carmignoto and Vicini 1992; Kew et al. 1998; Tovar and Westbrook 1999). Surface NMDARs are densely localized postsynaptically and more prominent at Schaffer collateral/commissural-fiber than mossy-fiber synapses (Takumi et al. 1999). Perisynaptic receptors surrounding the postsynaptic density region, are activated by high concentrations of glutamate. Extrasynaptic receptors comprise one-third of the total adult NMDAR population (Groc et al. 2009; Groc et al. 2006; Tovar and Westbrook 1999). Although the NR1 subunit plays a critical role in the release of assembled NR1/NR2 complexes from endoplasmic reticulum (Cull-Candy and Leszkiewicz 2004) and calcium-mediated inactivation of NMDARs (Zhang et al. 1998), the surface mobility (Groc et al. 2006), synaptic localization and function (Chung et al. 2004) of NMDAR are more determined by NR2 subunits through extensive interaction with postsynaptic density proteins, such as PSD-95, CaMKII, and other scaffold proteins (Wenthold et al. 2003).

NMDAR was also implicated in benzodiazepine dependence. NMDAR was downregulated in a well-established benzodiazepine withdrawal model (Van Sickle et al. 2002). The NMDA induced whole-cell currents and NMDAR mediated eEPSCs were decreased along with decreased mRNA and protein expression level of NR2B subunit in situ (Van Sickle et al. 2002). Anxiety-like behavior emerged only in Day 1 after 1 week flurazepam treatment, but when downregulation of NMDAR was blocked by MK-801 injection, anxiety was recurrent in day 2 (Xiang and Tietz 2007).
The goal of the present studies is to evaluate the possible mechanisms underlying the reduction in NMDA current in the FZP-withdrawn rats and its possible physiological significance. Whole-cell electrophysiological approaches in CA1 neuron in hippocampal slices, coupled with immunoblot techniques in postsynaptic density (PSD)-enriched fractions from CA1 minislices were used for this study. Since NR2B subunits are more mobile and experienced intensive internalization (Groc et al. 2006), they are expected to mediate the NMDAR downregulation which may counteract the AMPAR potentiation during FZP withdrawal.

Methods:

Animal:

All animal procedures were performed in accordance with protocols approved by University of Toledo Health Science Campus Animal Care and Use Committee and were consistent with the National Institute of Health animal care and use policy. Male Sprague-Dawley rats (Harlan, Indianapolis IN), P36-42 when euthanized, were first adapted 2 days to 0.02% saccharin vehicle, then offered FZP in vehicle for 1 week (100 mg/kg X 3 days; 150 mg/kg X 4 days) as their sole source of drinking water, followed by 1 or 2 days of drug withdrawal. Daily water consumption was monitored to adjust the drug concentration to offer the desired dose. Rats that did not reach a weekly average of 120 mg/kg/day were excluded. The oral bioavailability of flurazepam is such that this dosing regimen results in therapeutic brain concentrations of flurazepam in the 1.2 μM
range (0.6 μM in diazepam equivalents) similar to that achieved with intraperitoneal
ingjections without the side effects related to repeated injections (Gonsalves and Gallager 1985). Rats received saccharin water during the withdrawal period. Control rats received saccharin vehicle in parallel throughout the study. This dosing regimen reliably induces anxiety-like behavior in the elevated plus maze upon drug withdrawal (Van Sickle et al. 2004; Xiang and Tietz 2007). The expression of withdrawal-anxiety is associated with the presence or absence of depression of CA1 neurons NMDAR currents (Van Sickle et al. 2004; Xiang and Tietz 2007).

**Hippocampal slice preparation:**

Following decapitation, the hippocampus was rapidly dissected and transverse dorsal hippocampal slices (400 μm) were cut on vibratome in ice-cold, pregassed low-calcium artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 120; KCl 2.5; CaCl$_2$ 0.5; MgCl$_2$ 7.0; NaH$_2$PO$_4$ 1.2; NaHCO$_3$ 2; D-glucose 20; ascorbate 1.3; pH 7.4. The CA3 region was dissected from slices to prevent spread of epileptiform activity. Slices were maintained at room temperature for 15 min in gassed low-calcium ACSF, and then transferred to normal ACSF containing (in mM): NaCl 119; KCl 2.5; CaCl$_2$ 1.8; MgSO$_4$ 1.3; NaH$_2$PO$_4$ 1.25; NaHCO$_3$ 26; D-glucose 10; pH 7.4. Slices recovered at room temperature for >2 hr in ACSF prior to electrophysiological recording. During recording, slices were submerged and perfused at a rate of 2.5 ml/min with gassed ACSF at room temperature.
**Electrophysiological Recording:**

For NMDAR-mediated evoked EPSC recordings, hippocampal slices were continuously perfused with oxygenated ACSF and visualized on an upright Zeiss Axioskop. A stimulating electrode made from a gold-coated tungsten fiber was placed in the center of stratum radiatum, 50-100 μm from the recording electrode. Blind whole-cell patch-clamp recordings were made using borosilicate micropipettes (4-7 MΩ, WPI) from CA1 pyramidal neurons. The pipette solution contained (in mM): Cs methanesulfonate 132.5; CsCl 17.5; HEPES 10; EGTA 0.2; NaCl 8; Mg-ATP 2; Na3-GTP 0.3; QX-314 2; pH 7.2. Voltage-gated channels were inactivated by adjusting the holding potential to $V_H = +10$ mV in 10 mV increments. Stimulus intensity was determined by inducing half-maximal eEPSCs at $V_H = -20$ mV. Evoked EPSCs were recorded under voltage clamp ($V_H = +40$ mV) in the presence of 10 μM DNQX and 50 μM picrotoxin in ACSF. Ten min perfusion of ifenprodil (1 μM) was used to block NR2B subunit-containing NMDARs. Signals were amplified with an Axoclamp2A amplifier coupled to a 4-pole Bessel filter/amplifier (1 kHz, 100X, Cornerstone) and digitized online (20 kHz, Digidata 1200A, Molecular Devices) then stored on disk for later analysis.

To dissect AMPAR- and NMDAR-mediated currents, evoked EPSCs were recorded at a stimulus intensity to induce half-maximal response in the absence of Mg$^{2+}$ (NaCl 119; KCl 2.5; CaCl2 1.8; NaH$_2$PO$_4$ 1.25; NaHCO$_3$ 26; D-glucose 10; pH 7.4). First, the combined AMPAR- and NMDAR-mediated currents were recorded at $V_H = -40$ mV, in the presence of picrotoxin (50 μM). AMPAR-mediated components were obtained by 10
min application of APV (50 μM) and confirmed by further application of DNQX (10 μM). NMDAR-mediated responses reflected the difference between currents recorded with and without APV. The charge transferred by currents was measured by the area under each separate and combined curve (AUC).

Subcellular Fractionation for Immunoblots:

Subcellular fractionation was conducted at 0-4°C as previously described (Song et al. 2007). Briefly, rats were decapitated by guillotine and the brains were removed to bubbled icy ACSF. Hippocampi were quickly isolated from matched pairs of control and FZP-withdrawn rats and the CA1 region was microdissected and homogenized in ice-cold buffer containing (in mM): Tris 10, pH 7.4; Sucrose 320; EDTA 1; EGTA 1; NaF 5; Na orthovanadate 1; CsA 1 μM; Okadaic acid 0.5 mM and 1% protease inhibitor cocktail (Sigma, Pittsburgh, PA). Homogenates were centrifuged at 960 X g for 10 min to remove large debris. The supernatant (S1) was centrifuged at 10,000 X g for 30 min to obtain the crude membrane pellet (P2) and the cytosolic fraction (S2). The PSD-enriched fraction (P3) was obtained by incubating P2 pellets in Triton-homogenate buffer on ice for 20 min and centrifugation at 32,000 X g for 1 hr. Final pellets were sonicated in resuspension buffer (in mM): Tris 10, pH 8, EDTA 1, and 0.1% SDS. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Immunoblotting:

Subfractionated protein (15 μg per well) was mixed with sample buffer (Bio-RAD, Hercules, CA) plus 5% β-mercaptoethanol and running buffer (in mM): Tris base 25;
glycine 200; 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)-conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch, West Grove, PA), followed by enhanced chemiluminescence (ECL) (Denville Scientific, Metuchen, NJ). Primary antibodies used included: anti-NR1 (1:2,000 BD Biosciences, San Jose, CA), anti-NR2A (1:5,000 Millipore), anti-NR2B (1 μg/ml Millipore), and anti-actin (1:50,000, Millipore). Images of immunoblots were scanned and quantified with ImageJ. NMDAR signals were normalized to the corresponding actin signal.

**Data Analysis:**

Data were expressed as mean ± S.E.M. with n referring to number of samples. Statistical differences were assessed using unpaired Student's t test or ANOVA with Bonferroni's multiple comparison test correction, and were plotted using Prism software (GraphPad Software, La Jolla, CA). Statistical significance was set to p < 0.05.

**Results:**

**NR2B subunit-containing NMDARs mediate depression of NMDA currents after 2-day withdrawal:**

Previous studies in our lab showed that NMDAR-mediated evoked EPSCs currents elicited by NMDA application were decreased in CA1 neurons from 2-day FZP-withdrawn rats (Van Sickle et al. 2002). Our initial experiment here reproduced the
finding. The mean amplitude of NMDAR eEPSCs in the CON group was 491 ± 87 pA and 247 ± 43 pA in FZP group (p< 0.05, Fig 1A). Since levels of the NR2B subunit was decreased in 2-day FZP withdrawn rats (Van Sickle et al. 2002) and NR2B subunits are more mobile than NR2A subunits (Groc et al. 2006), it was hypothesized that a decrease in NR2B-subunit containing NMDAR are more likely to mediated the depression of NMDAR currents. Bath application (10 min) of 1 μM ifenprodil, an NR2B selective antagonist, eliminated the difference between NMDAR-mediated eEPSC amplitudes (CON, 257 ± 65 pA vs. FZP, 205 ± 29 pA, p>0.05, Fig 1A), indicating that NR2B subunit-containing NMDAR-mediated the downregulation. Representative traces of NMDAR-mediated eEPSCs are shown without ifenprodil (solid line) and with ifenprodil (broken line) (Fig 1B). The ifenprodil-sensitive component of eEPSCs, which represents the percentage of NR2B-containing NMDAR, was also calculated by the difference between the percentage change of amplitude before and after ifenprodil application. A significant decrease of the ifenprodil sensitive component was found in CA1 neurons from 2-day FZP-withdrawn rats (CON, 47.9 ± 8.5% vs. FZP, 9.5 ± 8.9%, p<0.05, Fig 1C). Finally, the average current mediated by NR2B-containing NMDAR in both CON and FZP-withdrawn rats was fitted with a one-exponential decay function. The result revealed a decreased in current amplitude, but similar kinetic properties (CON, τ = 0.15 Sec vs. FZP, τ = 0.14 Sec, Fig 1D).
Immunoblots revealed a decrease of NMDAR subunit expression in PSD-enriched subfractions from CA1 minislices of FZP-withdrawn rats:

Because the decreased of NMDAR-mediated eEPSCs is an indicator of functional change of synaptic NMDAR during FZP withdrawal, the level of NMDAR subunit expression was proposed to show a parallel change. To confirm this, a postsynaptic density (PSD)-enriched subcellular fraction was collected by stepwise centrifugation (Song et al. 2007). A significant decrease of NR2B subunit expression levels (CON, 100 ± 7.3% vs. FZP, 69 ± 6.6%, p<0.05, Fig 2A) was found in the PSD-enriched fraction collected from 2-day FZP withdrawn rats. An essential part of functional tetrameric NMDAR assemblies, the NR1 subunit was also examined in the same subfractions. Expression of the NR1 subunit was also reduced in FZP-withdrawn rats (CON, 100 ± 2.4% vs. FZP, 85 ± 3.9%, p<0.05, Fig 2B). Since NR2A and NR2B are the two major NR2 subunits in hippocampus, whether there is a compensatory increase in NR2A subunit levels, or a parallel decrease of NR2A subunits of a NR1/NR2A/NR2B heterotrimer were also taken into consideration. However the expression level of NR2A subunits in the PSD-enriched subcellular fraction was unchanged (CON, 100 ± 3.6% vs. FZP, 109 ± 9.3%, p=0.42, Fig 2C). Thus, the immunoblot results are consistent with the electrophysiology findings and strengthen the idea that the synaptic removal of the NR2B subunit, along with an obligatory NR1 subunit was underlying the depression of NMDAR currents in 2-day FZP-withdrawn rats.
Previous electrophysiological studies showed no significant change in NMDAR currents after 1-day FZP withdrawal. However, this finding does not exclude the possibility that an increase in NR2A subunits could replace NR2B subunits stabilizing total NR2 subunit expression, as with development (Cull-Candy et al. 2001). An immunoblot analysis of the levels of NMDAR subunits in PSD-enriched fractions from 1-day FZP withdrawn CA1 minislices revealed no difference among all three subunits (Fig 3).

**Relationship between AMPAR and NMDAR change during FZP withdrawal:**

The modification of NMDAR only happened in 2-day FZP withdrawn rats and pertained to NR2B-containing NMDAR. When considering the temporal sequence of AMPAR and NMDAR change, the upregulation of AMPAR was prior to NMDAR downregulation (Van Sickle et al. 2004). It seems the hyperactivity of AMPAR led to the further downregulation of NMDAR. And systematic injection of AMPAR antagonist GYKI-52466 at the onset of FZP withdrawal which prevented the upregulation of AMPAR, could also prevent the downregulation of NMDAR, giving strong support for the AMPAR contribution to NMDAR downregulation (Xiang and Tietz 2007). The progressive increased hyperactivity of AMPAR led to the downregulation of NMDAR, which may serve as a protective, negative-feedback role for neuronal over-excitation (Xiang and Tietz 2007). Therefore, we tried to see whether activation of AMPAR in 1-day FZP withdrawn rats, at this time point no obvious decrease of NMDAR was observed, could induce NMDAR’s downregulation. As expected, 30 min AMPA (1 μM) incubation significantly decreased the NMDAR-mediated eEPSC amplitude (Fig 4) and percentage.
of NR2B subunit (Fig 4) in the hippocampal slices from 1-day FZP withdrawn rats. In control rats, no significance was found for AMPA incubation.

**Downregulation of NMDAR during FZP withdrawal: a natural brake for glutamatergic over-excitation:**

The anxiety-like behavior in 1-day FZP withdrawn rats was accompanied with CA1 neuron hyperexcitability, which was evident from the significant increase in the frequency of extracellular, 4-aminopyridine (4-AP) induced spike discharges in slices (Van Sickle et al. 2004). But the anxiety-like behavior disappeared in 2-day FZP withdrawn rats without inference. Over-activation of AMPAR triggers the downregulation of NMDAR could act as negative feedback mechanism and avert the hyperactivity of glutamatergic transmission. Here, the evoked currents mediated by glutamate receptors were recorded at $V_h = -40 \text{ mV}$ in the presence of picrotoxin (50 μM). AMPAR-mediated currents were recorded with APV (50 μM) and APV sensitive components were calculated as NMDAR-mediated current. As shown in Fig 5, the average AMPAR-mediated evoked currents was increased (Fig 5B), but the decreased NMDAR current (Fig 5C) counteracted the AMPAR potentiation. Then average mix current (Fig 5A) recorded at -40 mV had the similar amplitude between control and 2-day FZP withdrawn rats. The charge transfer by combined, AMPAR- and NMDAR-mediated current showed the decreased NMDAR component can counteract the increased AMPAR component and made the total charge transfer mediated by glutamatergic synapses stable in 2-day FZP withdrawn rats.
Discussion:

**Regulation of NMDAR function:**

The dynamic regulation of NMDARs in numerous brain regions including in hippocampus (Lau and Zukin 2007) contributes significantly to activity-dependent synaptic remodeling making these excitatory receptors key postsynaptic players. NMDARs are linked with a group of scaffold and adaptor proteins in turn connected with various kinases, phosphatases and the cytoskeleton to form a large macromolecular signaling complex (Lau and Zukin 2007). Its high calcium permeability makes the NMDAR a hub of calcium-mediated signaling transduction. Upon activation, calcium-related signaling molecules, such as calmodulin, CaMKII and calcineurin exert different effects on synaptic transmission and plasticity (Xia and Storm 2005). On the other hand, transient calcium-dependent inactivation of NMDAR through nearby voltage-gated calcium channels or more effectively through NMDA channels themselves (Legendre et al. 1993), is an important step for keeping homeostasis of calcium from activity-dependent plasticity or excitotoxicity during sustained neuronal activity (Medina et al. 1999). Although NMDAR-centered macromolecular complexes are quite stable in the postsynaptic density (Ehlers 2003; Huh and Wenthold 1999), NMDAR function is subject to activity- and experience-dependent regulation by lateral movement and endocytosis (Lau and Zukin 2007). The lateral mobility of NMDARs is dependent on the NR2 subunit; NR2A-containing NMDARs more stable and NR2B-containing NMDARs more mobile within the synapse. Maturation of NMDARs is manifested by increased
insertion of NR2A subunits and decreased lateral mobility (Groc et al. 2006). Clathrin-mediated endocytosis can remove NMDARs from the cell surface for either recycling or degradation. Both NR1 and NR2 have conserved endocytic motifs in their proximal C-termini, necessary and sufficient to mediate endocytosis (Lau and Zukin 2007). In addition, the distal C-termini of NR2 subunits have various motifs associated with different rates of endocytosis. The NR2B subunit YEKL motif can bind with the clathrin adaptor protein 2 (AP2) and promote robust endocytosis; while for the NR2A subunit, a LL motif introduces a slow rate of endocytosis (Lau and Zukin 2007). Thus, both lateral movement and endocytosis can affect the synaptic retention of NMDARs and in turn affect its function.

**FZP-withdrawal-induced NMDAR downregulation:**

A role for NR2B subunit regulation was previously shown in another anxiety model which used Pavlovian contingencies to induce fear and anxiety. Fear conditioning results in LTP-like activation of AMPA receptors in the lateral amygdala. Blockade of NR2B-containing NMDAR with the NR2B-selective antagonist ifenprodil (Rodrigues et al. 2001; Zhang et al. 2008a; Zhang et al. 2008b) or genetic interference with overexpressed C-terminal of NR2B subunit (Zhang et al. 2008a) disrupted the acquisition of fear conditioning. Moreover, maintenance of fear conditioning was associated with decreased expression of NR2A and NR2B subunits proposed to guard against enhanced glutamatergic strength or to serve as a mechanism to rapidly reactivate emotional memories through rapid NMDAR recycling (Zinebi et al. 2003). A similar NR2-mediated
mechanism may be related to the regulation of anxiety-like behavior involving hippocampal circuits (Van Sickle et al. 2004). In 2-day FZP withdrawn rats, a 49.7% decrease in NMDAR-mediated evoked eEPSCs was found (Fig 1A). A similar-fold decrease in NMDA induced whole-cell currents was associated with a decrease in NR2B subunit mRNA and protein (Van Sickle et al. 2002) in situ. Given the previous findings and the greater mobility of synaptic NR2B subunits (Groc et al. 2006), we predicted that a reduction in NMDA function in FZP-withdrawn rats could be mediated by NR2B-containing NMDARs. Indeed, current mediated by NR2B-containing NMDARs, manifested by the ifenprodil-sensitive component, was significantly decreased (Fig 1C/D). Furthermore, NR2B-containing NMDAR underwent the NMDAR downregulation because blockade by ifenprodil eliminated the NMDAR eEPSC difference between control and 2-day FZP withdrawn rats (Fig 1A).

The findings of immunoblot studies of PSD-enriched subcellular fractions matched the electrophysiological findings in CA1 neurons from 1-day and 2-day FZP withdrawn rats. In 1 day FZP-withdrawn rats, there were no changes in NMDAR-mediated eEPSCs (Van Sickle et al. 2004) or of NMDAR subunit expression levels (Fig 3). The 1-fold reduction in ifenprodil currents in neurons from 2 day withdrawn rats were consistent with a decreased level of NR1 (Fig 2A) and NR2B subunit expression (Fig 2B). The possibility of a replacement of NR2B by NR2A subunits was excluded since NR2A expression levels (Fig 2C) were unchanged. The immunoblot results corroborated the electrophysiological findings that decreased NR2B subunit underlie NMDAR
downregulation. Hence, the removal of functional heteromeric NR1/NR2B receptors might serve as molecular basis for the decrease NMDAR eEPSCs during FZP withdrawal.

**Possible mechanism for NR2B removal:**

NMDAR responses are shaped by several mechanisms during and following normal or heightened receptor activity. For example, NR2 subunits fine-tune NMDAR functional expression with NR2B subunit-containing assemblies generating more prolonged currents than those which contain NR2A subunits (Monyer et al. 1994). In recombinant receptors a form of receptor desensitization, calcium-dependent inactivation mediated by calmodulin, transiently affects the interaction between the NR1 subunit and the actin cytoskeleton serving as a potential negative feedback mechanism to regulate NMDAR function (Ehlers et al. 1996; Zhang et al. 1998). In hippocampal cultures a single action potential was unable to result in inactivation of NMDAR-mediated EPSCs, whereas Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels or NMDAR, initiated by depolarizing steps selectively reduced NMDAR-mediated EPSCs (Medina et al. 1999; Rosenmund et al. 1995). While the latter effect was transient, α-actinin which served as the NR1 binding partner might also be involved in the tethering and subsequent relocalization of heteromeric NMDAR during synaptic activity (Wyszynski et al. 1997).

The removal of NMDAR from synapses requires several steps: 1) disruption of the interaction with scaffold proteins; 2) lateral movement; 3) clathrin-mediated internalization from extrasynaptic sites (Groc et al. 2009). α-Actinin can interact with the NR1 and NR2B subunit and may bridge the binding of the NMDAR with the actin
filament (Krupp et al. 1999; Wyszynski et al. 1997), to facilitate NMDAR clustering in dendritic spines (Rao et al. 1998). Ca\(^{2+}\) signaling through calmodulin by competing with \(\alpha\)-actinin to bind NMDAR, could in turn release the NMDAR from actin cytoskeleton. Since NR2B are more mobile (Groc et al. 2006) and thus more likely to internalize (Lavezzari et al. 2004), loss of the NR1’s interaction with actin filament could increase the removal of NR2B-containing NMDAR upon calcium accumulation (Fig 2).

Several indirect lines of evidence suggest that calcium influx may be elevated in CA1 neurons from FZP-withdrawn rats. First, electrophysiological evidence suggested that Ca\(^{2+}\)-permeable homomeric GluR1 AMPARs are responsible for the progressive potentiation of AMPAR currents at CA1 neuron synapses (Song et al. 2007). These findings were further supported by the increased synaptic expression of GluR1, but not GluR2 subunits measured using electron microscopic techniques (Das et al. 2008). After 1-day withdrawal, newly inserted AMPARs might also turn silent synapses into active synapses resulting in more calcium accumulation. These possibilities were supported by the findings that either systemic injection of AMPAR or NMDAR antagonists can block NMDAR downregulation in 2-day FZP withdrawn rats (Van Sickle et al. 2004; Xiang and Tietz 2007). The hypothesis that GluR1 homomers contribute to calcium accumulation was evaluated by activation of CA1 neuron AMPARs from 1-day FZP withdrawn rats (Fig. 4). Preincubation (0.5 hr) of hippocampal slices with 1 \(\mu\)M AMPA induced significant reduction in NMDA currents (Fig 4), at a time when changes in NMDA eEPSCs were undetectable in both groups (Van Sickle et al. 2004).
findings implied that additional calcium influx through activation of newly inserted calcium-permeable GluR1 homomeric AMPARs might play an important role to induce NMDAR downregulation. Importantly, voltage-gated calcium channel (VGCC) activation might also play an important role in calcium accumulation and thus regulation of CA1 neuron NMDAR function in FZP-withdrawn rats. A two-fold increase in VGCC-mediated current density emerged upon withdrawal from one week FZP treatment and lasted up to two days (Xiang et al. 2008).

**Physiological implication of NMDAR downregulation:**

NMDARs composed of NR2A or NR2B subunit have high conductance and are sensitive to Mg\(^{2+}\) blockade (Cull-Candy et al. 2001), but NR2B-containing NMDAR has much longer deactivation time span compared with NR2A (Cull-Candy et al. 2001), which means NR2B-containing NMDARs can introduce more calcium influx during synaptic transmission once they are activated. In fact, NR1/NR2B heterodimer could carry more charge for a single synaptic event than NR1/NR2A heterodimer (Erreger et al. 2005) and calcium imaging studies implied NR2B-containing NMDAR could introduce more calcium influx per unit of current (Sobczyk et al. 2005). Moreover, NR2B subunits with their long intracellular tail can bind to Ca\(^{2+}/\text{calmodulin-dependent protein kinase II (CaMKII)}\), and further recruit PSD95, SynGAP, nNOS, etc, to form a signaling complex at the postsynapse (Kennedy 2000). CDI was proposed to have significance in avoiding excitotoxicity during sustained neuronal activity (Medina et al. 1999), inactivation and/or removal of NR2B-containing NMDAR can significantly reduce calcium influx and its
downstream cascades. This could explain why blockade of NR2B-containing NMDAR could lead to the loss of fear conditioning, an anxiety model (Rodrigues et al. 2001), and it also could be the reason in the loss of anxiety like behavior in 2-day FZP withdrawn rats (Van Sickle et al. 2004). The hyperexcitability was present in 1-day withdrawn rats measured by 4-AP induced action potential frequency, which is parallel to anxiety-like behavior (Van Sickle et al. 2004). Once the NR2B-containing NMDAR was downregulated in 2-day FZP withdrawn rats, it will not only counteract the hyperactivity mediated by AMPAR upregulation when measured by charge transfer during glutamatergic transmission (Fig 5), but also lead to the disappearance of anxiety-like behavior (Van Sickle et al. 2004). Interestingly, similar NR2B downregulation was also found in synaptic site of striatal neuron from chronic amphetamine exposure rats (Mao et al. 2009) and nociceptive synapses of spinal cord during chronic peripheral inflammation hypersensitivity, along with the increased calcium permeable AMPAR (Vikman et al. 2008).

The NR2B subunit mediated NMDAR downregulation during FZP withdrawal may act as a compensatory feedback mechanism to protect the neuron from overactivation. Reduction of NMDAR would counteract the hyperactivity of AMPAR and tune down the glutamatergic synaptic transmission in 2-day FZP withdrawn rats (Fig 5). This negative feedback mechanism could act as the “fuse” to protect the neuron from over-activation during the FZP withdrawal.
References:


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Amp of NMDAR eEPSC (pA)

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B

- w/o ifen
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CON

FZP

Ifenprodil sensitive component (%)

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D

Amplitude of Ifenprodil-sensitive component (pA)

- CON: τ = 0.15S
- FZP: τ = 0.14S

Time (sec)

0.00 0.25 0.50 0.75 1.00
Fig. 1 NR2B subunits contributed to a reduction of NMDAR-mediated evoked EPSCs in CA1 neurons on Day 2

(A) The amplitude of NMDAR-mediated evoked EPSCs was decreased in CA1 neurons from 2-day FZP withdrawn rats, an effect abolished by ifenprodil, a NR2B subunit-selective antagonist. (B) Representative traces recorded before (solid line) and after (dotted line) ifenprodil. Upper panel: eEPSCs recorded in CA1 neurons from a control rat; Lower panel: eEPSCs recorded from a 2-day FZP-withdrawn rat. (C) The amplitude of the ifenprodil-sensitive component was significantly decreased in neurons from 2-day FZP-withdrawn rats. (D) Single-exponential decay fit of the average ifenprodil-sensitive current showed the similar decay time-constant (CON: $\tau=0.15$ Sec vs FZP: $\tau=0.14$ Sec), which suggested they were mediated by NR2B subunit. (* $p<0.05$)
Fig. 2 Decreased NR1 and NR2B subunit expression levels in PSD-enriched fractions from CA1 minislices on Day 2

Representative blots of NR1 (A), NR2A (B) and NR2B (C) subunit protein levels were shown in the left panel; quantitative analyses of signal optic density were shown in the histograms in the right panel. NR1 and NR2B subunit levels showed significant decreases in subfractions from 2-day FZP withdrawn rats (* p<0.05)
Fig. 3 NMDAR subunits expression levels in PSD-enriched subfractions were unchanged on Day 1

Representative blots of NR1 (A), NR2A (B) and NR2B (C) subunit protein levels were shown in the left panel; quantitative analyses of optical signal density were shown in the histograms in the right panel. No significance differences were found among any subunit levels between experimental groups.
Fig. 4 AMPA preincubation accelerated NMDAR downregulation mediated by the NR2B subunit in slices from 1-day FZP-withdrawn rats.

(A) Incubation (30 min) of hippocampal slices in 1μM AMPA led to a decrease of NMDAR-mediated eEPSC amplitude in CA1 neurons from 1-day FZP withdrawn rats, as in 2-day FZP withdrawn rats, but not in neurons from control, control+AMPA or 1-day FZP-withdrawn rats. (B) Blockade of NR2B-containing NMDAR with ifenprodil diminished the effect of AMPA incubation in slices from 1-day FZP withdrawn rats. (C) The ifenprodil-sensitive component was significantly decreased in slices from 1-day FZP withdrawn rats preincubated with AMPA. (D) Average traces of ifenprodil-sensitive currents in neurons from control (solid black line), control+AMPA (solid gray line), 1-day FZP (black dotted line) and 1-day FZP+AMPA (gray dotted line) slices indicated that AMPA incubation promoted a decrease in NR2B-containing NMDAR currents in 1-day FZP-withdrawn rats.
Fig. 5 Decreased NMDAR function counteracted the overexcitation mediated by AMPAR potentiation in CA1 neurons from 2-day FZP withdrawn rats

(A) Average traces of eEPSCs recorded in ACSF without Mg$^{2+}$ at $V_H = -40$mV using a stimulus intensity to elicit a half-maximal response. The combined current mediated by both AMPAR and NMDAR showed no differences between neurons from control and 2-day FZP-withdrawn rats. (B) When dissecting AMPAR-mediated current using APV, a significant increase of the charge transfer mediated by AMPAR was found in neurons from 2-day FZP withdrawn rats. (C) On the contrary, charge transfer mediated by NMDAR showed a significant decrease in neurons from 2-day FZP withdrawn rats. (D) While charge transfer mediated by combined AMPAR and NMDAR currents was unchanged in CA1 neurons from 2-day FZP withdrawn rats, AMPAR-mediated charge transfer increased and NMDAR-mediated charge transfer decreased (* p<0.05).
CONCLUSIONS:

1. AMPA receptor was potentiated in the 2-day FZP withdrawn rats, manifested as:
   a. The amplitude of AMPAR-mediated mEPSCs got ~30% increase, without the change of resting membrane potential, the frequency and kinetic properties;
   b. Estimated single-channel conductance derived from mEPSCs recording showed a ~2 fold increase;
   c. Increased slope of input-output curve

2. Postsynaptic CaMKII’s activity is required for the AMPAR potentiation, since blockade of CaMKII by KN-93 or autocamtide inhibitory peptide, either extracellularly or intracellularly, could reverse the AMPAR potentiation in 2-day FZP withdrawn rats.

3. The specific antagonist for GluR1 homomeric AMPAR, NAS, could block more AMPAR-mediated current in 2-day FZP withdrawn rats, which implied more GluR1 homomeric AMPAR presented in synapses.

4. Immunoblot of PSD-enriched subcellular fraction revealed the increased expression levels of total GluR1 and phospho-ser831 GluR1 in 2-day FZP withdrawn rats, along with the increased CaMKII expression level.

5. For the 1-day FZP withdrawn rats, only GluR1 expression level was increased, along with a ~15% increase of mEPSCs amplitude. No increased phosphor-ser831 GluR1 or single-channel conductance was found at this time point.
6. The expression of LTP was occluded in 2-day FZP withdrawn rats, measured by fEPSP (conducted by Mahmoud Mohamed) and whole-cell currents.

7. Behavior tests based on novelty preference paradigm did not find the significant difference between control and 2-day FZP withdrawn rats.

8. NMDAR-mediated eEPSCs were decreased in 2-day FZP withdrawn rats. It was mediated by NR2B subunit since ifenprodil, a NR2B specific antagonist, could eliminate the difference between control and 2-day FZP withdrawn rats.

9. Both NR2B mediated current and NR1/NR2B expression levels were decreased in 2-day FZP withdrawn rats, indicated the removal of NR2B-containing NMDAR from synapses during FZP withdrawal.

10. AMPA incubation of hippocampal slices from 1-day FZP withdrawn rats could accelerate NMDAR downregulation happened in the 2-day FZP withdrawn rats.

11. The downregulated NMDAR could counteract the upregulated AMPAR by measuring the charge transfer in the 2-day FZP withdrawn rats.
SUMMARY:

Benzodiazepines are allosteric modulators of the GABA_A receptor. By increasing GABA binding affinity and chloride channel open probability, BZs can facilitate GABA_A receptor-mediated inhibition (Macdonald and Olsen, 1994; Olsen and Tobin, 1990). BZs are widely prescribed as sedative, hypnotics, anticonvulsant and anxiolytics, but long term use of BZs is associated with a tendency to develop tolerance and dependence, which limits their clinical utility (Griffiths and Johnson, 2005).

Unlike BZ tolerance, which is directly linked to the dysfunction of the GABAergic system, BZ dependence may be related to changes in both GABAergic and glutamatergic systems (Allison and Pratt, 2003; Wafford, 2005). Both excitatory glutamate receptor-mediated AMPAR- and NMDAR-dependent mechanisms were previously shown to be involved in the expression of BZs dependence (Dunworth and Stephens, 1998; Koff et al, 1997; Steppuhn and Turski, 1993; Tsuda et al, 1998; Van Sickle et al, 2004; Xiang and Tietz, 2007).

Using a well-established animal model of BZ dependence (Das et al, 2008; Song et al, 2007; Van Sickle et al, 2002a; Van Sickle and Tietz, 2002b; Van Sickle et al, 2004; Xiang et al, 2008; Xiang et al, 2007), our lab previously found that hippocampal CA1 neuron glutamate receptors underwent two different types of modulation upon BZ withdrawal: 1) AMPAR potentiation manifested as: increased glutamate-induced whole-cell currents in dissociated CA1 neurons (Song et al, 2007); increased AMPAR-mediated
mEPSCs in slices (Van Sickle et al., 2002b; Xiang et al., 2007); a shift of the GluR1 subunit-mediated rectification index (Song et al., 2007); increased GluR1 subunit expression using immunoblot techniques (Song et al., 2007); and increased GluR1, but not GluR2 immunogold reactivity at CA1 synapses using EM techniques (Das et al., 2008). 2) Depression of NMDAR function was characterized by: decreased NMDA-induced whole-cell currents in dissociated CA1 neurons (Van Sickle et al., 2002a); decreased NMDAR-mediated eEPSCs (Van Sickle et al., 2004); and decreased NR2B mRNA and protein expression in situ (Van Sickle et al., 2004).

The appearance of anxiety-like behavior in FZP-withdrawn rats, measured using the elevated plus-maze, implied that the expression of anxiety was a self-limiting process since anxiety-like behavior was only measurable in 1-day, but not 2-day withdrawn rats (Van Sickle et al., 2004; Xiang et al., 2007). Although there were some clues about the relationship between the regulation of these two subtypes of glutamate receptors’ and anxiety from this previous work in our laboratory (Van Sickle et al., 2004; Xiang et al., 2007), the precise mechanisms that underlie the potentiation of AMPAR, and the depression of NMDAR function were still unclear.

This dissertation provides additional evidence to show how glutamatergic synaptic transmission was modified during BZ withdrawal and explored possible underlying mechanisms. Synapses are specialized structures which relay information from neuron to neuron or from neuronal to non-neuronal cells. The physiological significance of these functional connections are better understood for perception, behavior, and learning/
memory. Here we showed that synaptic AMPARs and NMDARs undergo different types of drug-induced modulation upon BZ withdrawal, i.e. AMPAR function was potentiated and NMDAR function was depressed. The first part of these studies further elucidated a CaMKII-mediated mechanism of AMPAR upregulation after BZ withdrawal. The dual roles of CaMKII, both to promote GluR1 subunit incorporation and subsequent GluR1 subunit phosphorylation at Ser\textsuperscript{831}, contributed to AMPAR potentiation and resembled the kinase’s action in activity-dependent plasticity. The activation of the CaMKII-mediated signal transduction pathway, which could lead to phosphorylation of the AMPAR GluR1 subunit and potentiation of glutamatergic transmission, appeared to be highly conserved in mediating both activity-dependent plasticity and drug-induced adaptations. The second series of studies also clarified that the depression of NMDAR function was mediated primarily by NR2B subunit-containing NMDARs, and that it could counteract AMPAR hyperactivity during FZP withdrawal. A homeostatic regulation hypothesis was proposed to explain the appearance of anxiety-like behavior in 1-day FZP-withdrawn rats and its disappearance of in 2-day FZP withdrawn rats.

The first manuscript was devoted to the study of AMPAR potentiation and its upstream kinase, CaMKII, during FZP withdrawal. First, AMPAR potentiation was supported by several pieces of experimental evidence: the amplitude of AMPAR-mediated mEPSCs was elevated in 2-day FZP withdrawn rat which agreed with previous findings (Van Sickle et al, 2002b); nonstationary noise analysis from these whole-cell recordings revealed a ~2 fold increase of single-channel conductance consistent with the amplitude
change; the slope of an input-output curve (derived from the plot of stimulus intensity versus AMPAR-mediated eEPSC amplitude) increased suggesting that the efficacy of synaptic transmission was increased and also corroborated other findings of increased synaptic strength following FZP withdrawal. In particular, the enhancement of single-channel conductance resembled what occurs to AMPAR function with other forms of synaptic plasticity, where CaMKII is a key player in mediating AMPAR potentiation. The importance of CaMKII for AMPAR potentiation during FZP withdrawal was then studied using a variety of CaMKII inhibitors. Both extracellular, bath application of the CaMKII inhibitor KN-93 and intracellular application of autocamtide inhibitory peptide II (AIP) reversed withdrawal-induced AMPAR potentiation, when evaluating both measures of mEPSC amplitude and estimated single-channel conductance.

The possible mechanism for CaMKII potentiation of AMPAR function was also explored. The GluR2 subunit has a dominant role to control expression of AMPAR conductance, yet only GluR2-lacking AMPARs can demonstrate increased single-channel conductance through Ser\textsuperscript{831} phosphorylation of the GluR1 subunit (Oh and Derkach, 2005). Both inward rectification (Song et al, 2007) and increased NAS blockade of AMPAR-mediated mEPSCs suggested an increased expression of GluR1 homomorphic AMPARs at CA1 synapses, a hypothesis supported using EM techniques (Das et al., 2008). To evaluate whether a similar sequence of events occurred during FZP withdrawal, the synaptic expression pattern of GluR1, phospho- Ser\textsuperscript{831} GluR1, CaMKII and phospho- Thr\textsuperscript{286} CaMKII was examined in 1-day and 2-day FZP-withdrawn rats by
immunoblotting PSD-enriched subcellular fractions of CA1 minislices. In 2-day FZP withdrawn rats, when the single-channel conductance of AMPARs was elevated, both GluR1 and phospho-Ser\textsuperscript{831} GluR1 had increased levels of expression, along with increased expression of CaMKII in the PSD-enriched subcellular fraction. On the contrary in 1-day FZP withdrawn rat when no increased AMPAR conductance was observed, only GluR1 subunit expression levels were increased. These findings offer a molecular basis for AMPAR potentiation during FZP withdrawal. Similar to what occurs during activity-dependent plasticity, the modulation of AMPARs could follow a two-step process during FZP withdrawal: increased synaptic incorporation of GluR1 homomeric AMPARs on day 1 of withdrawal, followed by CaMKII-mediated phosphorylation of Ser\textsuperscript{831} GluR1 leading to increased single-channel conductance on day 2. The LTP occlusion experiment, which was used to clarify the role of CaMKII to enhance synaptic transmission through the same mechanism as LTP (Lledo et al, 1995), was employed to evaluated possible consequences of CaMKII’s phosphorylation of GluR1. The expression of LTP was occluded in hippocampal slices from 2-day FZP withdrawn rats, measured by fEPSP recordings (carried out by Mahmoud Mohamed) and whole-cell eEPSC recordings. Despite the impaired LTP maintenance, an assessment of memory using a novelty preference paradigm indicated no significant differences in object recognition, place or contextual memory between control and 2-day FZP withdrawn rats. Although AMPAR potentiation showed a progressive increase in the first 2 days of withdrawal from FZP treatment, the depression of NMDAR function only occurred on
day 2 of withdrawal (Van Sickle et al, 2002a). NMDAR downregulation coincidently occurred with the disappearance of anxiety-like behavior in 2-day FZP-withdrawn rats. The NR2B subunit, which is required for the acquisition of fear conditioning (Rodrigues et al, 2001; Zhang et al, 2008a; Zhang et al, 2008b) and undergoes fast lateral movement and robust endocytosis (Groc et al, 2006; Lau and Zuki, 2007), was proposed as the molecular basis of the decreased NMDAR currents. NR2B-mediated components were dissected from NMDAR eEPSCs with the specific antagonist, ifenprodil and showed a significant decrease in CA1 neurons from 2-day FZP-withdrawn rats. The findings suggested that NR2B subunit-containing NMDARs mediated the depression of NMDAR function since blockade by ifenprodil could eliminate the differences in NMDAR currents between control and 2-day FZP withdrawn rats. Parallel to the electrophysiological findings, immunoblot analyses also revealed a decreased level of expression of the NR2B subunit in PSD-enriched subcellular fractions, along with decreased levels of the obligatory NR1 subunit. Unlike subunit changes associated with development, the replacement of the NR2B subunit by the NR2A subunit was excluded since the expression level of NR2A was unchanged.

Previous findings identified several sources that could lead to calcium accumulation in the postsynaptic CA1 neuron, which may contribute to AMPAR potentiation and NMDAR downregulation. During 1-day withdrawal from FZP treatment: 1) increased synaptic insertion of calcium-permeable GluR1 homomeric AMPARs (Das, et al., 2008; Song et al., 2007), 2) which could turn silent NMDAR-containing synapses into active
synapses containing homomeric AMPARs, 3) as well as increased voltage-gated calcium channel currents (Xiang et al., 2008), provide multiple routes for calcium influx. Further, activation of CaMKII on day 2 could accelerate calcium accumulation by increasing GluR1 homomeric AMPAR conductance (Oh and Derkach, 2005). As a possible consequence of calcium accumulation, calcium-dependent inactivation of NMDARs mediated by calmodulin, could affect the interaction between the NR1 subunit and the actin cytoskeleton. Calcium-dependent inactivation would not only decrease NMDAR current providing transient negative feedback regulation of NMDAR function but also initiate the removal of NR2B-containing NMDAR away from synapses. Newly inserted GluR1 homomeric AMPAR are likely critical for calcium accumulation since preincubation of hippocampal slices from 1-day FZP withdrawn rats with 1 \( \mu \text{M} \) AMPA to activate AMPARs accelerated downregulation of NMDARs at a time when NMDAR downregulation was not previously detected. This finding implied that depression of NMDAR function was secondary to increased AMPAR activity. Thus, GluR1-containing AMPARs are proposed to contribute to calcium accumulation; a hypothesis which will be tested in future experiments. As measured by the charge transfer of inward current the depression of NMDAR function could counteract AMPAR hyperactivity in 2-day FZP withdrawn rats, which could result in compensatory feedback regulation of CA1 neuron hyperactivity (Van Sickle et al, 2004) and a possible mechanism for the disappearance of anxiety-like behavior.
Taken together, a possible scenario is proposed to occur during FZP withdrawal, with calcium as the leading actor: in 1-day FZP withdrawn rats, the increased synaptic insertion of GluR1 subunit might form GluR1 homomeric AMPARs in asymmetric synapses and convert some silent, NMDAR-only synapses into active synapses. Enhanced depolarization through calcium permeable AMPARs, combined with the potentiation of voltage-gated calcium channel currents (Xiang et al, 2008), would provide additive calcium sources leading to calcium accumulation in postsynaptic neurons. Accumulation of calcium could reach a level to activate CaMKII on day 2. Active CaMKII would in turn phosphorylate the homomeric GluR1 subunit at Ser\(^{831}\) and significantly increase AMPAR conductance. The positive modulation of AMPAR single-channel conductance would render a greater degree of calcium influx through AMPARs. This LTP-like positive feedback signal could build-up postsynaptic calcium levels further on day 2. When calcium concentration becomes sufficiently high, calcium-dependent inactivation of NMDARs could be triggered. High concentration of calcium could bind with calmodulin, which would compete with \(\alpha\)-actinin for the binding of the NR1 or NR2B subunit of NMDAR and inactivate NR1/NR2B-containing NMDAR which would lead to the removal of NMDAR from synaptic region. Thus downregulation of NMDARs might act as a “fuse” to protect CA1 neurons from over-activation during FZP withdrawal. Unlike CaMKII-mediated, LTP-like AMPAR changes, which have the tendency to destabilize the activity of neural circuitry (Turrigiano and Nelson, 2004), this
downregulation of NMDAR function would tend to maintain the homeostasis of neural activity.
BIBLIOGRAPHY:


ABSTRACT:

BZ withdrawal can lead to adaptive modification of glutamatergic synaptic transmission in hippocampal CA1 neurons. Two subtypes of glutamate receptors, AMPAR and NMDAR, showed different patterns of modification upon BZ withdrawal. The possible mechanisms and intrinsic relationship of these two receptors during BZ withdrawal were studied in this dissertation.

AMPA-mediated mEPSC amplitudes were increased 30% in CA1 neurons with a 2-fold increase in single-channel conductance. The potentiated AMPAR-mediated synaptic transmission was also manifested by an increased slope of the input-output curve. CaMKII’s contribution to the potentiation was validated by pre-incubation of slices with the selective inhibitor, KN-93, or intracellular inclusion of AIP, both of which prevented the increases of AMPAR current amplitude and conductance. Increased NAS inhibition was consistent with synaptic incorporation of homomeric GluR1 AMPARs. In 1-day withdrawn rats, only GluR1 levels were increased in immunoblots of the PSD-enriched subcellular fraction from CA1 minislices consistent with increased mEPSC amplitude, but not conductance. In 2-day withdrawn rats, total, but not relative phospho-Thr286 CaMKII levels increased in the PSD-enriched subfraction in parallel with increased GluR1 and phospho-Ser831 GluR1 expression levels implying that CaMKII mediates AMPAR phosphorylation and increased channel conductance in FZP-withdrawn CA1 neurons. Whole-cell and field (f)EPSP recordings revealed that LTP expression, induced
by low-intensity theta burst stimulation, was impaired in CA1 neurons from FZP-withdrawn rats although no memory deficits were detectable using a novelty preference paradigm. The findings suggest that synaptic insertion and subsequent CaMKII-mediated phosphorylation of homomeric GluR1 AMPARs might contribute to BZ withdrawal-induced potentiation of AMPARs analogous to mechanisms underlying activity-dependent plasticity.

NMDAR function was depressed during BZ withdrawal. The contribution of the NR2B subunit to NMDAR downregulation was indicated both by the effect of ifenprodil on NMDAR currents and by the decreased expression level of both NR1 and NR2B, but not NR2A subunits in PSD-enriched fractions from 2-day FZP-withdrawn rats. The depression of NMDAR currents was secondary to AMPAR potentiation, since AMPA incubation could accelerate the reduction in NMDAR currents in 1-day FZP withdrawn rat, and could counteract the hyperactivity of AMPAR when total charge transfer was measured. Calcium-related homeostatic regulation through calcium-permeable AMPARs and L-type voltage-gated calcium channels was proposed to explain the possible CaMKII/calmodulin-mediated mechanisms underlying the enhancement of AMPA and depression of NMDAR currents, and their relationship to the appearance of withdrawal anxiety.