Protein kinase A alterations following chronic flurazepam treatment: implications for inhibitory and excitatory synaptic plasticity in rat hippocampal CA1

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Protein Kinase A Alterations Following Chronic Flurazepam Treatment: Implications for Inhibitory and Excitatory Synaptic Plasticity in Rat Hippocampal CA1

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

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Protein Kinase A Alterations Following Chronic Flurazepam Treatment: Implications for Inhibitory and Excitatory Synaptic Plasticity in Rat Hippocampal CA1

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2006
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INTRODUCTION

In 1957, while screening a compound produced years earlier for tranquilizing effects, Dr. Leo Sternbach discovered the first biologically active benzodiazepine (Randall et al., 1960; Sternbach, 1972). This class of drugs proved to have significant advantages over their immediate predecessors, providing effective anxiolysis, sedation, and seizure control without the respiratory suppression or physical dependence characteristic of barbiturates (Busto and Sellers, 1991; Gasser et al., 1975). Phase III testing was quickly completed, and the newest central nervous system (CNS) depressant, chlordiazepoxide (Librium), was marketed by Hoffman-LaRoche, Inc. in 1960. The development of analogues ensued, and the introduction of more potent (diazepam, Valium, 1963), short-acting (oxazepam, Serax, 1965), and water-soluble (midazolam, Versed, 1976) benzodiazepines soon followed. In the mid-1970’s, popular culture references to “Executive Excedrin,” and “Mother’s little helpers” abounded as the benzodiazepines became the most widely-prescribed and recognized drugs (Koerner, 1999). However, widespread availability soon led to concerns of benzodiazepine misuse. Liz Taylor admitted a liking for the combination of Jack Daniels and Valium, a preference also evinced by Elvis Presley’s post-mortem toxicology report. Nevertheless, when used in the appropriate clinical situation and patient population, benzodiazepines are safe and not commonly abused, remaining first-line drugs for the treatment of insomnia, anxiety, alcohol withdrawal, and status epilepticus.
The mechanism of benzodiazepine action was not elucidated until well after they had entered clinical use. Benzodiazepines increase inhibition in spinal cord neurons and in the cerebellum, and require endogenous γ-aminobutyric acid (GABA) for activity, data that suggested benzodiazepines potentiate GABAergic inhibition in the CNS (Costa et al., 1975; Haefely et al., 1975). These ideas were advanced with binding studies indicating that the prototype benzodiazepine, diazepam, binds with selectivity and high affinity to a stable protein in neuronal membranes (Braestrup and Squires, 1977; Mohler and Okada, 1977), identical to the GABA<sub>A</sub> receptor (Chang et al., 1981; Olsen, 1981; Schoch et al., 1985; Sigel and Barnard, 1984; Sigel et al., 1983). Moreover, the binding sites for benzodiazepines and GABA on the GABA<sub>A</sub> receptor complex are “coupled,” meaning binding of one ligand increases binding of the other. In this manner, benzodiazepines allosterically increase the affinity of the receptor for GABA, thus increasing the frequency of Cl<sup>-</sup> channel opening, and selectively enhance inhibitory neurotransmission; this is the basis for the sedative, anxiolytic, and anticonvulsant effects of this class of drugs (Olsen, 1981; Paul et al., 1981; Rogers et al., 1994).

As the mechanism of benzodiazepine action was being elucidated, their limitations in clinical practice were becoming apparent. Though not as pervasive in comparison to previous CNS depressants (Busto and Sellers, 1991; Khanna et al., 1998), tolerance and dependence, in fact, do occur with repeated exposure to the benzodiazepines and affect their clinical utility. Drug tolerance is characterized by reduced effectiveness upon repeated drug
administration, such that increasing doses are required to elicit equivalent results. Benzodiazepine tolerance restricts the usefulness of the agents as anticonvulsants to acute situations (Browne, 1976). Drug dependence often manifests as symptoms of withdrawal upon drug discontinuation (weight loss, insomnia, hyperactivity, anxiety, convulsions), and abatement of these symptoms with drug re-exposure (Schoch et al., 1993). Benzodiazepine dependence in humans is of notable concern when these drugs are used to treat patients with a history of substance abuse (Greenblatt and Shader, 1978; Griffiths and Weerts, 1997; Kalant et al., 1971; Rosenbaum, 2004; Woods et al., 1987).

To investigate the adaptive mechanisms occurring after chronic benzodiazepine exposure, presumably underlying tolerance and dependence, our laboratory utilizes a 1-wk treatment with the water-soluble benzodiazepine flurazepam (FZP). Rats are acclimated to saccharin water prior to offering FZP in a saccharin water vehicle (100 mg/kg x 3d, 150 mg/kg x 4d). Controls receive saccharin water throughout. This treatment reliably induces tolerance to the anticonvulsant effects of diazepam (Rosenberg et al., 1985, 1991), an expression accompanied by the in vitro uncoupling of benzodiazepine/GABA binding sites, downregulation of benzodiazepine binding, and downregulation of GABA<sub>A</sub> receptor mRNA and subunit protein (Chen et al., 1999; Chen and Tietz, 1995; Rosenberg et al., 1991; Tietz et al., 1986, 1989, 1993, 1999a, Zeng et al., 1995). Relatively large decreases in benzodiazepine receptor number occur in the hippocampal CA1 region of the rat brain following chronic
FZP treatment, an area that expresses a variety of GABA<sub>A</sub> receptor subunit subtypes, has well-defined GABAergic circuitry, and is important in several well common seizure models (Amaral and Witter, 1995; Maru et al., 2002; Oliver et al., 1977; Rosenberg and Chiu, 1981; Sieghart and Sperk, 2002; Tietz et al., 1986). In addition to the expected decrease in benzodiazepine responsiveness in the tolerant rat (Xie and Tietz, 1992; Zeng and Tietz, 1999) the principal cells of hippocampal CA1 region also display decreased postsynaptic GABA<sub>A</sub> receptor function (Poisbeau et al., 1997; Zeng et al., 1995; Zeng and Tietz, 1999). Exposure to the benzodiazepine antagonist, flumazenil (FLM), restores hippocampal CA1 neuron benzodiazepine sensitivity and GABAergic function in vitro, and reverses anticonvulsant tolerance in vivo (Tietz et al., 1999b). Thus, the rat hippocampal CA1 region provides an excellent substrate to investigate the GABAergic adaptations resulting from chronic benzodiazepine treatment and underlying the expression of anticonvulsant tolerance.

The changes that occur in the hippocampal CA1 region following chronic FZP treatment reflect use-dependent GABA<sub>A</sub> receptor regulation (Barnes, 1996): uncoupling of benzodiazepine/GABA binding sites, receptor internalization, and changes in GABA<sub>A</sub> receptor subunit expression – events that culminate in reduced postsynaptic responsiveness to GABA. A candidate denominator for these changes is suppression of protein kinase A (PKA)-mediated phosphorylation. The acute effects of PKA on GABA<sub>A</sub> receptor function vary depending on the method of PKA activation, the
GABA_α receptor subunit phosphorylated, and brain region involved (Brunig et al., 1999; Hinkle and Macdonald, 2003; McDonald et al., 1998; Nusser et al., 1999; Poisbeau et al., 1999). However, more sustained decreases in PKA activity cause an uncoupling of benzodiazepine/GABA binding, a reduction in GABA_α receptor subunit expression, and reduction in synaptic GABA_α receptor-mediated currents (Ali and Olsen, 2001; Hinkle and Macdonald, 2003; McDonald et al., 1998; Poisbeau et al., 1999; Thompson et al., 2000). Thus, the initial study described herein assesses whether or not PKA alterations occur in hippocampal CA1 neurons 2d after cessation of chronic FZP treatment, when GABAergic dysfunction and in vivo anticonvulsant tolerance coexist. The PKA activity in the soluble and insoluble fraction of CA1 hippocampal slices is compared between control and FZP-treated rats. The expression of PKA regulatory subunit RIIβ, which directs the PKA holoenzyme to membranes and cytoskeletal components (Colledge and Scott, 1999) also is assessed in these rats, as is the effect of endogenous activation of PKA on CA1 neuron GABAergic function.

The main findings of this study are as follows: 1) PKA activity is significantly decreased in the insoluble fraction of hippocampal CA1 neurons; 2) PKA-RIIβ subunit protein is decreased in the insoluble fraction of hippocampal CA1 neurons; and 3) in FZP-treated rats, miniature inhibitory postsynaptic current (mIPSC) amplitude is partially restored upon activation of PKA, while in control slices did not respond to such activation (Lilly et al., 2003). Notably, these changes are restricted to CA1, and not present in
adjacent areas of the hippocampus, thus temporally and spatially consistent with in vitro benzodiazepine tolerance, and the in vivo expression of benzodiazepine tolerance. The alterations we observe in hippocampal CA1 following chronic FZP treatment are consistent with a decrease in PKA activity. Acute inhibition of PKA elicits allosteric uncoupling of the benzodiazepine/GABA binding sites and the internalization of GABA<sub>A</sub> receptors, early steps in use-dependent regulation (Ali and Olsen, 2001; Barnes, 1996; Brown and Bristow, 1996). More prolonged decreases in PKA likely suppress GABA<sub>A</sub> receptor subunit protein expression by reducing cAMP-responsive element binding protein (CREB) mediated transcription of receptor subunits, an effect dependent on PKA activity and intact PKA-RIIβ (Bateson, 2002; Paolillo et al., 1999; Thompson et al., 2000). Collectively, decreases in PKA activity may then mediate both the direct in vitro manifestations of tolerance by uncoupling the benzodiazepine/GABA binding sites, and the GABAergic dysfunction in CA1 hippocampus by receptor internalization and decreased subunit gene expression, with reduced mIPSC amplitude a functional correlate of decreased synaptic receptor number (Nusser et al. 1998). Moreover, the partial restoration of mIPSC amplitude in FZP-treated rats upon activation of endogenous PKA is consistent with an acute increase in synaptic receptor number, an early step toward the eventual restoration of normal inhibitory function in hippocampal CA1 pyramidal cells (Ali and Olsen, 2001; Kittler et al., 2005; Nusser et al., 1997).
The insoluble fraction of PKA is directly or indirectly bound to structural proteins and cell membranes, and most likely represents that population of PKA impinging on synaptic receptor function. A-kinase anchoring proteins (AKAPs) comprise a functional class of proteins that localize selected kinases to discrete subcellular locations (Colledge and Scott, 1999; Diviani and Scott, 2001). Among these, AKAP150 has binding motifs for PKA-RIIβ and for components of inhibitory and excitatory post-synapses, including GABA_A receptor β subunits, PSD-95, and F-actin (Brandon et al., 2003; Colledge et al., 2000). However, the subcellular distribution of AKAP150 with regard to inhibitory and excitatory synapses had not been reported in rat brain. It was of interest to determine whether or not AKAP150 is proximate to synaptic profiles in hippocampal CA1, thus providing a source of local PKA that serves to modify ionotropic receptor function following chronic FZP treatment. The second study that is described was undertaken to address the location of AKAP150 in reference to symmetric and asymmetric junctions, which respectively indicate inhibitory and excitatory synapses in rat hippocampal CA1 (Gray, 1959). Confocal and electron microscopy are jointly utilized to assess the co-localization of AKAP150 with synaptic markers and to directly visualize the subcellular location of AKAP150-immunoreactivity.

The main findings of this study are as follows: 1) AKAP150-IR is concentrated near, but not within, excitatory postsynaptic densities; 2) AKAP150-immunoreactivity is not identified within or near profiles representing inhibitory post-synapses; 3) confocal immunofluorescence
microscopy reveals no overlap between AKAP150 and markers of inhibitory or excitatory post-synapses in rat CA1 hippocampus; and 4) significant overlap occurs with AKAP150 and trans-Golgi network-38 (TGN-38), a marker of the Golgi-apparatus. The lack of co-localization between excitatory synaptic markers and AKAP150 confirms the ultrastructural data, which depicts AKAP150-immunoreactivity near, but not within the excitatory post-synapse, similar to the distribution of AKAP150 in human hippocampus (Sik et al., 2000). However, this study does not provide light or electron microscopic evidence for the structural apposition of GABA\(_A\) receptor \(\beta\) subunits and AKAP150 at inhibitory synapses (Brandon et al., 2003). Being the primary determinant of PKA-RII\(\beta\) localization (Colledge and Scott, 1999; Diviani and Scott, 2001), yet not associated with inhibitory synapses, it is unlikely that AKAP150-tethered PKA affects GABA\(_A\) receptor function following chronic FZP treatment. Whether there is a functionally significant interaction between AKAP150 and GABA\(_A\) receptors in the Golgi-apparatus, or if another AKAP isoform scaffolds PKA near inhibitory synapses remains unclear.

Ongoing studies in our laboratory and others' have recently revealed changes in excitatory systems converging on CA1 pyramidal cells following chronic benzodiazepine treatment. For example, amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor number, subunit protein, and the amplitude of miniature excitatory postsynaptic currents (mEPSCs) are upregulated in hippocampal CA1 pyramidal cells (Izzo et al. 2001, Van Sickle
and Tietz 2002, Van Sickle et al. 2002). These changes occur in conjunction with GABAergic dysfunction, and likely contribute to pyramidal neuron hyperexcitability (Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Zeng et al., 1995; Zeng and Tietz, 1999). Behavioral studies have suggested that these changes are related to the expression of benzodiazepine withdrawal, which manifests as increased anxiety in this model (Izzo et al., 2001; Van Sickle et al., 2004).

Though the decrease in membrane-associated PKA activity in CA1 hippocampus after chronic FZP treatment is consistent with GABAergic dysfunction, the proximity of the AKAP150 to excitatory synapses in this brain region offers the possibility that PKA impinges on excitatory receptor changes occurring at this time point. Early in the FZP withdrawal period there is a detectable increase in PKA-mediated AMPA receptor subunit GluR1-Ser$^{845}$ phosphorylation, and this effect precedes more sustained increases in mEPSC amplitude (Song and Tietz, 2004; Van Sickle and Tietz, 2002). The PKA phosphorylation of this subunit increases both single-channel open probability and the number of synaptic AMPA receptors (Banke et al., 2000; Esteban et al., 2003; Lee et al., 2000; Nayak et al., 1998), both of which would favor the increased mEPSC amplitude we observe. In addition, the GluR1 subunit of the AMPA receptor is critical for activity-dependent changes in excitatory synaptic strength, and thus would expectedly be modified during excitatory synaptic plasticity (Lee et al., 2003; Mack et al., 2001; Passafaro et al., 2001). The third study was undertaken to compare the cytoplasmic and
synaptic content of GluR1 subunits in the hippocampal CA1 region from control and FZP-withdrawn rats using confocal and electron microscopic techniques. Tissue was collected from rats 2d after treatment cessation when mEPSC amplitude is elevated, CA1 pyramidal cells are hyper-excitable, and behavioral anxiety is demonstrable (Van Sickle and Tietz, 2002; Van Sickle et al., 2004).

The major findings of this study are as follows: 1) immunofluorescence visualized with confocal microscopy does not reveal a significant change in the cytoplasmic abundance of GluR1 subunits; 2) ultrastructural analysis of GluR1 expression in hippocampal CA1 pyramidal cells reveals a significant, 35% increase in the stratum radiatum of CA1 (apical dendrites) and an 20% increase in the stratum oriens of CA1 (basal dendrites), an effect that does not reach statistical significance; and 3) there is no difference in postsynaptic density length or width between control and treated rats. This study indicates that the increase in mEPSC amplitude in hippocampal CA1 pyramidal cells following cessation of chronic FZP treatment is at least partially accounted for by increased synaptic GluR1 expression.

The studies described herein provide support that PKA is involved in the coordinate changes occurring in excitatory and inhibitory neurotransmitter systems following chronic FZP treatment. The decrease in membrane-associated PKA activity is clearly consistent with manifestations of GABAergic dysfunction that occur following chronic FZP treatment (Lilly et al., 2003). However, the distribution of AKAP150, the scaffolding protein largely
responsible for membrane-associated PKA activity, is selectively proximate to excitatory, not inhibitory synapses in hippocampal CA1 (Lilly et al., 2005). At these excitatory synapses, there is physiological evidence of increases in AMPA receptor function, consistent with the finding of increased synaptic AMPA receptor GluR1 expression (Lilly et al., 2006). When considered within the framework of activity-dependent changes in excitatory tone, a reduction in local PKA activity may signal the impending recovery of normal excitatory function in CA1 pyramidal cells. Thus, PKA-mediated regulation of inhibitory and excitatory neurotransmitter systems may contribute to the synaptic modifications that characterize benzodiazepine tolerance and the expression of the benzodiazepine withdrawal syndrome.
LITERATURE REVIEW

The Rat Hippocampus

The hippocampus proper is a continuation of the cerebral cortex, but differs from it in fundamental organization, containing fewer layers and with projections that are largely unidirectional. The principal cells of the hippocampus are pyramidal-shaped glutamatergic neurons with their somata aligned in the stratum pyramidale (SP). In each of the hippocampal subfields (CA1, CA2, CA3), apical dendrites extend into the stratum radiatum (SR) and stratum lacunosum-moleculare (SL-M) while the basal dendrites and pyramidal cell axons course through the stratum oriens (SO). Inhibitory interneurons comprise the other cell population in the hippocampus, and are highly heterogeneous, discernable by the layer in which their somata reside, their afferent and efferent innervation and their neurochemical composition (Amaral and Witter, 1995). The figure below depicts a low powered micrograph of the rat hippocampus with its subregions denoted (left), and the architecture of a typical CA1 pyramidal cell (right).
The hippocampus is reciprocally connected to the frontal and parietal cortical areas, and to a number of subcortical structures including the amygdala, ventral tegmental area, thalamus, hypothalamus, and septal nuclei. It also receives serotonergic and noradrenergic input via the raphe nuclei and locus coeruleus, respectively. Fibers entering the dentate gyrus (perforant pathway) synapse on principal cells in the granule cell layer. The granule cells then project to the principal cells of the CA3 region by the “mossy fiber” pathway, and the CA3 pyramidal cells extend their processes to CA1 pyramidal neurons via the Schaffer collaterals, completing the “trisynaptic pathway.”

Principal neurons in CA1 provide the primary output of the hippocampal formation. In addition to receiving Schaffer collateral afferents from CA3 which synapse in both the SR and SO, they also receive recurrent CA1 collaterals that form synapses in SO (see Figure).

The excitatory postsynaptic potential in these cells is generated by activation of ionotropic AMPA- and n-methyl-D-aspartate (NMDA)-type glutamate receptors, which permit the flux of $\text{Na}^+/\text{K}^+$ and $\text{Ca}^{2+}$, respectively (Nusser, 2000; Takumi et al., 1999). CA1 pyramidal cells express a variety of AMPA and NMDA receptor subunits and their splice variants, and these combine to form functionally heterogeneous receptor populations (Lerma et al., 1994; Nusser et al., 1998b). These receptors often coexist at excitatory junctions where AMPA receptors respond quickly, depolarizing the adjacent synaptic membrane and thereby contributing to the relief of the voltage-
dependent NMDA receptor Mg\(^{2+}\) block. In the presence of depolarization and glutamate, NMDA receptors become Ca\(^{2+}\)-permeable and mediate the slow component of the excitatory postsynaptic potential.

Inhibition in the rat hippocampus is provided by GABAergic interneurons which comprise roughly 5% of all hippocampal neurons, but can contact up to 2000 pyramidal cells (Bernard and Wheal, 1994; Freund, 2003). The postsynaptic elements of inhibitory junctions on pyramidal cells contain ionotropic GABA\(_A\) receptors, which mediate most of the fast inhibitory neurotransmission in the hippocampus (and for that matter, the CNS). Upon presynaptic release of GABA, these receptors become Cl\(^-\)-permeable, hyperpolarizing the postsynaptic cell and reducing the likelihood of action potential propagation. The composition of these receptors has received much attention, as hippocampal CA1 pyramidal cells express multiple GABA\(_A\) receptor subunits which combine to form functionally divergent populations (Sieghart and Sperk, 2002). Unlike excitatory synapses, inhibitory junctions show a greater representation at the cell soma where they can shape and coordinate the frequency of pyramidal cell output. Characteristic oscillations in pyramidal cell activity define such behaviors as memory formation, anxiety, sleep, and seizures. Other interneurons converge on pyramidal cell dendrites and moderate the extent and pattern of excitatory neurotransmission originating there (Freund, 2003; Mann et al., 2005; Miles et al., 1996; Somogyi and Klausberger, 2005). In contrast to synaptically-mediated phasic inhibition, tonic inhibition also occurs in hippocampal CA1 neurons,
demonstrable in the presence of a GABA_A receptor antagonist as the requirement for a more positive holding current. Tonic inhibition is mediated by extrasynaptic GABA_A receptor complexes with high GABA affinity and prolonged conductance states (Bai et al., 2001; Scimemi et al., 2005). Interestingly, tonic inhibition in the hippocampus has been implicated in cognitive performance, and may prove to be a useful target in the management of age-related cognitive decline (Sieghart and Sperk, 2002).

Given the relatively advanced understanding we have of hippocampal structure and function, it is a popular model for studies addressing alterations in synaptic physiology as they relate to alterations in ionotropic receptor expression. Both the anticonvulsant and anxiolytic effects of benzodiazepines, for example, are related to their ability to modulate hippocampal activity (Fritschy and Brunig, 2003; McNaughton and Gray, 2000; Olsen et al., 1999; Sanchez et al., 2005). Moreover, many of the alterations associated with benzodiazepine tolerance and dependence are manifest in hippocampal CA1 neurons (Allison et al., 1999; Bateson, 2002; Tietz et al., 1999b; Van Sickle et al., 2004). Collectively, both the fund of knowledge available about hippocampal structure and function, the variety of ionotropic receptor combinations expressed, and the importance of the hippocampus to behaviors such as seizure and anxiety, make the hippocampus an excellent model for investigating synaptic alterations occurring with prolonged benzodiazepine exposure and withdrawal.
GABA<sub>A</sub> Receptors

The amino acid GABA is the primary endogenous inhibitory neurotransmitter in the CNS and present at up to 30% of synapses. It is the ligand for ionotropic GABA<sub>A</sub> receptors, the primary electrical conduits for fast inhibitory neurotransmission the CNS. Activation of these receptors permits the flow of Cl<sup>-</sup> down its electrochemical gradient, which results in hyperpolarization of the postsynaptic cell, reducing the probability of action potential propagation. The GABA<sub>A</sub> receptor is a target for many clinically useful agents, including the benzodiazepines which achieve their anxiolytic and anticonvulsants effects by potentiating GABAergic inhibitory neurotransmission.

The GABA<sub>A</sub> receptors are heteromers comprised of five subunits from eight subunit families (α, β, γ, δ, ε, π, θ, ρ), though only some of these (α, β, γ, δ) are widely expressed in the CNS. Multiple subtypes within these families have been cloned, including α1 - α6, β1 - β4, and γ1 - γ3, a diversity compounded by the existence of splice variants. These receptors share considerable structural homology with other members of the superfamily of ionotropic receptors that includes nicotinic acetylcholine receptors, glycine receptors, and serotonin receptors (Barnard et al., 1998). When expressed at the membrane surface, each subunit has a large extracellular N-terminus, four transmembrane segments, and a large intracellular loop between transmembrane segments 3 and 4. Ligand binding occurs at extracellular
pockets formed by the interaction between adjacent $\alpha$ and $\beta$ subunits (Macdonald and Angelotti, 1993; Olsen et al., 2004; Sieghart, 1995).

Given the number of different subunits in the GABA<sub>A</sub> receptor family, substantial diversity in receptor composition is possible. Electrophysiological, molecular modeling, and immunohistochemical studies have revealed that most native GABA<sub>A</sub> receptors are pentamers containing 2$\alpha$, 2$\beta$, and a $\gamma2$ or $\delta$ subunit, with the most abundant combination being $\alpha1\beta2\gamma2$ (Barnard et al., 1998; Farrar et al., 1999). However, there is evidence that a single GABA<sub>A</sub> receptor pentamer may contain different subtypes of the $\alpha$ and $\beta$ subunit families, and the $\gamma2$ subunit has both long and short splice variants (Li and De Blas, 1997; Sieghart and Sperk, 2002). Consideration of only the most abundant pentamer stoichiometry provides an appreciable number of subunit combinations with heterogeneity in structure, function, distribution, and sensitivity to allosteric modulators, protein kinases and phosphatases.

The GABA<sub>A</sub> receptors are anion-permeable channels, and upon activation permit the flux of Cl$^-$ (and to a lesser extent HCO$_3^-$) down its electrochemical gradient. By postnatal day 12, in rats there is an increase in the expression of a neuronal K$^+$/Cl$^-$ transporter, which reduces intracellular Cl$^-$ and establishes the gradient responsible for the GABA-mediated inhibitory postsynaptic currents in the adult CNS (Stein and Nicoll, 2003). Electrophysiological studies have provided parameters for assessing the strength of neurotransmission at inhibitory synapses. The amplitude of miniature inhibitory post-synaptic currents (IPSCs; recorded in the absence of
neuronal activity) reflects the number and conductance state of synaptic receptors. Alterations in receptor number and channel conductance underlie endogenous mIPSC amplitude variability and also apply to situations of synaptic GABAergic dysregulation (De Koninck and Mody, 1994; Otis and Mody, 1992; Zeng and Tietz, 1999). For example, the increase in mIPSC amplitude in principal cells of the hippocampus of kindled rats is based on an increase in synaptic receptor number (Nusser et al., 1998a; Otis et al., 1994). Conversely, the decrease in mIPSC amplitude in CA1 pyramidal cells following chronic benzodiazepine exposure is associated with decreased GABA<sub>A</sub> receptor subunit protein expression and decreased single channel conductance (Chen et al., 1999; Poisbeau et al., 1997; Zeng et al., 1995; Zeng and Tietz, 2000). Collectively, these findings implicate alterations in receptor number or channel conductance at the GABAergic postsynapse as the source of both inherent and acquired variations in inhibitory synaptic function.

Effects of Benzodiazepines on GABA<sub>A</sub> Receptors

The pharmacological effects of benzodiazepines are predicated on their interaction with GABA<sub>A</sub> receptors (Costa et al., 1975; Haefely et al., 1975). Benzodiazepines bind at the interface between α and γ subunits in the receptor complex, increasing the affinity of the receptor for GABA, and thereby increase the frequency of channel opening (Sigel and Buhr, 1997). By enhancing inhibitory neurotransmission in the CNS, benzodiazepines are
clinically useful as anxiolytics, anticonvulsants, and sedatives: effects mediated by specific GABA<sub>A</sub> receptor subtypes in discrete brain regions. In order for benzodiazepines to bind to the GABA<sub>A</sub> receptor, the complex must contain a γ subunit along with an α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, or α<sub>5</sub> subunit, that is, α<sub>4</sub>, α<sub>6</sub>, and δ-containing receptors are benzodiazepine-insensitive. The γ<sub>2</sub> subunit is the most abundant of the γ subunits, and mice with transgenic disruption of this gene exhibit a 95% decrease in benzodiazepine binding. These mice also exhibit significantly greater baseline and learned anxiety, underscoring the role of benzodiazepine-responsive GABA<sub>A</sub> receptors in this behavior (Crestani et al., 1999; Gunther et al., 1995).

In addition to the selectivity of benzodiazepines for certain GABA<sub>A</sub> receptor subtypes, the spectrum of their clinical effects is mediated by precise subunit combinations, and elegant studies have been designed to dissect these differences. Mice engineered to express an α<sub>1</sub> subunit with a mutation in the benzodiazepine binding site, rendering them benzodiazepine insensitive, have normal GABA sensitivity and normal cellular and subcellular distribution of the GABA<sub>A</sub> receptor complex, but α<sub>1</sub>-containing receptors cannot respond to diazepam. In these mice, diazepam’s sedative actions are eliminated and its anticonvulsant effects are blunted, but its anxiolytic effects remain (McKernan et al., 2000; Rudolph et al., 1999). This indicates that the anxiolytic effects of the benzodiazepines are likely mediated by α<sub>2</sub>, α<sub>3</sub>, or α<sub>5</sub> containing GABA<sub>A</sub> receptors. The same approach was subsequently applied to α<sub>2</sub> and α<sub>3</sub> subunits. In α<sub>2</sub> mutant mice, diazepam failed to show anxiolytic effects.
effects, while this effect was intact in α3 mutant mice (Low et al., 2000). Collectively, these studies indicate that the sedative and anticonvulsant actions of the benzodiazepines are mediated by α1-containing GABA A receptors, while the anxiolytic effects are mediated by α2-containing receptors. As discussed below, this segregation of clinical actions is a likely reflection of regional, as well as subcellular differences in the distribution of certain GABA A receptor subunits.

**GABA A Receptors in the Rat Hippocampus**

In the CA1 subfield of hippocampus, *in situ* hybridization and immunohistochemical studies have revealed the expression of a variety of GABA A receptor subunits (Sperk et al., 1997; Wisden et al., 1992). At the ultrastructural level, these subunits are associated with inhibitory synaptic profiles (γ2), or extra-synaptically (δ) and contribute to the phasic and tonic inhibition in CA1 hippocampus, respectively (Hutcheon et al., 2004; Nusser et al., 1996, 1997, 1998a; Scimemi et al., 2005). Within this region of the rat brain, all benzodiazepine-sensitive receptors contain a γ2 subunit coupled to at least one of the following α subunits α1, α2, or α5, although α5-containing receptors are likely extrasynaptic in complexes with δ subunits and are thus benzodiazepine unresponsive (Fritschy and Brunig, 2003; Nusser and Mody, 2002; Sieghart and Sperk, 2002; Somogyi et al., 1996). The subcellular distribution of synaptic GABA A receptors differs with respect to α subunits.
For example, \( \alpha 1 \)- and \( \alpha 5 \)- containing GABA\(_A\) receptors are distributed throughout CA1 pyramidal cell soma, dendrites and axon initial segments, while \( \alpha 2 \)-containing receptors are preferentially concentrated at the axon initial segment (Fritschy et al., 1998; Nusser et al., 1996). The GABA\(_A\) receptor \( \beta \) subunits are distributed differently with regard to cell type in CA1 hippocampus, with \( \beta 2 \) subunits expressed largely by interneurons, and \( \beta 1 \) and \( \beta 3 \) subunits at pyramidal cell synapses (Sperk et al., 1997).

There are presumptive functional consequences to the differential distribution of GABA\(_A\) receptor populations. The anticonvulsant effects of the benzodiazepines on \( \alpha 1 \)-containing receptors, for example, may be a reflection of their widespread distribution over CA1 pyramidal cells, which play an important role in certain forms of epilepsy (Krampfl et al., 2005), consistent with the epileptic phenotype of \( \beta 3 \) subunit (located on pyramidal cells) knockout mice, but not \( \beta 2 \) subunit (located on interneurons) knockout mice (Homanics et al., 1997; Sieghart and Sperk, 2002; Sur et al., 2001). The restriction of \( \alpha 2 \) subunits to the axon initial segment of CA1 pyramidal cells, where interneurons exert precise control over CA1 pyramidal cell output, is consistent with their role in theta rhythm and the expression of anxiety states (Buzsaki, 2002; Kopp et al., 2004; McNaughton and Gray, 2000).

*PKA-Mediated Regulation of GABA\(_A\) Receptor Function*
Phosphorylation of GABA<sub>a</sub> receptor complexes has various effects depending on the kinase employed and the substrate modified. PKA-mediated phosphorylation of the GABA<sub>a</sub> receptor has received significant attention with respect to the effects it has on receptor trafficking, channel conductance, and coupling of the benzodiazepine and GABA binding sites (Brandon et al., 2002). Each of the GABA<sub>a</sub> receptor β subunits contains a consensus sequence for PKA phosphorylation in its cytoplasmic loop (RRRXXSQLK; X being A in β1-2, and S in β3), however, not all β subunits are phosphorylated in situ. In transfected cell lines expressing different β subunits (α1βxγ2), PKA-mediated phosphorylation of a single residue on β1 (pSer<sup>409</sup>) or β3 (pSer<sup>409</sup>) subunit decreases GABA-mediated currents, while β2 subunit-containing receptors are not phosphorylated or functionally modified by PKA (McDonald et al., 1998). These findings were extended with patch-clamp studies of transfected cells expressing α1β1γ2 and α1β3γ2 receptors. In each of these receptor populations, PKA activation increased the fast component of receptor desensitization, but prolonged the duration of the GABA-mediated currents. This implicated the alteration in fast desensitization as the underpinning of the reduced GABA-mediated currents reported earlier, but also provided evidence that IPSCs are shaped by specific kinase-mediated phosphorylation events (Hinkle and Macdonald, 2003). These findings can be extrapolated to the native behavior of endogenous receptors. In hippocampal CA1 pyramidal cells, which express
both β1 and β3 subunit proteins, intracellular administration of the PKA catalytic subunit reduced the amplitude of mIPSCs, while stimulation of protein phosphatases 1 and 2A increased the amplitude of mIPSCs, consistent with basally phosphorylated and functionally suppressed GABA<sub>A</sub> receptors in this brain region (Poisbeau et al., 1999). Similar effects were reported in the striatum, which like CA1 hippocampus expresses β1 and β3 subunit proteins (Flores-Hernandez et al. 2000), and in the olfactory bulb which expresses β3 subunit-containing GABA<sub>A</sub> receptors (Brunig et al., 1999; Liu et al., 2000).

Effects of PKA on GABA<sub>A</sub> receptors are not relegated to acute modification of synaptic receptor currents. Protein Kinase A also affects GABA/benzodiazepine allosteric coupling, receptor trafficking, and gene expression. In contrast to the acute effects of PKA, however, more prolonged PKA activation seems to favor enhancement of GABA<sub>A</sub> receptor function. For example, in transfected cells expressing recombinant GABA<sub>A</sub> receptors (α1β1γ2), inhibition of PKA resulted in an uncoupling of the GABA/benzodiazepine binding sites and internalization of GABA<sub>A</sub> receptors, effects that could be reversed upon PKA activation (Ali and Olsen, 2001). Transcription of the α1 subunit is partially controlled by CREB, and increases in α1 subunit expression occur with elevated cAMP levels (Bateson, 2002; Thompson et al., 2000). Collectively, these studies indicate that although acute activation of the PKA cascade may reduce GABA<sub>A</sub> receptor function by altering synaptic channel desensitization, more prolonged PKA activity would
favor increased GABA_\alpha receptor function through increased membrane expression, increased GABA/benzodiazepine allosteric coupling, and increased subunit gene transcription. This interpretation is corroborated upon attempting to reconcile the effects of PKA activation on GABA_\alpha receptor currents discussed above, with those reported when recombinant \( \alpha 1 \beta 1 \gamma 2 \) receptors were expressed in cells with an endogenously high level of PKA activity (Angelotti et al., 1993).

**Controlling the Synaptic Expression of GABA_\alpha Receptors**

Changes in the synaptic expression of GABA_\alpha receptor subunits underlie the normal variation of mIPSC amplitude among different synapses and the alterations that occur in models of GABAergic dysfunction, including models of epilepsy and benzodiazepine tolerance. Synaptic GABA_\alpha receptors are constitutively cycled from the membrane to the subsynaptic compartment, where they may be degraded or re-expressed at the neuronal membrane (Kneussel, 2002; van Rijnsoever et al., 2005). These steps provide points for regulation, and candidate molecules have been implicated. As mentioned above, sustained activation of PKA favors the surface expression of GABA_\alpha receptors, likely by protecting the receptor complex from interacting with endocytotic machinery (Ali and Olsen, 2001; Kittler et al., 2005). Acute increases in synaptic GABA_\alpha receptors, however, may occur by other signaling cascades. For example, insulin-stimulated tyrosine kinase activity induces the translocation of GABA_\alpha receptors from intracellular
compartments to the membrane surface and increases mIPSC amplitude suggesting synaptic incorporation (Wan et al., 1997). In contrast, a rapid reduction in mIPSC amplitude occurred in hippocampal neurons exposed to brain derived neurotrophic factor, an effect that required activation of trkB and may occur by a PKC mediated decrease in the recycling of internalized receptors (Brunig et al., 2001; Connolly et al., 1999; Jovanovic et al., 2004). Others have identified molecules that interact with internalized GABA<sub>A</sub> receptors. For example, the ubiquitin-related protein PLIC-1 binds to GABA<sub>A</sub> receptor α subunits and prevents the degradation of internalized receptor complexes. When the interaction between PLIC-1 and α subunits is disrupted, a loss of receptor surface expression ensues. Factors that regulate the degradation of receptors complexes are beginning to be elucidated (Bedford et al., 2001; Kneussel, 2002). Clearly the membrane expression of GABA<sub>A</sub> receptors represents the integrated collaboration of signaling pathways controlling receptor exocytosis, endocytosis and degradation. Modulation at one or parallel junctures of this schemata may underlie the alterations in synaptic GABA<sub>A</sub> receptor expression implicated in various physiological and pathological processes.

**AMPA-type Glutamate Receptors**

The AMPA receptors belong to the family of ionotropic glutamate receptors, a family which also includes NMDA receptors and kainate receptors. Members of this family were initially distinguished based on
differential sensitivity to agonists and antagonists. Subsequent cloning of the individual receptor subunits substantiated these divisions and facilitated subunit and receptor modeling. Among these receptors, AMPA and NMDA receptors are the best characterized and often coexist at synapses in the CNS where they underlie fast and slow excitatory neurotransmission, respectively (Bekkers and Stevens, 1989; Dingledine et al., 1999; Racca et al., 2000).

A total of 4 AMPA receptor subunits have been cloned (GluR1-4), each of which can undergo alternative splicing and RNA editing, compounding the molecular diversity (Hollmann and Heinemann, 1994; Seeburg, 1996). There is considerable divergence in tertiary and quaternary structure between glutamate and GABA<sub>A</sub> receptors. Each glutamate receptor subunit contains two extracellular domains (including the N-terminus) that participate in ligand binding, three transmembrane domains that together with a lipophilic re-entrant loop contribute to the channel pore, and a cytoplasmic C-terminus that contains multiple phosphorylation sites and protein binding motifs (Ozawa et al., 1998; Roche et al., 1996; Sheng and Pak, 1999). These subunits combine to form AMPA receptors: the tetrameric homomers and heteromers responsible for fast excitatory neurotransmission in the CNS (Mayer, 2005; Wenthold et al., 1996; Wollmuth and Sobolevsky, 2004).

The profile of AMPA receptor Na<sup>+</sup>/K<sup>+</sup> currents, fast in rise and decay, distinguishes them from NMDA receptor-mediated Ca<sup>2+</sup> currents, which are slow in onset and decay. Many of the activity-dependent changes in synaptic
transmission selectively involve AMPA receptors, and are thus coincident with alterations in the fast component of excitatory neurotransmission (Isaac et al., 1996; Muller and Lynch, 1988; Turrigiano et al., 1998). Though initially thought impermeable to Ca\(^{2+}\), a subset of AMPA receptors with Ca\(^{2+}\) permeability has been identified. This functional heterogeneity is based on the subunit composition of the AMPA receptor. GluR2-containing receptors are impermeable to Ca\(^{2+}\) and represent the majority of AMPA receptors in the CNS. Conversely, GluR1 homomers and heteromers of GluR1/3, GluR1/4 display Ca\(^{2+}\) permeability (Dingledine et al., 1999; Geiger et al., 1995). This functional divergence has physiological significance. For example, Ca\(^{2+}\) influx through AMPA receptors is capable of altering subsequent excitatory currents and the expression of LTP (Gu et al., 1996; Jia et al., 1996).

**AMPA Receptors in the Rat Hippocampus**

The distribution of AMPA receptors in the rat CNS was first addressed with radioligand and in situ hybridization studies, which revealed high concentrations of AMPA receptors in the hippocampus, and differential expression of the various GluR subunits (Keinanen et al., 1990; Monaghan et al., 1984). Immunohistochemistry corroborated these findings, and demonstrated that GluR1 – GluR3 are highly expressed by hippocampal pyramidal neurons, while GluR4 levels are significantly lower and largely restricted to interneurons (Wenthold et al., 1996). These reports also provided ultrastructural evidence that the GluR subunits are highly
concentrated at excitatory synapses postsynaptic to glutamatergic axon terminals, but also associated with other cellular membranes, including the extrasynaptic membrane, the endoplasmic reticulum, and the Golgi apparatus (Baude et al., 1994, 1995; Craig et al., 1994; Gray, 1959; Nusser, 2000; Petralia and Wenthold, 1992; Takumi et al., 1999).

The presence of multiple AMPA receptor subunits in the hippocampus offers the possibility that receptors of various subunit combinations exist. Indeed, sequential immunoprecipitation of receptor complexes with subunit-specific antibodies has provided evidence for at least three subpopulations of AMPA receptors: heteromers of GluR1 and GluR2, heteromers of GluR2 and GluR3, and homomers of GluR1 (Wenthold et al., 1996). Accordingly, Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable channels have been identified in these cells, functionally substantiating the presence of GluR1 homomers (~8%) and GluR2-containing AMPA receptors, respectively (Lerma et al., 1994; Wenthold et al., 1996). Interestingly, GluR1 homomers are apparently restricted to the apical dendrites in hippocampal CA1, the functional significance of which is presently unclear (Bagal et al., 2005b).

Though functional AMPA receptors exist on the hippocampal CA1 pyramidal cell somata and dendrites (Jonas and Sakmann, 1992; Spruston et al., 1995), there is a clear gradient of AMPA receptor density from the soma to distal dendrites. This gradient is established developmentally, as AMPA receptors are first distributed diffusely throughout the cell membrane, later becoming more restricted to dendrites and their spines, and increasing in
concentration with distance from the cell body (Andrasfalvy and Magee, 2001; Craig et al., 1993; Megias et al., 2001; Petralia et al., 1999). The increase in synaptic conductance with distance from the cell soma blunts dendritic filtering, such that the synapses contacting distal dendrites elicit somatic potentials equivalent to those impinging on regions closer to the pyramidal cell soma (Andrasfalvy and Magee, 2001; Magee and Cook, 2000; Megias et al., 2001). Thus, changes in excitatory synaptic conductance occurring at points along the dendritic tree of CA1 pyramidal cells, such as those occurring with long-term potentiation (LTP) or chronic drug treatment, can be assessed by comparative recording of mEPSC amplitude at the cell soma (Davies et al., 1989; Van Sickle and Tietz, 2002). The mEPSC amplitude is related to both the conductance state (Banke et al., 2000; Luthi et al., 2004; Roche et al., 1996) and the number of synaptic AMPA receptors (Andrasfalvy and Magee, 2004; Isaac et al., 1996; Nusser et al., 1997; O'Brien et al., 1998), both of which may be regulated developmentally, during LTP, and during chronic drug exposure.

Activity-Dependent Alterations in Excitatory Synaptic Strength

Activity-dependent alterations in excitatory synaptic strength play critical roles during synaptogenesis and the subsequent plasticity that underlies learning, memory, and addiction (Asztely and Gustafsson, 1996; Ben-Ari et al., 1997; Carlezon and Nestler, 2002; Mayer and Westbrook, 1987). LTP is the most widely investigated model of activity-dependent
synaptic plasticity in the CNS, and represents the end result of coordinated changes in synaptic protein function, trafficking, and expression. Long-term potentiation is the increase in synaptic strength that occurs upon brief high frequency stimulation, such that the postsynaptic response to subsequent stimulation is potentiated, an effect that can last for hours, days or months. Long-term potentiation was first demonstrated in the hippocampus, a structure with longstanding implications in both memory formation and drug dependence (Bliss and Lomo, 1973; Nestler, 2002; Shors and Matzel, 1997). The mechanisms that underlie LTP are often implicated in other models of increased hippocampal excitatory tone, including that which occurs following cessation of chronic benzodiazepine treatment (Van Sickle et al., 2002, 2004; Van Sickle and Tietz, 2002).

The CA1 subfield of the hippocampus has a well-described glutamatergic input from CA3 principal cell axons. This tract, known as the Schaffer collateral – CA1 pathway, has received the most attention with regard to LTP (Bear and Malenka, 1994; Malenka and Bear, 2004). N-methyl-D-aspartate (NMDA) receptor activation and the concomitant Ca$^{2+}$ influx is essential for LTP in this region, and many of the manifestations of LTP are elicited by NMDA exposure or CaMKII activation (Abraham, 2003; Hayashi et al., 2000; Kauer et al., 1988; Malenka and Bear, 2004; Shi et al., 1999). Early studies indicated that upon induction of LTP at this synapse, there was both an increase in postsynaptic glutamate responsiveness, and a decrease in the rate of synaptic failure (i.e., the absence of a response
following afferent stimulation). These observations led to hypotheses favoring a presynaptic nidus of LTP in which glutamate release was altered, and hypotheses supporting postsynaptic increases in synaptic conductance as the foundation of LTP (Isaac et al., 1996; Malinow and Malenka, 2002). This debate was largely resolved with the discovery of “silent synapses.” Silent synapses are those excitatory cell junctions that contain NMDA receptors, but lack AMPA receptors. In CA1 hippocampus up to 25% of adult Schaffer collateral synapses remain without AMPA receptors (Kullmann, 1994; Nusser, 2000; Racca et al., 2000; Takumi et al., 1999). The finding that synapses could be converted from silent to non-silent explained the decreased rate of synaptic failures upon LTP induction, and established the postsynapse as the primary substrate of LTP (Isaac et al., 1996; Liao et al., 1995; Malinow and Malenka, 2002; Poncer, 2003).

The conversion of silent to active synapses in CA1 neurons requires an increase in the synaptic expression of AMPA receptors (Hayashi et al., 2000; Lledo et al., 1998; Shi et al., 1999). This increase in the synaptic expression, at least initially, is mediated by GluR1-containing AMPA receptors, which unlike GluR2-containing receptors, redistribute in a manner sensitive to synaptic activity. To illustrate this divergence, GluR1 and GluR2 were overexpressed in hippocampal cultures. The overexpression of GluR1 in these cultures confers the preferential assembly of GluR1 homomers, which remain confined to the dendritic cytoplasm unless LTP or increased CaMKII activity occurs, directing their redistribution to the synapse. In
contrast, when GluR2 subunits are overexpressed, the resulting GluR2 homomers replace resident synaptic AMPA receptors regardless of neuronal activity (Hayashi et al., 2000; Shi et al., 2001). In agreement with these findings, the surface expression of tagged GluR1 subunits in hippocampal culture is slow under normal conditions but rapidly increases with NMDA application, while GluR2 surface exocytosis is comparatively rapid and does not respond to NMDA exposure (Passafaro et al., 2001). When GluR1 and GluR2 are both present in the receptor complex, GluR1 dominates and determines the trafficking characteristics. Importantly, the activity-dependent incorporation of GluR1 homomers and GluR1/2 heteromers decreases synaptic failure rate, indicating that these receptors incorporate into silent synapses, while GluR2 containing receptors do not (Shi et al., 2001). Gene knockout models corroborate the importance of the GluR1 subunit to LTP: LTP cannot be induced in CA1 hippocampus in mice lacking the GluR1, but can be induced in mice lacking GluR2 and GluR3 subunits (Jia et al., 1996; Meng et al., 2003; Zamanillo et al., 1999). These data strongly implicate GluR1 subunit-containing AMPA receptors in the activity-dependent potentiation of excitatory neurotransmission in hippocampal CA1. Whether these are GluR1 homomers or GluR1/2 heteromers remains a point of investigation.

The conversion of silent to non-silent synapses may occur by direct insertion of GluR1-containing AMPA receptors into the postsynaptic density, or by translocation of these receptors from extra-synaptic to synaptic sites.
(Triller and Choquet, 2005). Indeed, AMPA receptors are associated with extrasynaptic membranes in CA1 hippocampus (Baude et al., 1995; Petralia and Wenthold, 1992), and extrasynaptic localization is significantly decreased in mice lacking the GluR1 subunit (Andrasfalvy et al., 2003). In hippocampal culture, GluR1 subunits appear to be initially inserted into the extrasynaptic neuronal membrane and only later do they overlap with the synaptic markers. Moreover, when LTP is induced chemically (in the absence of neuronal activity), GluR1 surface expression increases, but synaptic expression remains unchanged. These data support a two-step process in which the extrasynaptic expression of GluR1-containing receptors occurs prior to their synaptic expression. This is in contrast to GluR2 subunits, which appear to be directly inserted into the post-synapse, co-localizing with other synaptic proteins (Oh et al., 2005; Passafaro et al., 2001).

The heterogeneity in receptor trafficking between GluR1- and non-GluR1-containing AMPA receptors is based largely on differences in their cytoplasmic tails. Chimeric GluR1 subunits that express the C-terminal region of GluR2 traffic like GluR2 subunits, and are constitutively expressed at the postsynaptic membrane. Chimeric constructs of GluR2 subunits containing the GluR1 C-terminus act like GluR1 subunits, and are retained in the cytoplasm. Moreover, when both are present on the AMPA receptor complex, the presence of GluR1 determines trafficking behavior (Shi et al., 2001). The terminal 3 amino acids of the GluR1 subunit represent a consensus sequence for a PDZ binding domain, a domain important for the
anchoring of synaptic receptors (Malinow and Malenka, 2002; Sheng and Pak, 1999). More proximally, the C-terminus includes serine substrates for both CaMKII (Ser^{831}) and PKA (Ser^{845}). These residues are phosphorylated in hippocampal slices upon activation of their respective kinases (Barria et al., 1997; Mammen et al., 1997b; Roche et al., 1996), with subsequent potentiation of AMPA receptor function (Banke et al., 2000; Derkach et al., 1999). Although phosphorylation of both Ser^{831} and Ser^{845} occurs during LTP (Lee et al., 2000), and both CaMKII and PKA activity are required for normal LTP, only direct PKA-mediated GluR1 phosphorylation appears to be necessary (Huang et al., 1995; Silva et al., 1992). Indeed, the effects of CaMKII appear to be mediated by regulation of protein-protein interactions, as mutating the PDZ-domain, but not the CaMKII phospho-site on the GluR1 subunit, eliminates the CaMKII-mediated increases in synaptic AMPA receptor delivery and disrupts normal LTP (Hayashi et al., 2000; Shi et al., 2001). In contrast, either inhibition of the PKA pathway or substitution of alanine for Ser^{845} abolishes CaMKII-mediated increases in synaptic GluR1 and the induction of LTP in hippocampal slices (Esteban et al., 2003). Collectively, these studies support a model of LTP in which increases in afferent activity causes PKA-mediated phosphorylation of GluR1 subunits, resulting in their translocation to extrasynaptic neuronal membranes. Increases in Ca^{2+} concentration via NMDA receptor opening activates local CaMKII, which enables the interaction of these receptors with subsynaptic binding proteins and recruits GluR1-containing AMPA receptors to synaptic
sites, providing potentiation of synaptic responsiveness. Continuous
replacement of synaptic AMPA receptors by GluR2-containing receptors
stabilizes the increased synaptic strength, and along with increased synthesis
of GluR subunits serves the long-lasting changes in synaptic strength
(Esteban et al., 2003; Ju et al., 2004; Oh et al., 2005; Passafaro et al., 2001;
Shi et al., 2001; Vickers et al., 2005).

In addition to the alterations in receptor distribution that occur upon
channel phosphorylation, changes in synaptic receptor function also result
from increased phosphorylation (Song and Huganir, 2002). CaMKII-mediated
GluR1-Ser\textsuperscript{831} phosphorylation increases single AMPA receptor channel
conductance (Derkach et al., 1999; Derkach, 2003). PKA-mediated GluR1-
Ser\textsuperscript{845} phosphorylation increases AMPA receptor channel open time in
transfected kidney and dissociated hippocampal cells (Banke et al., 2000).
Although not as pervasive as a change in receptor number, these
mechanisms do contribute to various forms of LTP, including those that occur
in the hippocampus (Luthi et al., 2004).

**Protein Kinase A and A-Kinase Anchoring Protein 150**

Although phosphorylated proteins were identified over 100 yr ago, the
process of protein phosphorylation was not clarified until much later. In fact,
the idea that the phosphate entity on a protein was the product of enzymatic
transfer was not definitively resolved until the 1950s, when substantial
increases in phosphoserine residues were reported upon co-incubation of
casein with mitochondrial extracts (Burnett and Kennedy, 1954). Clarification of the molecules involved soon followed, as cAMP was identified as a cofactor, and an adenosine triphosphate (ATP) and cAMP-dependent phosphotransferase was purified (Fischer and Krebs, 1955; Rall and Sutherland, 1958; Walsh et al., 1968). The discovery and characterization of G-proteins and their coupled receptors elucidated events preceding cAMP production and kinase activation (Lefkowitz, 2004; Pawson and Scott, 2005). Collectively, these findings provided the basic framework for our present understanding of PKA signaling, beginning with the formation of cAMP by G-proteins linked to adenylate cyclase, and culminating in the dissociation of PKA catalytic subunits with subsequent transfer of phosphate residues from ATP to cognate serines and threonines.

The effects of protein phosphorylation obviously depend on the substrate, but broadly include alterations in tertiary structure with consequent effects on enzyme activity and protein-protein interactions. The effects also may be short-lived and serve to alter the post-translational function of a protein, such as the modification of ion currents by reversible phosphorylation of synaptic channels (Banke et al., 2000; McDonald et al., 1998), or have longer effects due to increases in gene transcription mediated by the cAMP responsive transcription factor CREB. Both ion channel phosphorylation and CREB activation have been implicated in the synaptic plasticity underlying learning and drug dependence (Carlezon et al., 2005; Esteban et al., 2003; Nestler, 2004; Wolf et al., 2004).
The inactive PKA tetramer is comprised of a regulatory subunit dimer and two catalytic subunits. Two categories of regulatory subunits have been cloned (RI and RII) each with two members (RIα, RIβ, RIIα, RIIβ), and four catalytic subunits have been identified (Cα, Cβ, Cγ, PRKX). In the rat brain, RIIβ and Cβ are responsible for most PKA activity, although all other subunits are present with the exception of Cγ (Bregman et al., 1989; Skalhegg and Tasken, 1997; Taylor et al., 1990). Upon binding of cAMP to the regulatory subunits, the catalytic subunits dissociate becoming active phosphotransferases. Although minor differences exist in the tissue distribution of catalytic subunits, they do not show differences in substrate recognition or ATP binding, nor do they preferentially associate with specific regulatory subunits. In contrast, regulatory subunits differ in binding affinity and subcellular distribution, differences largely based whether they are of the RI or RII subtype. RI-containing tetramers (PKA-RI) dissociate and become catalytically active at lower cAMP concentrations, a quality that tends to favor the association of catalytic subunits with RII-containing forms (PKA-RII) imparting a greater relative stability to the tetramer. This latter point may have additional relevance considering the different subcellular distributions of RI- and RII-containing tetramers, as PKA-RII is highly concentrated in insoluble cellular elements, while the PKA-RI tetramers are soluble and cytosolic (Brandon et al., 1998; Skalhegg and Tasken, 1997; Stein et al., 1987; Tasken and Aandahl, 2004). There also are apparent differences in the signaling pathways associated with the regulatory subunits. For example,
RIIβ-containing tetramers are critical for transducing increases in cAMP to CREB activation and subsequent gene transcription. This was illustrated by comparing cortical and cerebellar neuron cultures, the former expressing PKA-RIIβ, and the latter does not. The PKA-RIIβ-containing cells required greater increases in cAMP to dissociate into regulatory and catalytic subunits (based on a lower cAMP affinity), and more efficiently (10x) increase the nuclear accumulation of catalytic subunits and CREB activity. These differences could be mitigated upon antisense suppression of RIIβ in cortical cells, or with the expression of RIIβ and its anchoring protein (AKAP150) in cerebellar cells (Colledge and Scott, 1999; Paolillo et al., 1999). Thus, it was suggested that PKA-RIIβ-containing tetramers are preferentially responsible for the increases in CREB-mediated transcription following sustained cAMP elevation at the neuronal membrane. Anchoring molecules are responsible for the subcellular localization of PKA, and may serve as the basis of this apparent selectivity within the PKA signaling pathway.

_A-kinase Anchoring Proteins_

The AKAPs comprise a large family of functionally associated proteins, which serve to bind various protein kinases and phosphatases and recruit them to specific subcellular locations. A number of AKAPs have been identified, including those that localize to mitochondria, microtubules, synaptic vesicles, and ion channels such as glutamate receptors and calcium channels (Diviani and Scott, 2001; Edwards and Scott, 2000). Of particular interest to
regulation of synaptic function is AKAP150, a protein that directly binds PKC and PP2B, in addition to the RII subunit of PKA (Colledge and Scott, 1999; Wong and Scott, 2004). AKAP150 is distributed throughout the dendritic regions of rat hippocampal CA1, and enriched in the PSD fraction. AKAP150 interacts directly with the actin cytoskeleton, and disruption of actin polymerization eliminates the membrane expression of this protein (Carr et al., 1992; Gomez et al., 2002). *In vitro* studies reveal that AKAP150 interacts by a central domain with PSD-95 and SAP97, proteins that are restricted to the excitatory post-synapse and linked through PSD-95, Dlg, and Zo-1 (PDZ) domains to glutamate receptor subunits. Being near the plasma membrane, the enzymes associated with AKAP150 can respond to increases in cAMP and Ca\(^{2+}\) elicited by activation of transmembrane receptors. A consequence of increased PKA activity near the excitatory PSD, for example, is glutamate receptor phosphorylation which acutely increases synaptic currents and modulates receptor trafficking, both of which contribute to synaptic plasticity (Esteban, 2003; Luthi et al., 2004).

More recently it was suggested that AKAP150 also interacts with components of the inhibitory post-synapse. PKA-mediated phosphorylation of GABA\(_A\) receptors affects both receptor function and trafficking (Brandon et al., 2002; Hinkle and Macdonald, 2003; McDonald et al., 1998), and a local pool of PKA molecules, recruited by AKAP150 is conceivable. In fact, it was shown that AKAP150 interacts with GST-fusion constructs containing the cytoplasmic domain of GABA\(_A\) receptor β subunits. Moreover,
immunoprecipitation of GABA<sub>A</sub> receptor β subunits from rat brain pulls down AKAP150, and co-transfection of AKAP150 and GABA<sub>A</sub> receptors in COS7 cells increases the β subunit phosphoserine content (Brandon et al., 2003). The same study also reported overlap (albeit limited) of AKAP150 and GABA<sub>A</sub> receptor β subunits in hippocampal culture.

Given the evidence for presence of AKAP150 at both excitatory and inhibitory synapses in hippocampal CA1, determining the actual synaptic distribution of this protein in situ became important. Combined light and electron microscopic studies revealed that there is a concentration of AKAP150 proximate to asymmetric excitatory synapses in hippocampal CA1. In contrast, AKAP150 does not co-localize with markers of inhibitory synapses (gephyrin, GABA<sub>A</sub> receptor β subunits) or reside near inhibitory synaptic junctions (Lilly et al., 2005). This distribution is similar to that reported in the human hippocampus with the respective ortholog AKAP79 (Sik et al., 2000), and provides structural evidence for AKAP150 anchored PKA near excitatory, but not inhibitory, synapses. The process by which PKA is distributed near inhibitory synapses remains unclear (Brandon et al., 2002).

**PKA and AKAP150 are Relevant to Alterations of Synaptic Function**

The CNS functions by transferring electrical and chemical information from one neuron to another, and alterations in this synaptic activity are thought to be responsible for learning, memory, and drug dependence (Nestler, 2002). The LTP in the hippocampal CA1 region is the most widely
studied example of synaptic plasticity, and provides a substrate for investigating the role of signaling molecules in neuronal adaptations (Malenka and Bear, 2004). The LTP is divided into two phases, an early phase in which post-translational protein modification is primary, and a later phase that requires gene transcription and protein synthesis (Lynch, 2004; Nguyen et al., 1994). There is significant and compelling evidence that the cAMP/PKA signaling pathway involved throughout these processes. Forskolin or G-protein coupled receptor stimulated adenylate cyclase increases hippocampal cAMP and augments LTP (Lisman et al., 2002; Lisman and Grace, 2005), while pharmacological inhibition or genetic disruption of adenylate cyclase impairs both early and late synaptic potentiation (Wu et al., 1995). Direct inhibition of PKA also blunts early and late LTP, an effect corroborated with mice lacking the PKA catalytic subunit (Brandon et al., 1995; Otmakhova et al., 2000; Qi et al., 1996). The effects of PKA on early phases of LTP are likely mediated through direct phosphorylation of the AMPA receptor GluR1 subunit on Ser$^{845}$, increasing its surface expression (Esteban et al., 2003; Oh et al., 2005; Passafaro et al., 2001), while the role of PKA in the later phases of synaptic potentiation are through CREB activation. The role of CREB in this model is supported in transgenic models where mice lacking normal CREB exhibit normal LTP induction, but lack the late phase of LTP (Abel et al., 1997; Ahmed and Frey, 2005; Bourtchuladze et al., 1994).

Despite the well-established role of PKA in short- and long-term alterations in synaptic function, identifying the specific isoforms involved in
these processes has been less straightforward. Mice that do not express PKA-RIβ exhibit disrupted LTP in hippocampal CA3, but not hippocampal CA1 neurons (Brandon et al., 1995; Huang et al., 1995), likely related to the different types of LTP in these brain regions (Malenka and Bear, 2004). Considering the preference of RII-containing PKA tetramers for insoluble cellular elements (Pawson and Scott, 1997; Stein et al., 1987), and their relative abundance in excitatory synaptic spines (Lilly et al., 2005), a reasonable hypothesis is that PKA-RII is a key player in the modification of excitatory synaptic strength in hippocampal CA1 neurons. There is, in fact, preliminary evidence for this. For example, disrupting the interaction of AKAP150 and PKA RII, or mutating the PDZ binding domain of the glutamate receptor subunits blunts cAMP-induced phosphorylation of the AMPA receptor GluR1 subunit on Ser\textsuperscript{845} (Colledge et al., 2000), an obligate step in the induction of LTP (Esteban et al., 2003). Moreover, when the AKAP150/PKA-RII interaction is disrupted in hippocampal cultures using a competitive peptide, AMPA receptor currents become attenuated, and GluR1 pSer\textsuperscript{845} levels decrease due to unopposed PP2B activity also tethered near the synapse by AKAP150 (Rosenmund et al., 1994; Tavalin et al., 2002). These studies support the idea that synaptic GluR1 subunits are basally phosphorylated in hippocampal CA1 by an AKAP150 associated PKA, and that this phosphorylation serves to maintain channel function (Banke et al., 2000; Lee et al., 2000; Mammen et al., 1997a). Moreover, the increase in synaptic AMPA receptor number during LTP is accompanied by an
upregulation of AKAP150 mRNA, which may recruit additional PKA molecules in effort to maintain elevated synaptic function (Genin et al., 2003). Nevertheless, despite the availability of PKA-RII knockout lines and a peptide competitive for the AKAP150-RII interaction, the role of AKAP150-anchored PKA-RII in LTP has not been clearly addressed.

Although there is not clear evidence for AKAP150-anchored PKA at inhibitory spines, there is evidence that PKA activation impinges on inhibitory receptor function and trafficking. Similar to the effects on excitatory synaptic function, activation of PKA appears to enhance inhibitory synaptic function, while inhibition of PKA may coordinately induce receptor uncoupling, receptor internalization, and reduced gene expression. Though the specifics of these processes are outlined elsewhere herein, a suppression of local PKA activity is a candidate mechanism for the coordinate decreases in GABA<sub>A</sub> receptor protein expression and mIPSC amplitude in CA1 hippocampus following chronic FZP treatment (Ali and Olsen, 2001; Bateson, 2002; Chen et al., 1999; Lilly et al., 2003; Tietz et al., 1999a; Zeng and Tietz, 1999).

**Benzodiazepine Tolerance and Withdrawal**

The actions of benzodiazepines are predicated upon their interaction with GABA<sub>A</sub> receptors. Upon binding to the GABA<sub>A</sub> receptor complex, benzodiazepines increase the affinity of the receptor for its endogenous ligand (GABA), a phenomenon called allosteric coupling (Costa et al., 1978). Benzodiazepine binding causes an increase in the frequency of Cl⁻ channel
opening, and potentiation of inhibitory neurotransmission in the CNS. Benzodiazepines replaced the barbiturates as anticonvulsants, anxiolytics, and sedatives and remain widely prescribed in the treatment of anxiety and sleep disorders.

Compared to their non-selective predecessors, the benzodiazepines exhibit a safer side effect profile, a product of their interaction with a specific binding site. Characterization of this binding site enabled the synthesis of a specific antagonist, flumazenil, which provides added safety in the event of accidental or intentional overdose. Elucidating other characteristics of the benzodiazepine/GABA<sub>A</sub> receptor interaction holds promise for the development of more selective agents. For example, benzodiazepines only bind to the subset of γ subunit-containing GABA<sub>A</sub> receptors that also contain an α1, α2, α3, or α5 subunit in the complex. Though the γ2 subunit is distributed throughout the rat brain, and contributes to 95% of all benzodiazepine binding sites, the α subunits are distributed with variable density in brain regions controlling seizure genesis, anxiety and sleep cycles, and specific receptor populations responsible for the spectrum of benzodiazepine effects have been identified (Gunther et al., 1995; Sperk et al., 1997; Wisden et al., 1992). The development of benzodiazepine site ligands specific for certain α subunit-containing receptors is ongoing.

With prolonged benzodiazepine exposure, even at clinical doses, benzodiazepine dependence can occur, an effect that has partially limited the clinical utility of these agents (Griffiths and Weerts, 1997). Drug dependence
is a symptom complex that in humans includes the physiological manifestations of (1) tolerance, and (2) a withdrawal syndrome, along with psychosocial manifestations related to drug seeking. Unlike stimulant drugs of abuse such as cocaine and amphetamine which are positively reinforcing due to their effects on midbrain dopamine neurotransmission, benzodiazepine dependence, especially in the clinical setting, is thought to be rooted in the attempted avoidance of the withdrawal syndrome (Nestler, 2004; Woods et al., 1992). Drug tolerance is the reduced effectiveness upon repeated exposure, or the need for escalating doses to achieve a similar effect. Drug withdrawal refers to the emergence of untoward symptoms occurring upon cessation of a drug, and is specific to the particular agent of abuse. For benzodiazepines the withdrawal phenomenon includes anxiety, insomnia, altered sensorium, and may include seizures. Importantly, the features of the withdrawal phenomenon can be dissociated from the condition for which the benzodiazepines were initially prescribed (Allison and Pratt, 2003). Moreover, both tolerance and the withdrawal syndrome can be reproduced in animal models, and changes in inhibitory and excitatory systems, discussed sequentially below, have been identified that correlate with these manifestations of benzodiazepine dependence.

Benzodiazepine Tolerance

Drug tolerance is broadly divided into pharmacokinetic tolerance, in which the metabolisms of the drug is increased resulting in lesser amount of
the agent at the site of action, and pharmacodynamic tolerance, in which there is an alteration in the amount or nature of the drug target. Benzodiazepine tolerance involves alterations in their drug target, and is thus properly referred to as functional, rather than dispositional (Rosenberg et al., 1991). The acquisition of tolerance occurs with a different time course depending on the drug used, the dose administered and the behavior measured. Mechanisms that result in reduced drug sensitivity include a decrease in the number of drug binding sites, a change in benzodiazepine/GABA allosteric coupling, and altered GABA<sub>A</sub> receptor subunit expression that results in decreased target function.

Benzodiazepine tolerance is commonly manifested <em>in vitro</em> by decreased ability to stimulate GABA-mediated Cl⁻ flux or a decreased ability to prolong mIPSC decay. <em>In vivo</em> benzodiazepine tolerance is often reported as an attenuated ability to suppress the chemically-induced seizures or to provide anxiolysis in behavioral paradigms of anxiety (File, 1985; Li et al., 1993; Rosenberg et al., 1991; Tietz et al., 1999b; Xie and Tietz, 1992; Yu et al., 1988). A decrease in the number of benzodiazepine binding sites has been implicated in the expression of benzodiazepine tolerance. Following chronic FZP treatment, a decrease in the benzodiazepine receptor number was identified in the rat hippocampus and cortex, an effect that required prolonged FZP exposure (at least 1 wk), and recovered within 24 h. The time-course of these changes was consistent with some measures of benzodiazepine tolerance, but abated while anticonvulsant tolerance
persisted (Rosenberg and Chiu, 1981; Rosenberg et al., 1985; Tietz et al., 1986), suggesting that simple receptor downregulation is not the primary mechanism of benzodiazepine tolerance. Moreover, a decrease in benzodiazepine binding is not a consistent finding in all models of benzodiazepine tolerance (Allison and Pratt, 2003; Gallager et al., 1984).

Binding of benzodiazepines and GABA to the GABA<sub>A</sub> receptor are reciprocally coupled; that is binding of one enhances binding of the other, an effect that relies on motifs in the transmembrane domains of the γ2 subunit, and is regulated by the type of α subunit in the complex (Costa et al., 1978; Jones-Davis et al., 2005; Puia et al., 1991; Tallman et al., 1978). A change in the coupling between these binding sites might contribute to the reduced effectiveness of benzodiazepines upon prolonged administration. In insect-derived Sf9 cells expressing transfected GABA<sub>A</sub> receptor subunit proteins, 24 h incubation with diazepam results in the functional uncoupling of the GABA/benzodiazepine binding sites (Ali and Olsen, 2001). In cultured cortical neurons, 10 d exposure to FZP or diazepam results in the in vitro tolerance of these agents to potentiate whole-cell GABA currents and GABA stimulated Cl<sup>-</sup> uptake, associated with uncoupling of the benzodiazepine and GABA binding sites (Hu and Ticku, 1994; Prasad and Reynolds, 1992). Using an animal model and chronic 3-4 wk benzodiazepine treatments, others have demonstrated functional uncoupling in cortical and hippocampal regions concordant with tolerance to the ability of benzodiazepines to potentiate whole-cell GABA currents in vitro, and ability to suppress PTZ-induced
seizures in vivo (Chen and Tietz, 1995; Gallager et al., 1984; Rosenberg et al., 1991; Tietz et al., 1989). However, in some models in vitro allosteric uncoupling is not apparent, though behavioral tolerance is demonstrable in these models (Brett and Pratt, 1995; Stephens and Schneider, 1986).

Given the brief time course in which GABA/benzodiazepine uncoupling can occur (hours) and recover (minutes), both post-translational receptor modifications and receptor internalization have been evaluated as potential mechanisms. In some models, there is coordinate GABA/benzodiazepine uncoupling and decreased receptor number offering the possibility that uncoupling merely represents the internalization of GABA_A receptors where the membrane-impermeable GABA is denied access in binding assays. The finding that GABA_A receptors associated with endocytotic machinery (clathrin-coated vesicles) are uncoupled, and that this population increases with chronic benzodiazepine treatment provided correlative support for this possibility (Tehrani and Barnes, 1997; Tehrani et al., 1997). However, uncoupling can occur without a change in receptor number, and likely precedes receptor internalization (Barnes, 1996; Brett and Pratt, 1995; Hu and Ticku, 1994; Tietz et al., 1986, 1989). A role for the post-translational modification of receptor protein was suggested by studies in recombinant systems, where PKA inhibition elicits an uncoupling of the benzodiazepine/GABA binding sites, and PKA activation results in recoupling (Ali and Olsen, 2001), although other kinases, most notably PKC, have been
implicated in these processes (Brown and Bristow, 1996; Gao and Greenfield, 2005; Johnston et al., 1998).

Alterations in GABA\textsubscript{A} receptor subunit protein also have been implicated as an underpinning of reduced benzodiazepine sensitivity following chronic exposure. Like the reports of receptor number and coupling however, some discrepancies are apparent. An overall decrease in protein expression would result in fewer synaptic receptors barring changes in trafficking. Moreover, selective decreases in subunit expression might affect benzodiazepine sensitivity, allostERIC coupling or receptor trafficking. For example, the preferential expression of δ subunits in place of γ subunits would result in a greater proportion of benzodiazepine-insensitive GABA\textsubscript{A} receptors, and substitution of α\textsubscript{2} or α\textsubscript{3} subunit for α\textsubscript{1} might affect benzodiazepine binding and the functional coupling between GABA/benzodiazepine binding sites (Gunther et al., 1995; Luddens and Wisden, 1991; Puia et al., 1991). Moreover, the grouping of genes on adjacent chromosomal regions (e.g., chromosome 4: α\textsubscript{2}, α\textsubscript{4}, β\textsubscript{1}, γ\textsubscript{1}; chromosome 5: α\textsubscript{1}, β\textsubscript{2}, γ\textsubscript{2}; chromosome 15: α\textsubscript{5}, β\textsubscript{3}, γ\textsubscript{3}) offers the possibility that subunits forming discrete receptor populations are under similar transcriptional control. Following 14-d diazepam treatment, there were a reported increases in some (α\textsubscript{4}, α\textsubscript{5}, β\textsubscript{1}, γ\textsubscript{3}), and decreases in other (γ\textsubscript{2}) subunits in the rat cortex, partially consistent with coordinated gene regulation and induced expression of α\textsubscript{4}, benzodiazepine-insensitive, GABA\textsubscript{A} receptors (Darlison et al., 2005; Holt et al., 1996). However, changes in subunit gene expression are inconsistent
between models and often do not follow gene cluster-specific regulation. For example, although a 4-wk FZP treatment is associated with an immediate reduction in the β2, β3, and γ2 subunit mRNAs in rat hippocampus, 1-wk and 2-wk treatments fail to alter γ2 levels, despite the presence of demonstrable benzodiazepine tolerance. Moreover, with 1-wk FZP treatment there are decreases in α1 and β3 subunit mRNA and protein in rat hippocampus, while β2 subunit mRNA is increased, inconsistent with a role for “cluster-specific” GABA_A receptor subunit gene regulation in benzodiazepine tolerance (Chen et al., 1999; Tietz et al., 1993, 1999a; Zhao et al., 1994a, b). These discrepancies indicate that although regulation of specific subunit genes may contribute to the expression of benzodiazepine tolerance, it is not likely required. This notion is strengthened by instances were in vitro and in vivo manifestations of benzodiazepine tolerance occur in the absence of changes in subunit gene expression, or in a manner temporally or spatially inconsistent with changes in subunit gene expression (Brett and Pratt, 1995; Holt et al., 1996, 1999; Pratt et al., 1998; Zhao et al., 1994a, b).

Considering the above studies as a whole, it is evident that benzodiazepine tolerance in vivo can exist in the absence or presence of receptor downregulation, uncoupling, and changes in receptor protein expression depending on differences in the route, length and intensity of administration, the time after treatment cessation, and the brain region analyzed. The mechanisms underlying benzodiazepine tolerance can arguably best be framed within the model of “use-dependent GABA_A receptor
regulation.” This model submits a framework for the behavior of the GABA_A receptor in the presence of excess agonist (GABA) or positive modulators, including the benzodiazepines, and identifies four sequential steps: (1) desensitization, (2) uncoupling and internalization of receptors, (3) receptor degradation, and (4) suppression of subunit gene expression (Barnes, 1996). It is probable that prolonged and intense treatments would invoke later elements of this schemata, while less intensive treatments may operate on the earlier steps. Moreover, brain regions or GABA_A receptor populations that are less benzodiazepine sensitive may show GABA/benzodiazepine uncoupling, while those targets that are more sensitive may show earlier and more advanced use-dependent downregulation (Brett and Pratt, 1995; Rosenberg and Chiu, 1979, 1981; Tietz et al., 1986). This may account for the early expression of sedative and anticonvulsant benzodiazepine tolerance (α1-mediated benzodiazepine action), while anxiolytic tolerance (α2-mediated benzodiazepine action) typically requires more intense treatments (File, 1985; Puia et al., 1991). Moreover, downregulation of more benzodiazepine-sensitive GABA_A receptors, in more benzodiazepine-sensitive brain regions would not only result in benzodiazepine tolerance, but also would predispose to endogenous GABAergic dysfunction associated with chronic benzodiazepine treatment, a consistent finding in the rat hippocampus following 1-wk FZP treatment (Zeng and Tietz, 1997; Zeng et al., 1994; Zeng and Tietz, 1999).
Benzodiazepine Withdrawal

The second element of animal models of drug dependence is the withdrawal phenomenon. With particular respect to the benzodiazepines, the withdrawal phenomenon plays an integral role in the acquisition of drug dependence, as avoidance of withdrawal, rather than an effort to procure the pleasurable effects of the benzodiazepines, underlies the psychosocial manifestations related to drug-seeking in humans. This benzodiazepine withdrawal syndrome is most notably characterized by anxiety, although seizures can also occur, both of which are replicable in animal models (Allison and Pratt, 2003; Woods et al., 1992).

A hypothesis for the expression of benzodiazepine withdrawal has been proposed, and can be integrated with the use-dependent regulation of GABA<sub>A</sub> receptors. As discussed above, with prolonged benzodiazepine exposure, GABA<sub>A</sub> receptor degradation or decreased GABA<sub>A</sub> receptor subunit gene transcription likely ensues to blunt the ongoing enhancement of inhibitory neurotransmission (Barnes, 1996). In conjunction with the inhibitory changes, elements mediating excitatory neurotransmission are also upregulated. Upon cessation of benzodiazepine treatment, these alterations may contribute to region-specific neuronal hyperexcitability that portends the expression of a withdrawal syndrome (Allison and Pratt, 2003; Stephens, 1995). In support of this idea, administration of AMPA and NMDA receptor antagonists mitigate the acquisition and expression, respectively, of benzodiazepine withdrawal-induced anxiety and seizures (Steppuhn and Turski, 1993). The viability of
this hypothesis rests on two elements: (1) the demonstration that glutamatergic upregulation occurs in regions of GABAergic dysfunction, and (2) brain regions exhibiting these coordinate changes impinge on the circuits that mediate behaviors manifest by the withdrawal syndrome. The following paragraphs will address these points in sequence.

Decreased synaptic GABAergic function is apparent in CA1 hippocampus and persists for 2d after cessation of 1-wk FZP treatment, a paradigm that reliably produces anticonvulsant tolerance and manifestations of benzodiazepine withdrawal (Poisbeau et al., 1997; Zeng et al., 1995; Zeng and Tietz, 1999). This change is restricted to the CA1 region of hippocampus, and does not extend to adjacent regions such as the dentate gyrus, a selectivity possibly based on differences in benzodiazepine receptor number or GABA<sub>A</sub> receptor subunit gene expression (Soria et al., 1995; Sperk et al., 1997; Wisden et al., 1992; Young and Kuhar, 1979). Accompanying the GABAergic dysfunction in this paradigm is an enhancement of excitatory neurotransmission. Rat hippocampal CA1 pyramidal cells exhibit increased mEPSC amplitude and synaptic GluR1 expression, that like the GABAergic dysfunction, requires chronic treatment and is restricted to CA1 pyramidal cells (Lilly et al., 2006; Van Sickle and Tietz, 2002). These glutamatergic changes are concordant with the hippocampal CA1 pyramidal cell hyperexcitability and the expression of benzodiazepine-withdrawal anxiety. Moreover, the increase in mEPSC amplitude and the expression of anxiety is abolished with a single injection of a selective AMPA receptor antagonist.
immediately upon treatment cessation (Van Sickle et al., 2004). Importantly, other models also have noted a correlation between the upregulation of AMPA receptor subunits and the expression of anxiety during benzodiazepine withdrawal (Izzo et al., 2001).

Glutamatergic hyperfunction in hippocampal CA1 neurons is a likely contributor to the neuronal hyperexcitability that is temporally consistent with the expression of benzodiazepine withdrawal-associated anxiety. One of the efferent targets of the glutamatergic hippocampal CA1 pyramidal cells is the septal nucleus (Walaas and Fonnum, 1980). Neuronal activity within the septal nucleus is positively correlated with the expression of anxiety. For example, ablation of the septal nucleus encourages rat exploration in the elevated plus maze, the behavioral counterpart to anxiety in this model. Pharmacologically decreasing the output of septal neurons with glutamate receptor antagonists or benzodiazepines also blunts the expression of anxiety (Menard and Treit, 2000; Pesold and Treit, 1996). Septal activity is coupled to anxiety by its reciprocal connection to the hippocampus, where it can induce theta activity, an oscillation frequency of CA1 pyramidal neurons that is associated with models of anxiety, including withdrawal from the benzodiazepines (Amaral and Witter, 1995; Poisbeau et al., 1997). In fact, a common feature of anxiolytic agents, regardless of whether they act on the GABAergic, serotonergic, dopaminergic, or noradrenergic transmitter systems, is that they increase the threshold for the septal induction of hippocampal theta rhythm (McNaughton and Gray, 2000). Further correlative
evidence includes the finding that the anxiolysis provided by direct intra-hippocampal benzodiazepine infusion is mitigated by a infusion of glutamate into the septal nucleus (Menard and Treit, 2001).

Together, the increased glutamatergic neurotransmission in the hippocampal CA1 region during benzodiazepine withdrawal occurs following prolonged enhancement of inhibitory GABAergic function, supporting the idea that mechanisms to maintain a homeostatic balance of excitatory and inhibitory neurotransmission have been invoked. These changes were consistent with CA1 pyramidal cell hyperexcitability and the expression of withdrawal associated anxiety, and support hypotheses implicating the septo-hippocampal pathway in the manifestations of drug-withdrawal-induced anxiety.

An Animal Model of Benzodiazepine Dependence: The Expression of Tolerance and Withdrawal

During 1-wk FZP treatment, rats that ingest an average of 100 mg/kg x 3 d and 150 mg/kg x 4 d exhibit brain benzodiazepine levels (~160 ng/g brain tissue) comparable to that achieved with a 5 mg/kg diazepam injection (144 ng/g brain tissue), or the implantation of diazepam-filled silastic capsules (Brett and Pratt, 1995; Gallager et al., 1985; Gonsalves and Gallager, 1988; Pratt et al., ; Xie and Tietz, 1992). In contrast to diazepam, FZP is a water-soluble benzodiazepine, allowing chronic oral administration via the drinking water without the problems inherent in daily injections or the implantation of
drug-eluting capsules. Chronic FZP treatment elicits *in vitro* and *in vivo* manifestations of benzodiazepine tolerance, and when treatment is discontinued, rats express a benzodiazepine withdrawal syndrome that manifests as increased anxiety (Rosenberg et al., 1991; Tietz et al., 1999b; Van Sickle et al., 2004).

Alterations in synaptic function in hippocampal CA1, but not adjacent areas such as the dentate gyrus, occur in tandem with the behavioral manifestations of tolerance and withdrawal. The rat hippocampus exhibits a high density of benzodiazepine-sensitive GABA_A receptors which shape pyramidal neuron excitability and exert *in vitro* effects related to their anxiolytic and anticonvulsant actions (McNaughton and Gray, 2000; Oliver et al., 1977; Wolf and Haas, 1977; Young and Kuhar, 1979). Chronic FZP treatment alters the ability of the benzodiazepines to modulate hippocampal neurotransmission, and there are respective decreases in GABA_A receptor number, GABA/benzodiazepine coupling, GABA_A receptor subunit expression and inhibitory synaptic function associated with the expression of benzodiazepine tolerance (Rosenberg and Chiu, 1981; Tietz et al., 1986, 1999b). Indices of GABAergic dysfunction in hippocampal CA1, including reduced mIPSC amplitude and decreased subunit protein expression, are prevented with acute administration of a benzodiazepine antagonist at treatment cessation, which also prevents the expression of *in vitro* and *in vivo* benzodiazepine tolerance (Tietz et al., 1999b). There also are changes in excitatory neurotransmission in hippocampal CA1 pyramidal neurons (but
again, not the adjacent dentate gyrus) following cessation of chronic FZP treatment. These alterations include increases in AMPA receptor-mediated mEPSC amplitude, increased synaptic GluR1 subunit expression, and pyramidal neuron hyperexcitability (Lilly et al., 2006; Van Sickle and Tietz, 2002; Van Sickle et al., 2004). These findings are consistent with the expression of a withdrawal syndrome, an idea strengthened by studies in which the withdrawal syndrome and the increase in mEPSC amplitude are prevented by administration of an AMPA receptor antagonist at cessation of 1-wk FZP treatment. Taken together, the in vitro changes in hippocampal neurotransmission after 1-wk of FZP treatment correlates with the in vivo expression of benzodiazepine tolerance and withdrawal, and thus provides an excellent model to study these behavioral manifestations.
Role of protein kinase A in GABA<sub>A</sub> receptor dysfunction in CA1 pyramidal cells following chronic benzodiazepine treatment<sup>1</sup>

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Abbreviations Used: GABAR, GABA<sub>A</sub> receptor; BZ, benzodiazepine; FZP, flurazepam; DG, dentate gyrus; mIPSC, miniature inhibitory postsynaptic current; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; PKA, protein kinase A; AKAP, A-kinase anchoring protein; VIP, vasoactive intestinal polypeptide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; QX-314, lidocaine N-ethyl bromide quaternary salt; CREB, cAMP-responsive element binding protein.
ABSTRACT

One-week treatment with the benzodiazepine (BZ) flurazepam (FZP), results in anticonvulsant tolerance, associated with reduced \( \text{GABA}_A \) receptor (GABAR) subunit protein and mIPSC amplitude in CA1 neurons of rat hippocampus. Since protein kinase A (PKA) has been shown to modulate GABAR function in CA1 pyramidal cells (Poisbeau et al. 1999), the present study assessed whether GABAR dysfunction is associated with changes in PKA activity. Two days after 1-week FZP treatment, there were significant decreases in basal (-30%) and total (-25%) PKA activity and a 40% reduction in PKA RII\( \beta \) protein in the insoluble fraction of CA1 hippocampus. The soluble component of CA1 showed a significant increase in basal (100%) but not total PKA activity. Whole-cell recording \textit{in vitro} showed a 50% reduction in mIPSC amplitude in CA1 pyramidal cells, with altered sensitivity to PKA modulators. Neurons from FZP-treated rats responded to 8-bromo-cAMP with a significant increase (31%) in mIPSC amplitude. Likewise, vasoactive intestinal polypeptide (VIP), an endogenous PKA activator, caused a significant 36% increase in mIPSC amplitude in FZP-treated cells. Neither agent had a significant effect on mIPSC amplitude in control cells. This study supports a role for PKA in GABAR dysfunction after chronic FZP treatment.

Keywords: \( \text{GABA}_A \) receptor, flurazepam, PKA, VIP, hippocampus, tolerance
Running Title: Role of PKA in BZ-induced GABAR dysfunction
GABAₐ receptors (GABARs) are pentameric ion channels, comprised of subunits from seven subunit families [α(1-6), β(1-3), γ(1-3), δ(1), ε(1), θ(1), π(1)] (Sieghart 1995, Whiting et al. 1999). In the adult CNS, GABA binding results in Cl⁻ influx which hyperpolarizes the postsynaptic neuron decreasing neuronal excitability. Since GABARs are the major fast inhibitory neurotransmitter receptors in the CNS, they are targets for a variety of pharmacological agents (Sieghart 1995). For example, benzodiazepines (BZs) positively modulate GABARs by increasing GABA affinity and thus the frequency of channel opening (Lavoie and Twyman 1996). Consequently, BZs have been used clinically as anxiolytics, sedative-hypnotics and anticonvulsants. Their usefulness as anticonvulsants, however, is limited by the development of functional tolerance (e.g. Gonsalves and Gallager 1988, Rosenberg 1995, Tietz et al. 1999b, Izzo et al. 2001).

Using a 1-week flurazepam (FZP) treatment, we have identified a number of in vitro correlates of BZ anticonvulsant tolerance in vivo, which are prominent in the rat hippocampal CA1 area and less apparent in the dentate gyrus (DG). In this model, BZ anticonvulsant tolerance in vivo is accompanied by alterations in CA1 pyramidal cells including: loss of zolpidem-induced prolongation of miniature inhibitory postsynaptic current (mIPSC) decay, uncoupling of BZ/GABA binding sites, and downregulation of GABAR subunit proteins (Chen et al. 1995, Zeng and Tietz 1999, Chen et al. 2001).
These measures are accompanied by a reduced mIPSC amplitude at hippocampal CA1 pyramidal neurons (Poisbeau et al. 1997, Zeng and Tietz 1999), evidence of GABAR dysfunction after chronic BZ exposure. Such changes have implications not only for subsequent BZ effectiveness, but also for normal GABAergic function in CA1 pyramidal cells, and fall within the context of use-dependent GABAR regulation (Barnes 1996). Notably, exposure to the BZ antagonist flumazenil, which reverses anticonvulsant tolerance in vivo (Gonsalves and Gallager 1988), coordinately reverses in vitro tolerance to zolpidem, GABAR subunit protein downregulation, and the reduction in mIPSC amplitude (Tietz et al. 1999b), underscoring the association between these phenomena.

Chronic exposure to BZs also induces changes in the excitatory systems converging on CA1 pyramidal cells. Namely, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor function, subunit protein, and receptor number are upregulated, while NMDA receptor function and subunit protein are downregulated (Izzo et al. 2001, Van Sickle and Tietz 2002, Van Sickle et al. 2002) after chronic BZ treatment. Although excitatory receptor changes are likely more related to BZ dependence than BZ tolerance (Gray et al. 1999, Izzo et al. 2001), the mechanisms and significance of these synchronous changes remain under investigation.

Both GABAR subunit expression and function can be regulated by protein kinases (Brandon et al. 2002). Protein kinase A (PKA), for example, supports the allosteric coupling between the GABA and BZ binding sites in
recombinant systems (Ali and Olsen 2001). PKA can also increase or decrease GABAR function, effects likely contingent on the type of β subunit expressed, the extent of receptor phosphorylation, and the manner of PKA activation (McDonald et al. 1998, Nusser et al. 1999, Poisbeau 1999, Brunig et al. 1999). cAMP levels are also implicated in GABAR subunit gene expression, a phenomenon at least partially dependent on PKA activity (Thompson et al. 2000).

PKA is a tetrameric serine/threonine kinase and in the CNS is comprised of 2 regulatory (RIα, RIIα, RIβ, or RIIβ) and 2 catalytic (Cα or Cβ) subunits (summarized in Doskeland et al. 1993). The association of the various regulatory isoforms with A-kinase anchoring proteins (AKAPs) targets PKA to discrete cellular compartments and confers spatial specificity (Colledge and Scott 1999, Diviani and Scott 2001). Activation of G-protein linked receptors positively coupled to adenylate cyclase increases cellular cAMP levels, and activates PKA. One such class of receptors includes the vasoactive intestinal peptide (VIP) receptors (VPAC1, VPAC2, and PAC1), which are present throughout most of the adult CNS, and reach appreciable density in the hippocampus (Brenneman and Gozes 2000, Harmar and Lutz 1994, Harmar et al. 1998). Moreover, hippocampal VIP receptors likely predominate at GABAergic synapses on CA1 neurons, as GABA and VIP co-localize in a subclass of interneurons that selectively impinge on the spine-free somata and proximal dendrites of pyramidal cells (Ascady et al. 1996).
Anticonvulsant tolerance *in vivo* is accompanied by changes in GABAR structure and function that are predominately evident in CA1 area of hippocampus and not DG (Poisbeau et al. 1997, Chen et al. 1999, Tietz et al. 1999a). Likewise, GABARs on CA1, but not DG, neurons are sensitive to regulation by PKA (Poisbeau et al. 1999). The present study was designed to determine whether changes in CA1 neuron GABAergic function following 1-week flurazepam treatment (1) are associated with selective alterations in PKA activity and (2) can be regulated by manipulation of the PKA system in these neurons. To this end, we evaluated endogenous hippocampal PKA activity and PKA isoform expression levels within cellular fractions containing either membrane associated receptors or soluble substrates. We also evaluated the effect of PKA activation on GABAR mIPSC amplitude in CA1 pyramidal cells from control and FZP-treated rats.
MATERIALS AND METHODS

Benzodiazepine Treatment

After a 2 day acclimation period, adult male Sprague-Dawley rats (initial weight 185-225 g) were offered FZP in 0.02% saccharin water (FZP-treated) or saccharin water vehicle (controls) for 1 week. The goal for the FZP group was an average dose of 100 mg/kg/day for the first three days, and 150 mg/kg/day for the following four days. Rats that did not achieve a weekly average of 100 mg/kg/day were excluded. After drug removal, rats were given saccharin water for 2 days prior to testing. Previous studies have indicated that this treatment results in a reduction in diazepam's effect to suppress pentylenetetrazol induced seizures without affecting baseline seizure threshold, a tolerance that persists at least 2 days after FZP treatment (Rosenberg 1995, Tietz et al. 1999b). Moreover, 2 days after the end of treatment, residual FZP is not detectable in the hippocampus (< 3 ng FZP and metabolites/g hippocampus, Xie and Tietz 1991), and does not confound electrophysiological studies. For acute treatment, rats were given 2.5 mg/kg of the active FZP metabolite (desalkyl-FZP) by gavage in an emulsion of peanut oil, water, and acacia (4:2:1) 2 days before testing. Control rats received emulsion only. This dose was chosen because it results in levels of BZ activity (0.57 M diazepam equivalents) similar to that found in the hippocampus of rats after 1-week FZP treatment (0.57 mM diazepam equivalents, Xie and Tietz, 1992).
Hippocampal Slice Preparation

Rats were decapitated and transverse dorsal hippocampal slices (500 µm) were cut on a vibratome (Pelco 101, Ted Pella, Inc., Redding CA) in ice-cold pregassed (95% O₂/5% CO₂) buffer containing: 120 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO₄, 1.2 mM NaH₂PO₄, 2.4 mM CaCl₂, 26 mM NaHCO₃ and 10 mM D-glucose (288 mOsm, pH 7.2-7.4).

PKA Activity Assays

Hippocampal slices were prepared as described and areas CA1 and DG were microdissected and homogenized 10 times by hand in a 0.5 ml Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh PA, USA) containing 200 µl extraction buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml aprotonin, 1 mg/ml leupeptin, 0.5 mM PMSF). After centrifugation (14,000 x g, 5 min at 4°C) the supernatant (soluble) was collected and the pellet (insoluble) was resuspended in extraction buffer containing 0.1% Triton X-100. Kinase activity in these fractions was assessed in the absence (basal) or presence (total) of 5 M cAMP. Assays were carried out on 5 µl of sample at 37°C for 5 min. PKA activity was measured by [³²-P]ATP incorporation into biotinylated kemptide, subsequently immobilized on avidin-coated filters (Promega, Madison WI, USA). Negative control (5 l dH₂O in place of homogenate) values were subtracted from each sample and activity was standardized to protein content.
determined by BCA protein assay (Pierce, Rockford IL, USA). Data were expressed as pmol ATP transferred/min/g protein. Values for control and treated rats were analyzed using a paired t-test with a significance level of p \leq 0.05.

**Determination of cAMP Levels**

A 10 µl sample of unfractionated homogenate prepared for PKA activity determination (above) was placed in 0.5 ml of ice cold 100% ethanol, incubated on ice for 5 min, centrifuged at 5000 x g for 15 min and the supernatant collected. The ethanol was evaporated in a centrifugal evaporator, and the pellets were resuspended in immunoassay buffer (Cayman Chemical, Denver CO, USA) and stored at 4°C. cAMP levels were determined by competitive enzyme immunoassay (Cayman) and expressed as nmol cAMP/mg protein. Data for control and treated rats was analyzed using a paired t-test with a significance level of p \leq 0.05.

**Determination of mRNA Levels by Semi-quantitative RT-PCR**

Total RNA was extracted from isolated whole rat hippocampi using the Trizol™ method (Invitrogen, Carlsbad CA, USA). The RNA was DNase treated and first strand DNA was synthesized using oligo-dT primers (Invitrogen). PCR reactions contained: 1 µg cDNA, 0.1 µM forward and reverse primers, 1x PCR buffer, 0.2 mM dNTPs, 1 mM MgCl₂, and 2.5 units of
Platinum Taq polymerase (Invitrogen) in a total volume of 50 µl. PCR primers, annealing temperature, and cycle number are listed in Table 1. Cycle parameters included an initial denaturation step (95ºC for 2 min) followed by 30 sec intervals of denaturation (95ºC), annealing (Table 1), and extension (72ºC). Pilot studies were performed with each set of primers to assess linearity of product formation with respect to cycle number, and care was subsequently exercised to ensure all products were within linear range at the terminal cycle. Samples were resolved on a 1.5% agarose gel containing 0.008% ethidium bromide. Bands were visualized at 610 nm on a Typhoon Imager (Amersham Biosciences, Piscataway NJ, USA). Densities, normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) content, were compared with UnScanIt-gel software (Silk Scientific Co, Orem UT, USA). Data for control and treated rats was analyzed using Student’s t-test with a significance level of p < 0.05.

**Immunoblotting**

Slices were prepared as for PKA activity assays but after crude fractionation the supernatant was collected and the pellet resuspended in 100 µl of extraction buffer. A BCA protein assay was carried out prior to storage at -70ºC. Twenty to 40 µg protein from each CA1 and DG was resolved on a 10% SDS-gel (Protean III, Biorad, Hercules CA, USA) at 150 V. The gel was transferred (95 V for 3.5 hr at 4ºC) to nitrocellulose and blocked for 60 min at
room temperature in TBST (50 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% milk + 1% BSA (RIIβ) or 5% milk alone (Cβ). The membrane was immunoblotted for PKA RIIβ (1:5000, Chemicon, Temecula CA, USA) or PKA Cβ (1:1000, Santa Cruz, Santa Cruz CA, USA) diluted in blocking solution. After rinsing in TBST (6 x 5 min) membranes were incubated with anti-rabbit-HRP (1:100,000, Santa Cruz) diluted in blocking solution, rinsed (6 x 5 min) and developed for chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Actin immunoblotting (mouse anti-actin, 1:2000, Santa Cruz) was used as an internal standard for protein loading, and was either performed concurrently or after stripping and reprobing the nitrocellulose (0.5 M NaCl, 0.5 M acetic acid x 5 min, 1 N NaOH x 2 min). Care was exercised to ensure the chemiluminescent signal was linear with respect to loaded protein. Data for control and treated rats are expressed as proportion of actin and were analyzed using Student’s t-test with a significance level of p ≤ 0.05.

mIPSC Recording

Slices were prepared as described above and maintained at room temperature in gassed buffer for 1 to 3 hr prior to recording. GABAR-mediated action potential-independent mIPSCs were recorded in CA1 pyramidal neurons in the presence of: 1 μM tetrodotoxin (Na+-channel blocker), 50 μM DL-2 amino-5 phosphonovaleric acid and 10 μM 6,7-dinitroquinoxaline-2,3-dione (glutamate receptor antagonists) using whole-cell
voltage clamp techniques as described previously (Zeng and Tietz 1999). Patch pipettes (4-7 MΩ), were pulled from borosilicate capillaries (nonfilamented, 1.5 mm A, Sutter Instruments, Novato CA, USA) with a Flaming-Brown electrode puller (P-97, Sutter), and filled with internal solution: 130 mM CsCl, 1.0 mM EGTA, 0.5 mM CaCl₂, 2.0 mM MgCl₂, 2.0 mM lidocaine N-ethyl bromide quaternary salt (QX-314), 2.0 mM ATP, and 10.0 mM HEPES (pH 7.2). Cs⁺ and QX-314 were included to eliminate GABA$_B$ receptor-mediated events and spontaneous firing of CA1 pyramidal neurons, respectively. Neurons were voltage clamped (-70 mV) in continuous mode with an AxoClamp 2A amplifier (Axon Instruments, Foster City CA, USA). The current output was low-pass filtered (10 kHz), offset and amplified 10,000 fold. The signal was continuously monitored on-line (PClamp 6.0 Software, Axon Instruments), digitized (Digidata 1200, Axon Instruments) and stored on VCR tape for later analysis. Recorded events above the level of background (−2.0 pA) with a duration of > 3 ms were detected and averaged with Strathclyde CDR and SCAN software (J. Dempster, University of Strathclyde, Glasgow Scotland) or MiniAnalysis software (Synaptosoft, NJ, USA).

Effects of 8-bromo cAMP and VIP on mIPSC amplitude

Baseline mIPSC activity was recorded for at least 5 min in each cell. After baseline was established, increasing concentrations of 8-bromo cAMP (250 – 1000 µM), or VIP (0.03 – 1 µM) were superfused for 8 min each. mIPSC amplitude was recorded for the final 2 min of each increment in concentration.
The effects of 8-bromo cAMP and VIP on mIPSC amplitude were grouped by concentration and analyzed by ANOVA with post-hoc analysis using the method of Sheffe’. The confidence level was \( p < 0.05 \).
RESULTS

PKA activity in the insoluble fraction of hippocampal CA1 is modified following chronic FZP treatment

Two days following 1-week FZP treatment, hippocampal tissue was prepared as described in the Methods. PKA activity was assessed in the absence and presence of 5 µM cAMP, to reflect basal and maximal PKA activity, respectively. Pilot studies indicated that basal and stimulated values could be reduced 99% and 95% respectively, by co-incubation of sample with protein kinase inhibitor (10 µM, data not shown). Positive control samples (10 ng PKA catalytic subunit) were, at a minimum, 6-fold greater than the highest value in each experiment (data not shown). In the soluble CA1 fraction there was a significant (p < 0.05) increase in basal PKA activity (CON: 0.88 ± 0.25, FZP 1.75 ± 0.40 pmol ATP/min/µg protein), however, cAMP-activated PKA activity was not altered (Fig. 1a). Chronic FZP treatment resulted in a statistically significant reduction in both basal (CON: 0.65 ± 0.13, FZP 0.47 ± 0.04 pmol ATP/min/µg protein) and stimulated (CON: 3.72 ± 0.32, FZP 2.88 ± 0.32 pmol ATP/min/µg protein) PKA activity in the insoluble CA1 fraction (Fig. 1b). There was no effect of FZP treatment on PKA activity in the soluble or insoluble DG fractions. Moreover, 2 days after acute desalkyl-FZP treatment, PKA activity was not significantly changed in the soluble (CA1 basal: CON 3.31 ± 0.27, FZP 3.43 ± 0.41; total: 6.90 ± 0.62, 7.72 ± 0.95; DG basal: 3.73 ± 0.80, FZP 3.40 ± 0.35; total: 7.08 ± 0.68, 6.34 ± 1.07) and
insoluble (CA1 basal: CON 0.28 ± 0.09, FZP 0.25 ± 0.05; total: 0.90 ± 0.15, 0.90 ± 0.08; DG basal: 0.26 ± 0.09, FZP 0.29 ± 0.09; total: 1.10 ± 0.40, 1.00 + 0.17) fractions (n = 3 rats/group).

A transient increase in cAMP levels causes the release of PKA catalytic subunits and subsequent kinase activity. More prolonged increases can cause the induction of multiple PKA isoform mRNAs (Tasken et al. 1991). Thus, it was of interest to determine whether the alteration in PKA activity in the hippocampus following chronic FZP treatment was associated with a change in resting cAMP levels. To this end, we performed cAMP assays on isolated CA1 and DG tissue (Fig. 2). Chronic FZP treatment did not affect the cAMP level in the hippocampal CA1 area (CON 0.52 ± 0.21, FZP 0.59 ± 0.12 pmol cAMP/mg protein). Additionally, a trend toward a decrease in cAMP in the DG (Con 1.24 ± 0.45, FZP 0.8 ± 0.41 pmol cAMP/mg protein) did not reach statistical significance. Collectively, these findings suggested that a change in resting cAMP levels was not responsible for the change in PKA activity.

**Chronic FZP treatment did not affect mRNA levels of the predominant hippocampal PKA isoforms**

To further explore the basis for altered basal PKA activity following 1-week FZP treatment we performed a comparison of hippocampal PKA mRNA levels. Figure 3 illustrates that 2 days following chronic FZP treatment there was no significant change in the mRNA levels of the predominant cytosolic
(RI\(\alpha\) and RI\(\beta\)) PKA subunits. Additionally, the predominant PKA subunit associated with the insoluble fraction (RII\(\beta\)) was also unchanged. Likewise, the level of \(C\beta\), the most commonly expressed catalytic subunit in the rat brain, was not altered in the rat hippocampus. Notably, these experiments used mRNA isolated from the whole hippocampus, since microdissection of specific regions would have increased the likelihood of contamination and excessive RNAase activity. To specifically address regional changes PKA isoform expression in parallel with the regional change in PKA activity, we measured PKA RII\(\beta\) and \(C\beta\) protein in microdissected CA1 and DG.

**Chronic FZP treatment changed PKA isoform protein levels**

Because the mRNA samples for RT-PCR were collected from whole hippocampus (see methods), and as such may not pick up specific regional differences in PKA isoform expression, we chose to examine the protein expression of PKA subunit RII\(\beta\) and \(C\beta\) in CA1 and DG of hippocampus. RII\(\beta\) is of particular interest because it is the major regulatory PKA isoform in the brain, and is localized to the membrane cytoskeleton, i.e. the same fraction in which consistent decreases in PKA activity were shown (Fig. 1b). An alteration in RII\(\beta\) levels may have implications for kinase targeting or sensitivity to cAMP (Skalhegg and Tasken 1997, Colledge and Scott 1999). \(C\beta\) was also investigated because of its relative abundance in the rat brain.
Although a change in PKA RIIβ mRNA was not detected in whole hippocampus (Fig. 3), chronic FZP treatment resulted in a significant 40% decrease (n = 5 rats/group) in RIIβ protein in CA1, but did not affect RIIβ protein levels in the DG. Whether PKA RIIβ mRNA and protein are regulated in parallel in this region of the hippocampus could best be assessed by in situ hybridization studies. When hippocampal Cβ protein levels were compared between control and FZP-treated rats, there was no change in the expression of this subunit in CA1 or DG regions of the hippocampus.

**PKA modulators differentially affected mIPSC amplitude in control and FZP-treated rats**

Spontaneous, inward mIPSCs were recorded in symmetrical Cl⁻ solutions in CA1 neurons that were voltage clamped at –70 mV. As reported previously (Poisbeau et al. 1997, Zeng and Tietz 1999), mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats was significantly reduced in comparison to control cells (CON -22.6 ± 1.7, FZP –11.4 ± 1.5 pA, Fig 5b and 6a). 8-bromo-cAMP, a membrane permeable cAMP analog, potentiates GABAR currents in acutely dissociated DG neurons in the μM range (Kapur and Macdonald, 1996). In the present study, there was no significant effect of 8-bromo-cAMP on mIPSC amplitude in CA1 pyramidal cells in control slices, though at the highest concentrations, there was a trend toward a decrease in mIPSC amplitude (Fig. 5a and 5c). However, when 8-bromo-cAMP was superfused onto hippocampal slices from FZP-treated rats, there was a
significant concentration-dependent increase in mIPSC amplitude (500 µM, p < 0.05, Fig. 5b). Importantly, mIPSC frequency was not altered at concentrations up to 500 µM.

VIP is contained in subsets of GABAergic interneurons that synapse on CA1 pyramidal cell somata and proximal dendrites and is released in an activity-dependent manner, eliciting postsynaptic increases in cAMP and subsequent activation of PKA (Ascady et al. 1996, Giachetti et al. 1977, Brenneman et al. 2000). To determine whether the effect of 8-bromo cAMP on GABAR mIPSC amplitude in slices from FZP-treated rats could be replicated with an endogenous modulator of PKA, we assessed the effect of VIP (0.03 - 1 µM) on GABAR function. Again the baseline mIPSC was significantly reduced in cells from FZP-treated rats (CON -24.7 ± 3.4, FZP – 14.3 ± 2.9 pA). In agreement with the effects of 8-bromo-cAMP, VIP significantly increased mIPSC amplitude (30 nM, p < 0.05) in CA1 neurons from FZP-treated rats (Fig. 6b). The maximal effect of VIP resulted in full restoration of mIPSC amplitude in FZP-treated cells. After this point VIP showed a trend to inhibit mIPSCs in both control and FZP neurons, although this effect did not reach statistical significance. Although a previous report described presynaptic effects of VIP (Wang et al. 1997), VIP did not significantly alter mIPSC frequency at the concentrations used, which were well below that used in the former study. This suggests that the alteration of GABA mIPSCs by VIP was a postsynaptic phenomenon.
There are 3 VIP receptors in the rat CNS (VPAC1, VPAC2, and PAC1) with different affinities for the peptide (IC$_{50} = 1$, 3 and 1000 nM respectively, Harmar et al. 1998). We explored the possibility that the differential effect of VIP on mIPSC amplitude was associated with an alteration in VPAC2 expression. Reasons for the focus on VPAC2 include VPAC2’s abundance in CA1 hippocampus (Sheward et al. 1995), the relative lack of VPAC1 mRNA in the hippocampus (data not shown) and the low affinity of PAC1 for VIP (Harmar et al 2000). Since a specific antibody to VPAC2 is unavailable, we compared the transcript levels of VPAC2 by semi-quantitative RT-PCR. There was no effect of chronic FZP treatment on VPAC2 receptor mRNA expression (96% of control, Fig. 6c, n = 5/group). Concurrent studies indicated that PAC1 expression was also unaltered (data not shown). This finding suggests that changes in VPAC2 receptor mRNA do not underlie the enhanced sensitivity of FZP-treated cells to VIP.
DISCUSSION

The main findings of the present study indicate that the reduction in GABAR mIPSC amplitude in CA1 neurons following chronic FZP treatment is accompanied by decreased PKA activity and decreased PKA RIIβ protein in the insoluble fraction of CA1. Moreover, when PKA activity was elevated in vitro by 8-bromo-cAMP or VIP, GABAR mIPSC amplitude was partially restored. These findings suggest that the reduced GABAR mIPSC amplitude at this time point may be related to a change in endogenous PKA activity. In addition, restoration of local PKA activity could serve as a means to restore GABAR function in the BZ tolerant rat.

Interestingly, others have shown that RIIβ subunit disruption occurs with a reduction in PKA activity and catalytic subunit protein (Brandon et al. 1998). In the present study the reduction in CA1 PKA activity occurred with reduced PKA RIIβ but not Cβ subunit protein. The type of regulatory subunit contained in the PKA holoenzyme affects cAMP sensitivity and subcellular localization (Skalhegg and Tasken 1997). Thus, a change in the level of one regulatory subunit may have considerable effects on both the likelihood of substrate phosphorylation and the proximity of substrates. Although not assessed in the present study, the Cα isoform has been detected in CA1 of mouse hippocampus (Cadd and Mcknight 1989), where it may comprise part of the RIIβ holoenzyme in the normal animal.
Alterations in hippocampal PKA activity due to chronic psychoactive drug treatments have previously been reported, concomitant with changes in PKA isoform expression (e.g. Dwivedi et al 2002, Mori et al. 1998). Chronic clozapine treatment, for example, significantly decreased PKA activity in both the soluble and insoluble fractions of hippocampus and was accompanied by a reduction in hippocampal GABAR expression (Dwivedi et al. 2002, Farnbach-Pralong 1998). In the present study, there was a significant reduction in membrane associated PKA activity paralleled by a reduction in PKA RIIβ protein in the CA1 region of hippocampus, but not the DG. Notably, these changes occurred after chronic, but not acute, FZP treatment, and were temporally (evident 2 days after chronic FZP treatment) and spatially (prominent in CA1, but not DG) consistent with the reductions in GABAR subunit protein and mIPSC amplitude (Poisbeau et al. 1997, Chen et al. 1999, Tietz et al. 1999a, 1999b). Thus, PKA may play a role in the GABAR alterations induced by chronic FZP.

Phenomena that are common to chronic BZ treatments and models of BZ tolerance include an uncoupling of GABA/BZ binding sites, internalization of the GABAR, and subsequent alterations in subunit expression (Barnes 1996). Others have indicated that the preliminary steps, i.e. BZ induced uncoupling and GABAR internalization, are mimicked by inhibition of PKA, and reversed by PKA activation (Ali and Olsen 2001, Brown and Bristow 1996). Hence, the reduction in mIPSC amplitude, which likely reflects a loss of functional receptors at the postsynaptic membrane (Nusser 1998,
Poisbeau et al. 1997), could be a result of decreased local PKA activity (Fig. 1b). Changes more persistent than those resulting from receptor internalization might be expected to result from alterations in subunit gene expression. Like other models of BZ tolerance (Heninger et al. 1990, Holt et al. 1996, Impagnatiello et al. 1996, Costa et al. 2002), the FZP treatment used here causes selective reductions in GABAR subunit expression, notably of $\alpha_1$ and $\beta_3$ subunit mRNA and protein (Chen et al. 1999, Tietz et al. 1999a). Transcription of the $\alpha_1$ subunit gene is partially controlled by cAMP-responsive element binding protein (CREB; Bateson et al. 2002), which likely subserves the PKA-dependent increases in $\alpha_1$ expression induced by increased cAMP levels (Thompson et al. 2000). Interestingly, the ability of cortical neurons to transduce cAMP increases to CREB activation relies on the intact expression of the PKA RII$\beta$ subunit (Paolillo et al. 1999), which was significantly reduced following chronic FZP treatment (Fig. 4). If reduced PKA activity and RII$\beta$ protein results in a suppression of CREB-mediated transcription, this scheme may provide a mechanism for the selective downregulation of $\alpha_1$ subunit in hippocampal CA1 neurons following chronic FZP treatment, and the associated functional sequelae. Thus, PKA may play a critical role in both the transient and sustained reductions of GABAR function characteristic of use-dependent regulation (Barnes 1996), by regulating receptor internalization and GABAR gene expression, respectively. The reduced GABAR mIPSC amplitude is likely a functional manifestation of these GABAR reductions (Nusser et al. 1998).
The sensitivity of GABARs from FZP-treated animals to PKA modulators suggests that, apart from the role PKA may play in the acquisition of GABAR dysfunction following chronic FZP treatment, PKA activation may be important for the recovery of GABAR function in the FZP-treated rat. To screen the sensitivity of GABARs to PKA modulators we utilized both 8-bromo-cAMP and VIP, an endogenous modulator of PKA activity. In CA1 of hippocampus, VIP is present in a subset of GABAergic interneurons that synapse on CA1 pyramidal cell somata and proximal dendrites (Ascady et al. 1996). Similar to the present findings, postsynaptic effects of VIP on GABAR function have been described in dissociated bipolar and retinal ganglion cells, where VIP increased GABAR currents in a PKA-dependent fashion (Veruki and Yeh 1992, 1994).

The acute effects of PKA on GABAR function have been investigated in a wide variety of cell types and brain areas. However, there is not an absolute consensus regarding the acute effects of PKA and its modulators on GABAR function. Nonetheless, the effects reported so far are largely attributed to phosphorylation of the $\beta_1$ and $\beta_3$ subunits (Brandon et al. 2002, McDonald et al. 1998), which are both expressed in CA1 pyramidal cells (Wisden et al. 1992, Tietz et al. 1999a, Pirker et al. 2000, Chen et al. 1999). In recombinant systems, forskolin caused a direct phosphorylation of $\beta_1$ subunits and subsequent reduction of GABAR currents, while $\beta_3$ phosphorylation resulted in an enhancement of GABAR function (McDonald et al. 1998). The latter findings were subsequently extended to olfactory
granule neurons (Nusser et al. 1999). However, others demonstrated reduced GABAR currents in olfactory granule neurons when PKA activity was indirectly enhanced by agonism of the D1-dopamine receptor (Brunig et al. 1999), a phenomenon also evident in the striatum and accompanied by increased phosphorylation of the β1/3 subunit (Flores-Hernandez et al. 2000). Both the subunit composition of the GABAR and the mechanism of PKA stimulation account for some of the reported discrepancies regarding the effects of PKA on GABAR function. Although there was a trend toward reduced mIPSC amplitude in control cells with greater PKA activation, these effects only approximated those reported previously with intracellularly applied PKA (Poisbeau et al. 1999). This discrepancy may reflect different methods of PKA activation. Alternatively, GABARs in control cells may exhibit greater basal phosphorylation and be less responsive to PKA activation.

Because PKA’s capacity to modify GABAR function is partially based on the subunit complement of the GABAR, the sensitivity of CA1 pyramidal cells from FZP-treated rats to PKA modulators may be a reflection of altered subunit composition. For example, the increased β2 and the decreased β3 subunit expression two days after cessation of chronic FZP treatment (Chen et al. 1999, Tietz et al. 1999a) could conceivably unmask the functional effects of β1 subunit-mediated phosphorylation on CA1 pyramidal cells. However, this would arguably reduce GABAR function in neurons (McDonald et al. 1998, Poisbeau et al. 1999), and the present study indicates that PKA
activation in FZP-treated cells causes a preliminary increase in GABAR function. Another possibility is that chronic FZP treatment results in a change in the endogenous phosphorylation of the GABAR. Poisbeau et al. (1999) reported that GABARs in CA1 pyramidal cells, but not DG neurons, are under the tonic control of PKA-mediated phosphorylation, which serves to suppress GABAR function. An increase in β2 mRNA following FZP treatment could result in the expression of GABAR isoforms less amenable to endogenous phosphorylation, but perhaps more sensitive to changes in PKA activity. In this manner, both the altered GABAR subunit expression and local reductions in PKA activity may act in tandem to predispose CA1 neuron GABARs to modulation by PKA. Nevertheless, PKA activation in these neurons reportedly serves to depress GABAR function, and the present study indicates that PKA activation in fact increases mIPSC amplitude in CA1 neurons from FZP-treated rats.

Perhaps the most cogent explanation for the early effect of PKA modulators on CA1 neurons from FZP-treated rats is that the effects of PKA are solely a reflection of rapid changes in the number of functional GABARs at the neuronal membrane. Correlates of chronic BZ treatment, including GABA/BZ uncoupling, reduced mIPSC amplitude, and the reductions in GABAR subunit protein can be reversed by acute exposure the BZ antagonist flumazenil. Although restoration of some measures occurs within minutes (Klein et al. 1994, Ali and Olsen 2001), and others within hours (Gonsalves and Gallagher 1988, Tietz et al. 1999b), neither time course precludes a
phosphorylation event as a potential intermediary. For example, while inhibition of PKA has implications for the uncoupling and internalization of GABARs, activation of PKA may serve to shuttle GABARs to the membrane (Ali and Olsen 2001), partially restoring mIPSC amplitude (Poisbeau et al. 1997, Nusser et al. 1998). Hence, like the acquisition of GABAR dysfunction (receptor internalization followed by suppression of gene transcription), the restoration of GABAR function may be biphasic, i.e. an early phase dependent on the translocation of assembled GABARs from cytosol to membrane, and a later phase that requires gene transcription and translation. As PKA has implications for both the former (Ali and Olsen 2001, Angelotti et al. 1993) and the latter (Thomson et al. 2000), it is a candidate signaling molecule for the use-dependent regulation of GABARs (Barnes et al. 1996).

Although reduced PKA activity and RIIβ protein in the hippocampal CA1 region appears related to the GABAR dysfunction after chronic FZP treatment, it is possible that these changes are also related to alterations in excitatory amino acid receptor function. Chronic benzodiazepine treatment alters hippocampal excitatory receptor function, such that there is a general increase of AMPA receptor number and function, and a decreased NMDA receptor number and function. These alterations are likely in vitro manifestations of BZ dependence and not BZ tolerance (Izzo et al. 2001) and in our model GABAR, AMPAR and NMDAR changes exist simultaneously (Tietz et al. 1999b, Van Sickle et al. 2002, Van Sickle and Tietz 2002). Because PKA also modulates excitatory receptor function in CA1 neurons
(e.g. Banke et al. 2000), where an RIIβ binding AKAP has been localized to excitatory synapses (Sik et al. 2000), PKA may also be a candidate for the coordinate regulation of excitatory and inhibitory systems following chronic BZ treatment.

In summary, the presence of alterations in PKA activity in the hippocampal CA1 area, but not the DG, supports previous work indicating the different responses of these neurons to protein kinases and to regulation by chronic FZP treatment (Poisbeau et al. 1997, 1999, Tietz et al. 1999a, Chen et al. 1999, Van Sickle and Tietz 2002). These studies support an association between the acquired GABAR dysfunction following chronic FZP treatment and the reduced local PKA activity and RIIβ expression in CA1 pyramidal cells. Collectively, suppressed PKA activity is likely related to the FZP-induced GABAR dysfunction, and subsequent elevation of PKA activity may be important for the return to normal function in CA1 pyramidal cells.
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Fig. 1  Chronic FZP treatment alters hippocampal PKA activity.  (a) The soluble fraction of CA1 exhibits greater basal PKA activity following 1-week FZP treatment (199% of control) but there is no effect on total, i.e. cAMP stimulated PKA activity in CA1 area of hippocampus. Moreover, soluble PKA activity in the dentate gyrus was not significantly affected.  (b) The insoluble fraction of hippocampal CA1 exhibits less basal and less total PKA activity (72% and 77% of control, respectively) following 1-week FZP treatment. There is no statistically significant effect of FZP treatment on PKA activity in the DG.  $n = 6$ rats/group.
Fig. 2 Chronic FZP treatment does not affect hippocampal cAMP levels. cAMP levels were assayed in CA1 and DG two days after the end of 1-week FZP treatment. Alterations in PKA activity (Fig. 1) are not accounted for by an alteration in endogenous cAMP levels. CA1: 86% of control; DG: 65% of control. *n = 6 rats/group*.
Fig. 3 Chronic FZP treatment does not affect PKA isoform mRNA expression in the hippocampus. Total mRNA was isolated from rat hippocampus as described in Methods. Top: Rlα, RIIα, RIIβ and Cβ mRNA levels were compared between FZP-treated and control rats using semi-quantitative RT-PCR: Rlα, (110% of control), RIIα (90% of control), RIIβ (98% of control), Cβ (120% of control). One-week FZP-treatment did not significantly affect the transcript levels of these PKA isoforms. n = 5 rats/group. Bottom: Representative bands depicting control and FZP-treated samples.
Chronic FZP treatment altered PKA isoform protein levels in hippocampal CA1 area. Insoluble fraction of hippocampal (a) CA1, but not (b) DG, exhibited a significant 40% reduction in PKA RIIβ protein 2 days after 1-week FZP treatment. PKA Cβ isoform levels in (a) CA1 or (b) DG were unaffected by chronic FZP treatment. $n = 5$ rats/group. Bottom: Representative bands from Western Blots depicting RIIβ and Cβ levels from control and FZP-treated samples.
Fig. 5 8-bromo-cAMP positively modulated mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats. (a) Representative traces of mIPSC events before and during superfusion of increasing concentrations (250-1000 µM) 8-bromo-cAMP. (b) Baseline mIPSC amplitude, averaged across 5 min recording, was significantly reduced (50% of control) 2 days following chronic FZP treatment. These same cells were subsequently exposed to 8-bromo-cAMP (CON: 10 cells/10 rats, FZP: 9 cells/9 rats). (c) The membrane permeable cAMP analog, 8-bromo-cAMP, significantly increased mIPSC amplitude in FZP-treated (+30% at 500 µM, p ≤ 0.05), but not control,
pyramidal cells. At the highest concentration (1000 µM), mIPSC amplitude was partially suppressed by 8-bromo-cAMP in control cells.

Fig. 6 VIP positively modulated mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats. (a) mIPSC amplitude, averaged across 5 min recording, was significantly reduced (58% of control) 2 days following chronic FZP treatment. These same cells were subsequently exposed to VIP (CON: 8 cells/8 rats, FZP: 7 cells/7 rats). (b) Like 8-bromo-cAMP, VIP increased mIPSC amplitude in cells from FZP-treated (36% at 30 nM, p < 0.05), but not control rats. (c) VPAC2 mRNA expression (top) assessed by RT-PCR in control and FZP-treated rats relative to β-actin mRNA levels (bottom). The
differential susceptibility of FZP-treated cells to VIP is not accompanied by a change in VPAC2 mRNA (96% of control level). A representative comparison is shown. $n = 5 \text{ rats/group}$
Table 1. Primers and annealing conditions for RT-PCR studies.

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Synaptic and Subcellular Localization of AKAP150 in Rat Hippocampal CA1 Pyramidal Cells: Co-localization with Excitatory Synaptic Markers

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Abbreviations

VGLUT1, vesicular glutamate transporter 1; VGAT, vesicular GABA transporter; GluR1-4 glutamate receptor subunits 1-4; TGN38, trans-Golgi network glycoprotein 38; AKAP, A-kinase anchoring protein; GABA\(_A\), \(\gamma\)-aminobutyric acid receptor type A; PKA, protein kinase A; PKC, protein kinase C; PP2B, protein phosphatase type B; PSD, postsynaptic density; GABARAP, GABA\(_A\) receptor associated protein; RACK1, receptor for activated C-kinase-1; LTP, long term potentiation; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL-M, stratum lacunosum-moleculare.
ABSTRACT

Excitatory and inhibitory ionotropic receptors are regulated by protein kinases and phosphatases, which are localized to specific subcellular locations by one of several anchoring proteins. One of these is the A-kinase anchoring protein (AKAP150), which confers spatial specificity to protein kinase A and protein phosphatase 2B in the rat brain. The distribution of AKAP150 was examined at rat hippocampal CA1 pyramidal cell asymmetric and symmetric postsynaptic densities and with respect to the distribution of markers of excitatory (VGLUT1, GluR1) and inhibitory receptors (VGAT, GABA_A receptor β2/3 subunits, gephyrin) and the Golgi marker, TGN38. AKAP150 was close to asymmetric synapses, consistent with numerous molecular and biochemical studies suggesting its interaction with components of the excitatory postsynaptic density. In contrast, we did not find AKAP150-immunoreactivity associated with inhibitory synapses in rat CA1 neurons, despite reports demonstrating an in vitro interaction between AKAP150 and GABA_A receptor β subunits, and the reported co-localization of these proteins in rat hippocampal cultures. There was some overlap between AKAP150 and GABA_A receptor β2/3-immunoreactivity intracellularly in perinuclear clusters. These findings support previous work indicating the integration of kinase and phosphatase activity at excitatory synapses by AKAP150, but do not support a role for selective targeting of AKAP150 and its accompanying proteins to inhibitory synapses.
**Key Words:** protein kinase A, calcineurin, postsynaptic density, GABA<sub>A</sub> receptors, receptor phosphorylation, receptor targeting.

AKAPs are a group of functionally related proteins that bind the regulatory subunits of PKA, and target the holoenzyme to discrete subcellular locations (Colledge and Scott 1999; Diviani and Scott 2001). The various AKAP isoforms can be discerned by their specific molecular interactions and subcellular distribution (Colledge and Scott 1999; Edwards and Scott 2000). AKAP150 (human AKAP79) interacts with the regulatory (RIIβ) subunit of PKA, PKC, and PP2B (calcineurin). Moreover, this AKAP isoform is believed to target its associated proteins to neuronal membranes and synapses, through domains capable of interacting with postsynaptic scaffold proteins including PSD-95, SAP-97, and F-actin, as well as the intracellular loops of GABA<sub>A</sub> receptor subunits (Carr et al. 1992; Colledge et al. 2000; Gomez et al. 2002; Brandon et al. 2003). Thus AKAP150 may confer spatial specificity to serine/threonine kinases and phosphatases at synaptic sites *in situ*.

Studies in recombinant and native neuronal systems have indicated that the cytoplasmic tails of GluR1, GluR2, and GluR4 AMPA receptor subunits are substrates for PKA, PKC, and CaMKII (Roche et al. 1996; Barria et al. 1997; McDonald et al. 2001). PKA modulates AMPA receptor currents via phosphorylation of GluR1-containing receptors at Ser845 (Greengard et al. 1991; Roche et al. 1996; Banke et al. 2000). In the hippocampus, PKA-mediated phosphorylation of Ser<sup>845</sup> is permissive for synaptic incorporation of AMPA receptors during LTP induction and required for LTP maintenance.
(Nayak et al. 1998; Lee et al. 2000; Esteban et al., 2003). In addition, it is the AKAP150-anchored PKA and PP2B that are responsible for the phosphorylation and dephosphorylation of Ser845 on GluR1 (Tavalin et al. 2002).

Similarly, GABA\textsubscript{A} receptors are regulated by protein phosphorylation (Swope et al. 1999; Olsen and Macdonald 2002; Brandon et al. 2002). The cytoplasmic loops of GABA\textsubscript{A} receptor β1-3 subunits contain consensus PKA substrate sequences (Moss et al. 1992; McDonald and Moss 1994), and increases or decreases in GABA\textsubscript{A} receptor function have been reported depending on the β subunit residue phosphorylated (McDonald et al. 1998; Nusser et al. 1999; Poisbeau et al. 1999; Hinkle and MacDonald 2003). The effects of phosphorylation on GABA\textsubscript{A} receptor function may be mediated by PKA tethered to the receptor by AKAP150. Indeed, AKAP150 and GABA\textsubscript{A} receptor β subunits co-immunoprecipitate from whole rat brain lysates, and co-localize in rat hippocampal culture (Brandon et al. 2003).

Modulation of receptor trafficking and function via receptor phosphorylation could occur locally at synapses or at a distance from synaptic sites, likely directed by the precise subcellular localization of kinase/phosphatase anchoring complexes. Unfortunately the exact location of AKAP150 in neurons is uncertain. In one electron microscopy study (Sik et al., 2000) AKAP 79, the human ortholog of rodent AKAP150, was found postsynaptically in proximity to excitatory (asymmetrical) synapses in the human hippocampus. However, a light microscopy study (Glantz et al., 1992)
failed to reveal AKAP150 immunoreactivity associated with excitatory synapses in rat hippocampal neurons. Moreover, despite the wealth of biochemical information relating AKAP150 to GABA\textsubscript{A} receptor phosphorylation, neither of these studies found AKAP150 immunoreactivity at, or in close proximity to, inhibitory synapses. Therefore in this study, the distribution of AKAP150 was assessed in rat CA1 neuron synapses using both preembedding immunohistochemistry and dual immunofluorescence with markers of excitatory (VGLUT1, GluR1) and inhibitory (VGAT, GABA\textsubscript{A} receptor β2/3 subunits, gephyrin) synapses.
MATERIALS AND METHODS

Tissue preparation

Eight adult male Sprague-Dawley rats (250-500 g, Harlan, Indianapolis, IN), were perfused under anesthesia (120 mg/kg Na pentobarbital, i.p.) via the left ventricle with 0.9% NaCl containing 4% sucrose followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) with or without 0.25% glutaraldehyde for light (LM) and electron microscopic (EM) studies, respectively. For LM studies, blocks of tissue containing the hippocampus were post-fixed (1-6 hr at 4°C), equilibrated in 15% sucrose in PB, then frozen-sectioned in the coronal or sagittal plane at 40 µm on a sliding microtome. Tissue intended for electron microscopic studies was blocked and sectioned at 50 µm on a vibratome® (Pelco101, Ted Pella Inc., Redding, CA). All sections were collected in PBS (0.01 M, pH 7.4) and stored at 4°C until further processing.

Immunohistochemistry

Antibodies. The following antibodies were used: goat anti-AKAP150 (N-19, Santa Cruz, Santa Cruz, CA), rabbit anti-GluR1 (R. Wenthold, NIDCD, Bethesda, MD), mouse anti-gephyrin (mAb 7a, Boehringer Mannheim, Indianapolis, IN), guinea pig anti-VGLUT1 (Chemicon, Temecula, CA), mouse anti-GABA receptors 2/3 (mAb341, bd-17, Chemicon), rabbit anti-VGAT (Chemicon), and mouse-anti-TGN-38 (Affinity Bioreagents, Golden, CO).
**LM Immunohistochemistry.** Free-floating sagittal sections (40 µm) were slide-mounted (Colorfrost®/Plus slides, Fisher Scientific, Pittsburgh, PA), blocked for 30 min in 10% normal horse serum diluted in PBS-T (0.01 M PBS + 0.1% Triton X-100), and incubated overnight at 4°C in anti-AKAP150 (1:200), rabbit anti-GluR1 (1:1000, 1 µg/ml) or mouse anti-GABA<sub>A</sub> receptor β2/3 (1:100, 10 µg/ml). After rinsing (3 x 5 min, 1 x 10 min PBS-T) tissues were exposed to HRP-conjugated biotin-sp-IgG raised in donkey, against goat (AKAP150), rabbit (GluR1), or mouse (GABA<sub>A</sub> receptor β2/3). Sections were rinsed, developed 5-10 min with a nickel enhanced DAB (diaminobenzidine) reaction (Vector Labs, Burlingham CA), dehydrated and cleared with xylene, then coverslipped with Permount™ (Fisher). Slide-mounted sections were visualized on a lightbox (Imaging Research Inc., St. Catherine, Ontario, Canada) under constant illumination and images were acquired with a high resolution CCD camera (Sierra Scientific, Sunnyvale CA) using NIH image (v. 1.59) software.

**Double Immunofluorescence.** After 30 min in 10% normal horse serum diluted in PBS-T (0.01 M PBS + 0.1% Triton X-100), free-floating sections were incubated overnight at 4°C in anti-AKAP150 (1:100-1:200) alone or in combination with one of the following: rabbit anti-GluR1 (1:1000), mouse anti-gephyrin (1:100), guinea pig VGLUT1 (1:4000), mouse anti-GABA<sub>A</sub> receptor β2/3 (1:100), rabbit anti-VGAT (1:200), or mouse anti-TGN38.
After rinsing (3 x 5 min, 1 x 10 min PBS-T) tissues were exposed to Alexa anti-goat–568 (1:200, Molecular Probes, Eugene, OR) alone or in combination with the appropriate secondary antibody: Alexa anti-mouse-488 (1:200, Molecular Probes), Alexa anti-rabbit-488 (1:200, Molecular Probes), or anti-guinea pig–FITC antibodies (1:50, Jackson Labs). Sections were then rinsed and mounted on glass slides coated with 0.5% gelatin/0.05% chrome alum and coverslipped with Vectashield (Vector). Immunoreactivities were analyzed with an Olympus FX confocal microscope. High magnification was achieved using a Plan APO Olympus 60x objective lens (1.4 numerical aperture, theoretical resolution 0.17 \( \mu m \)). Cross-talk between the emission of different fluorochromes at 488 nm with confocal microscopy was minimized as previously described (Geiman et al. 2002). Controls included the omission of the primary or secondary antibody in both single- and double-labeling experiments. These controls verified lack of significant auto-fluorescence, lack of secondary antibodies interactions with naïve tissue, and the lack of secondary antibody interactions with primary antibodies of different species.

In addition, AKAP150 antiserum specificity was tested by pre-absorption with the 19-amino acid immunizing peptide (20 \( \mu g \) antiserum and 20 \( \mu g \) peptide for 60 min at room temperature), before being applied to tissue sections. No staining was observed following preabsorption of the primary AKAP150 antiserum.
Preembedding immunohistochemistry. Vibratome sections (50 µm) were exposed to 1% sodium borohydride in PBS for 30 min, rinsed 6 x 5 min in PBS, and incubated in 10% normal chicken serum (Vector) for 3 hr. Sections were then incubated in goat anti-AKAP150 (1:50) overnight with agitation at 4°C. Tissues were rinsed (3 x 5 min; 1 x 10 min) and incubated for 3 hr at room temperature in anti-goat-biotin (Vector), rinsed (3 x 5 min; 1 x 10 min) and exposed to avidin-biotin-peroxidase complex (Vector) for 2 hr. After rinsing, the immunoreactive sites were visualized with a DAB reaction (0.005% DAB with 0.01% H₂O₂), rinsed again, fixed for 60 min in 2.5% glutaraldehyde, and washed in 0.1 M Tris-maleate buffer (TMB, pH 7.4) overnight. Sections were then silver intensified with a silver nitrate solution (6 min), rinsed in TMB (3 x 5 min), treated with 0.05% gold chloride (5 min), rinsed, incubated with 2.5% sodium thiosulfate (2 min), rinsed in TMB, followed by PBS, then fixed with 0.5% osmium tetroxide for 20 min. To prepare for embedding, sections were dehydrated through an ascending series of ethanols (50%, 2 x 70%, 2 x 95%, 3 x 100%, 10 min each), propylene oxide (2 x 10 min), 50% epon/araldite resin in propylene oxide (overnight) and then 100% resin (6 hr). Tissues were flat-embedded in between glass coverslips coated with formen-trenmittel (Electron Microscopy Sciences, Ft. Washington, PA) and the resin polymerized at 55°C for 3 days. Areas of CA1 were excised from cured resin, mounted in EM capsules for recutting, ultrathin sections obtained and collected on nickel grids. The sections were analyzed in a Philips 201 electron microscope. Digital images
Sections were scanned at low power in the electron microscope. When reaction product was observed, it was examined at higher power and its relationship to subcellular structures was recorded. Although peroxidase immunohistochemistry is not quantitative, this method provided a qualitative assessment of the subcellular distribution of AKAP150.

Colocalization analysis. Dual fluorescence colocalization was assessed using Image Pro Plus software v. 5.1 as previously described (Alvarez et al., 2004). At single confocal planes both colors in dual immunofluorescence images were superimposed, and 17 mm² regions of interest were analyzed from the dendritic (SO and SR) and somatic (SP) layers of CA1 hippocampus. Each color immunofluorescence was thresholded to specifically detect immunoreactive objects. Threshold-segmented images for each color of immunofluorescence where superimposed and the number of objects showing co-localization compared to the total number of objects captured with each immunoreactivity. Immunoreactivities that showed less than 2% co-localization could arise because random partial overlap of close by structures, and were not considered significant.
RESULTS

Preliminary experiments with the anti-AKAP150 antiserum revealed that this protein is heterogeneously distributed in rat brain. AKAP150 immunoreactivity (AKAP150-IR) was most pronounced in the striatum and olfactory tubercle. The cortex and hippocampus expressed moderate levels, whereas midbrain and hindbrain were relatively devoid of AKAP150-IR (Fig. 1A). This pattern of expression was similar to that previously reported using a different antiserum against AKAP150 (Glantz et al., 1992). Moreover, all staining was eliminated when the antiserum was pre-absorbed with the immunizing peptide (Fig. 1C).

Within the hippocampus, AKAP150-IR was distributed over both neuronal and glial elements (Fig. 1B, 1D). The glial staining was most obvious at the surface of the tissue section, and when shorter post-fixation was utilized. The significance of this staining is presently unclear. AKAP150-IR was not uniform in all regions. For example, CA1 and dentate gyrus showed stronger immunoreactivity than CA3. In addition, basal dendritic regions (SO) displayed greater immunoreactivity than did apical dendritic regions (SR; Fig. 1B). At higher magnification, AKAP150-IR was punctate (Fig. 1E), and when superimposed on NeuN-immunolabeled CA1 pyramidal cell somata revealed that most AKAP150-IR was located in the cytoplasm, while a smaller proportion of puncta were close to the cell surface (Fig. 1E, 1F).
The subcellular distribution of AKAP150 was further examined with electron microscopy and silver-intensified ABC peroxidase preembedding immunolabeling. AKAP150-IR appeared as clusters of silver particles over a weak diffuse DAB precipitate that frequently was not well resolved in metal-contrasted sections. Most AKAP150-IR was found in dendritic spines, usually in spine cytoplasm or the membrane region outside the PSD (postsynaptic density; Figs. 2A-C). Some AKAP150-IR was found in other dendritic regions and also on glial profiles (arrowheads, Fig. 2D). Although multiple CA1 layers were analyzed, there was no indication of AKAP150-IR in close association with symmetric, presumed inhibitory synapses. Control sections, including those from the thalamus (a region that does not express AKAP150) and those not exposed to AKAP antibodies showed no reaction product in neuronal or glial elements.

Although the ultrastructural studies indicated enrichment of AKAP150 in association with excitatory, but not inhibitory, synapses in CA1 of rat hippocampus, it was important to reevaluate these findings with light microscopy, where less stringent and membrane permeabilization could be used, allowing for increased detection sensitivity. First, immunofluorescent labeling for AKAP150 was compared to markers of presynaptic (VGLUT1) or postsynaptic elements (GluR1) of excitatory synapses. Preliminary observations confirmed the robust hippocampal expression of VGLUT1 reported elsewhere (Fig. 3A; Herzog et al. 2001). AKAP150-IR did not overlap with VGLUT1 staining in any hippocampal layer suggesting that AKAP150 is
not localized presynaptically. However, there were regions where VGLUT1-IR was apposed to AKAP150-IR in the cellular and dendritic layers of CA1 (arrowheads, Fig. 3B, C). GluR1 immunoreactivity was also strong in the rat hippocampus, and also reflected the pattern previously reported (Petralia and Wenthold, 1992) with a relatively dense distribution in the CA1 region (Fig. 3D). Although there was occasional overlap of AKAP150 and GluR1, less than 1% of AKAP clusters were associated with GluR1 immunoreactivity (Fig. 3D-F). This is perhaps a reflection of the subsynaptic distribution of AKAP150 noted with electron microscopy.

To assess whether AKAP150 was present at rat hippocampal CA1 inhibitory synapses, dual immunofluorescence studies were carried out with AKAP150 and markers of inhibitory terminals (VGAT) and postsynaptic elements (gephyrin and GABA<sub>A</sub> receptor β2/3 subunit). VGAT is present in the synaptic vesicles of inhibitory terminals, and in the rat hippocampus clearly demarcates the cellular somatic layer of CA1 (Fig. 4A). VGAT immunoreactivity in CA1 somatic (Fig. 4B) or dendritic (Fig. 4C) layers did not co-localize with AKAP150-IR in these layers. Interneurons in CA1 dendritic layers often exhibited perinuclear AKAP clusters usually more prominent than those evident in pyramidal neurons. Gephyrin, a marker of inhibitory PSDs (Sassoë-Pognetto and Fritschy 2000), likewise exhibited more pronounced staining in the somatic layer of CA1 (Fig. 4D). As the gephyrin antibody is more sensitive to fixation, a shorter post-fixation (1 hr) was used to assess overlap. Glial staining for AKAP was appreciably greater in the lighter-fixed
tissue. There was no significant enrichment of AKAP-immunoreactivity (i.e. <1 % colocalization) associated with gephyrin clusters in either the cellular (Fig. 4E, F, G), or dendritic layers of CA1.

In hippocampal cell cultures co-localization between of AKAP150 and β2/3 immunoreactivities have been reported (Brandon et al., 2003). We utilized a monoclonal antibody to GABA_A receptor β2/3 subunits in conjunction with the antiserum to AKAP150 to explore this possible interaction in intact hippocampus. Again, less than 1% of AKAP150 clusters were associated with β2/3 immunoreactivity (Fig. 4, J). Interestingly, the limited overlap detected was largely cytoplasmic and distributed in clusters around the nuclear periphery (Fig. 4J). Whether this overlap is functionally significant is presently unclear.

AKAP150-IR was clearly associated with intracellular structures surrounding the nucleus, where it co-localized with TGN38, a transport protein specifically located in the Golgi apparatus (Fig. 5A-C). In the CA1 region, 10% of AKAP150 clusters contained also TGN-38 immunoreactivity. This co-localization was most apparent in the prominent Golgi apparatus displayed by hilar interneurons (Fig. 5D-F).
DISCUSSION

This study extends previous studies that describe AKAP150 immunoreactivity in multiple layers of the hippocampal CA1 region and provides the first direct ultrastructural evidence that AKAP150 immunoreactivity is associated with excitatory synaptic profiles in rat hippocampal CA1 pyramidal cells, in situ. Using both electron and light microscopic immunochemical techniques, we found no evidence for the presence of AKAP150 at inhibitory synapses in the rat hippocampus, suggesting that there is no anatomical basis for a stable interaction between AKAP150-anchored PKA and GABA\_A receptor \( \beta \) subunits at the synapse. The identification of AKAP150 in clusters around the the nucleus and in association with a marker of the Golgi apparatus offers the possibility that the AKAP150 plays a role in the trafficking of membrane targeted proteins.

AKAP150 is present throughout rat hippocampal formation, but restricted to CA1 in human hippocampus. Notwithstanding these differences, here we report that the ultrastructural distribution of AKAP150 in the rat CA1 region is similar to the distribution of its ortholog in CA1 of human hippocampus (Sik et al. 2000). Our findings are consistent with multiple biochemical and molecular studies that support the association of AKAP150 and excitatory synapses (Bregman et al. 1989; Coghlan et al. 1995; Klauck et al. 1996; Colledge et al. 2000). Moreover, the findings provide a direct structural correlate for functional studies that implicate the PKA-AKAP150-PP2B complex in regulating short- and long-term changes in hippocampal
excitatory receptor function (Rosenmund et al. 1994; Abel et al. 1997; Tavalin et al. 2002).

Asymmetric synapses contain NMDA- and AMPA-type glutamate receptors embedded in a prominent membrane thickening comprised of multiple proteins, the PSD (Gray, 1959, Kennedy 1997, Walikonis et al. 2000). Scaffolding molecules, like those in the AKAP family, are proposed to play a role in recruiting signaling molecules to the postsynaptic membrane (Colledge and Scott 1999; Edwards and Scott 2000; Diviani and Scott 2001). However, in the present immunofluorescence studies, most AKAP150 clusters were not associated with GluR1 clusters. Ultrastructural analysis revealed that AKAP150 immunoreactivity was clearly associated with dendritic spines, subjacent to the post-synapse, consistent with the lack of co-localization between AKAP150 and GluR1. The immunoperoxidase label offers the advantage of greater sensitivity, but the reaction product tends to diffuse, and therefore lacks the spatial resolution of immunogold labeling. However, little or no immunoreactivity was detected in PSDs suggesting that diffusion of the reaction product from these structures is unlikely to have caused errant localization of AKAP150-immunoreactivity in the cytoplasm. Unfortunately we were unable to obtain adequate labeling using this antibody with post-embedding techniques. It is possible that AKAP150-immunoreactivity could have been masked by reduced antibody accessibility or steric hindrance with the macromolecular PSD complex. Using immunogold, Sik et al. (2000) also reported AKAP79 immunoreactivity near,
but not within PSDs. Considering the similarity between the ultrastructural distribution of AKAP79/150 immunoreactivity in the human and rat hippocampal CA1 regions reported by Sik et al. and in this study using two different immunocytochemical electron microscopic methods and different antibodies, we conclude that AKAP150 may be preferentially located in cytoplasmic regions in proximity to excitatory synapses, but not within the PSD.

Molecular studies have indicated that AKAP150 is linked to the GluR1 subunit by the postsynaptic protein SAP97 (Colledge et al. 2000) and maintains AMPA receptor function by locally recruiting PKA (Rosenmund et al. 1994; Tavalin et al. 2002). In contrast, yotiao, an alternate AKAP isoform, directly interacts with the NR1 subunit of the NMDA receptor and active protein phosphatase 1, suppressing NMDA receptor function by promoting NR1 dephosphorylation (Westphal et al. 1999; Edwards and Scott 2000). Although PKA bound to AKAPs may be inactive (Faux et al. 1999, Edwards and Scott 2000), disruption of the AKAP150-PKA interaction suppresses AMPA receptor function and phosphorylation on Ser845 (Rosenmund et al. 1994; Tavalin et al. 2002). The subsynaptic localization of AKAP150 may allow for local sequestration of PKA, which upon dissociation from AKAP150 is catalytically active and required to maintain the phosphorylation of synaptic GluR1 subunits. When excitatory synaptic strength is increased following LTP induction, AKAP150 is upregulated (Genin et al. 2003), perhaps
recruiting more PKA. This might increase excitatory synaptic function and receptor incorporation (Lee et al. 2000, Estaban et al. 2003).

There is ample evidence for PKA-mediated regulation of GABA<sub>A</sub> receptors (McDonald et al. 1998; Poisbeau et al. 1999; Nusser et al. 1999; Olsen and Macdonald 2002; Hinkle and Macdonald, 2003). Moreover, Brandon et al. (2003) reported an association between AKAP150 and GABA<sub>A</sub> receptor β subunits, and co-localization of AKAP150 and β2/3 in rat hippocampal culture. However, we did not find AKAP150 at inhibitory synapses in CA1 of rat hippocampus. This finding is consistent with the absence of AKAP79 at inhibitory synapses in the human hippocampus (Sik et al. 2000), and fails to provide a structural basis for local regulation by AKAP150-anchored PKA of GABA receptors at the synapse. Similarly, RACK1 and PKC co-immunoprecipitate with cortical GABA<sub>A</sub> receptor β subunits, interactions that are necessary for the PKC-mediated attenuation of whole-cell GABA currents in cultured neurons (Brandon et al. 2002). However, neither PKC nor RACK1 have been identified immunohistochemically in association with inhibitory synapses in the CNS. Unlike excitatory synapses, inhibitory profiles do not exhibit a prominent PSD, are not associated with spines, and in the hippocampus are concentrated at the cell soma and proximal processes (Megias et al. 2001). In the absence of an anchoring protein, one possibility is that the receptors at inhibitory synapse PSDs are regulated by cytoplasmic changes in kinase and phosphatase activity, rather than being controlled by compartmentalized aggregates of
regulatory enzymes, including PKA and PKC in the PSDs. Alternatively, post-translational modifications of synaptic GABA\textsubscript{A} receptors may occur at the synapse through recruitment of kinases and phosphatases by scaffolding molecules yet to be identified.

The perinuclear staining of AKAP150 and its overlap with the Golgi-apparatus marker TGN38 is intriguing. A similar pattern of Golgi-staining was observed in a variety of cell lines with an alternate AKAP isoform, AKAP350. Notably, AKAP350 has multiple splice variants, including the NMDA receptor binding AKAP, yotiao (Schmidt et al. 1999). It is interesting to speculate that the Golgi- and receptor-associated AKAP variants regulate excitatory tone by affecting receptor trafficking and synaptic receptor function, following transcription of a single gene. The presence of AKAP150 immunoreactivity in perinuclear clusters and in association with TGN38 may indicate a Golgi-associated function for AKAP150. Like AKAP150, GABARAP binds to GABA\textsubscript{A} receptor subunits \textit{in vitro} and co-localizes with GABA\textsubscript{A} receptors in neuronal culture. Additionally, GABARAP is found in the Golgi apparatus and now thought related to the intracellular trafficking of GABA\textsubscript{A} receptors (Kneussel et al. 2000; Kittler et al. 2001). An alternate possibility is that AKAP150 is clustered at the Golgi-apparatus in transit to the sub-synapse, where it tethers local signaling molecules. Albeit limited, the co-localization between AKAP150 and the GABA\textsubscript{A} receptor $\beta$2/3 subunit in perinuclear clusters offers the possibility that the previously reported interaction between
AKAP150 and GABA<sub>A</sub> receptors (Brandon et al. 2002) in part occurs in the Golgi apparatus.

In summary, the present study indicates that AKAP150 immunoreactivity is present in proximity of excitatory synapses in rat hippocampal CA1 neurons. Together with previous functional studies pertaining to the effect of AKAP150 and its associated kinases on excitatory receptor function, these findings confirm the existence of an endogenous pool of AKAP150 in the proximity of synaptic excitatory ionotropic receptors. Conversely, we did not find AKAP150 immunoreactivity in association with inhibitory synapses in rat CA1 pyramidal cells.
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References


Figure 1.
**Figure 1.** AKAP150 immunohistochemistry. A. Saggital section of rat brain depicting AKAP150-IR. Note the relatively intense staining in the striatum and olfactory tubercle, as well as in some layers of both cortex and hippocampus. B. AKAP150 is distributed throughout all but the most distal dendritic layers (SL-M), and in the CA1 region stains basal dendrites (SO) more intensely than the apical dendrites (SR). C. Pre-absorbing AKAP150 antiserum with the antigenic peptide eliminates the immunoreactivity. D. CA1 of rat hippocampus. Glial immunoreactivity was more prominent at the tissue surface, and the staining became predominantly punctate at deeper levels (E). F. High magnification of the CA1 pyramidal cell layer, demonstrating that AKAP150-IR (red) is present both at the cell periphery and in the cytoplasm, with clusters that surround the nucleus (arrowheads, inset enlarged 50%). In this panel an antibody against the nuclear antigen NeuN(green) demarcates nuclear and cell boundaries. Scale bars = 5 mm (A), 1 mm (B, C), 200 µm (D), 100 µm (E) and 37.5 µm (F).
Figure 2.
Figure 2. Electron micrographs of AKAP150-IR in rat hippocampal CA1 neurons. Most immunoreactive profiles were found in proximity of asymmetric post-synapses. AKAP150-IR was concentrated in the neck of dendritic spines (reaction product denoted by arrowheads in A, B, and C), proximate to postsynaptic densities (arrows in A, B, and C). No immunoreactivity was observed in the vicinity of symmetric, presumed inhibitory, synapses. Immunoreactivity was also noted in glial elements, associated with glial filaments (arrowhead in D). Scale Bars = 0.5 μm.
Figure 3.
**Figure 3.** Immunohistochemistry of AKAP150 and Excitatory Synaptic Markers. A. VGLUT1-IR in the CA1 region. B. High magnification image of CA1 SP. Note that VGLUT1 (green) and AKAP150 (red) do not overlap, but are interspersed (see arrowheads) in both this layer, and in dendritic layers (SR. panel C). D. GluR1-IR in the CA1 region. E. High magnification image of GLUR1-IR in CA1 SP. Note the GluR1-IR (green) is present throughout the pyramidal cell cytoplasm, and partially overlaps with AKAP150 (arrowheads, lower right) (F), enlarged 200% in G (arrowheads). Scale bars = 100 µm (A, D); 25 µm (B-C, E-F).
Figure 4.
Figure 4. Immunohistochemistry of AKAP150 with Inhibitory Synaptic Markers. A. VGAT-IR in the CA1 region. B. High magnification photomicrograph of CA1 SP. Although the VGAT (green) and AKAP150 (red) clusters are interspersed, a common overlapping pattern is not easily discernable. C. Image from SR depicting a VGAT immunoreactive interneuron, with AKAP-IR arranged around the nucleus (arrowhead). D. Gephyrin-IR in the CA1 region. When higher magnification images of gephyrin (E) and AKAP150 (F) in SP of CA1 are overlaid (G), puncta representing these proteins do not co-localize. H. GABA_A receptor β2/3-IR in CA1 of hippocampus. Note the presence of numerous well-stained interneurons in SP. I. High magnification image depicting β2/3-IR (green) and AKAP150-IR (red) in the pyramidal cell layer of CA1. Panel I is enlarged 200% in J. Although limited, the overlap that is present between β2/3 and AKAP150 is more obvious in interneurons, and present within cytoplasmic clusters (arrowheads). Scale bars = 100 μm (A, D, H); 25 μm (B, C, E-G, I).
Figure 5.  A. AKAP150-IR in the CA1 pyramidal cell layer of the hippocampus.  B. TGN38-IR at the same plane as in (A), depicting the Golgi apparatus of pyramidal cells (arrowheads) and interneurons (arrows).  C. When overlaid, there are multiple areas of colocalization over both pyramidal cells (arrowheads) and interneurons (arrows), although those on interneurons are more apparent.  D. AKAP150-IR in the hilus of the rat dentate gyrus.  E. TGN38-IR at the same plane as in (D), depicting the extensive Golgi apparatus in hilar interneurons.  F. When overlaid AKAP150 and TGN38 colocalize in these interneurons (arrowheads). Scale bars = 25 μm.
Altered AMPA receptor GluR1 subunit expression in rat hippocampal CA1 during benzodiazepine withdrawal

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Abstract

Mechanisms of increased synaptic strength at glutamatergic synapses have been described in hippocampal CA1 pyramidal neurons, with particular regard to long-term potentiation and associative learning. Similar mechanisms may operate during withdrawal from drugs of abuse, and underlie the increase hippocampal CA1 neuron excitability during withdrawal from prolonged flurazepam (FZP) administration. As the GluR1 subunit of the AMPA receptor (AMPAR) is critical for activity-dependent alterations in excitatory strength, we sought to determine whether alterations in GluR1 subunit distribution and synaptic expression were apparent during FZP-withdrawal, and might underlie the reported increase in AMPAR-mediated miniature excitatory postsynaptic current (mEPSC) amplitude. Confocal analysis revealed a small (5%) non-significant (p = 0.40) reduction in cytoplasmic GluR1 subunit content in rats undergoing FZP-withdrawal. Electron microscopic analysis of apical and basal dendritic regions in hippocampal CA1 revealed a significant 35% increase in synaptic GluR1 subunit expression per µm of PSD length in the stratum radiatum (p = 0.02) without a change in asymmetric synapse length or width. The trend toward increased GluR1 subunit expression (20%) in the stratum oriens was not significant (p = 0.10). These findings provide evidence that increased synaptic GluR1-containing AMPAR expression is associated with increased excitatory neurotransmission in rat hippocampal CA1 neurons during FZP-withdrawal. Taken together with previous work from our laboratory, these
findings also support the idea that increases in synaptic strength that occur in models of drug dependence and activity-dependent plasticity operate on similar mechanisms.
1. Introduction

Benzodiazepines (BZs) bind to an allosteric site on the GABA<sub>A</sub> receptor (GABAR) and increase the frequency of Cl<sup>-</sup> channel opening to facilitate inhibitory neurotransmission in the central nervous system (Korpi et al., 2002). The BZs are effective anxiolytics, hypnotics, and anticonvulsants, yet prolonged exposure to these drugs results in both tolerance and dependence, phenomena that limit their clinical utility (Argyropoulos and Nutt, 1999; Griffiths and Weerts, 1997). The anxiety associated with BZ withdrawal is associated with increased hippocampal CA1 pyramidal cell excitability and increased AMPAR expression at the light microscopic level (Izzo et al., 2001; Van Sickle et al., 2002; Van Sickle et al., 2004).

Ionotropic AMPARs are tetrameric cation-permeable membrane channels, responsible for the majority of fast excitatory synaptic transmission in the central nervous system (CNS). AMPARs are heteromers of at least four subunit subtypes (GluR1-4), and the rat hippocampus expresses high levels of GluR1, GluR2, and GluR3 subunits, which combine to form three subpopulations of AMPARs: GluR2/3 heteromers, GluR1/2 heteromers, and GluR1 homomers (Lerma et al., 1994; Petralia and Wenthold, 1992; Wenthold et al., 1996). Electrophysiological data supports a differential localization of native receptor assemblies, with GluR1 homomers apparently restricted to the stratum radiatum (SR, apical dendrites) and absent in the stratum oriens (SO, basal dendrites) of hippocampal CA1 (Bagal et al., 2005b). Other differences
among the AMPAR subpopulations include Ca\textsuperscript{2+} permeability and control of membrane delivery. For example, the activity-dependent conversion of ‘silent’ to active AMPAR-containing synapses that occurs upon induction of LTP in hippocampal CA1 is critically dependent on insertion of GluR1-containing AMPARs. In fact, GluR1 subunits are largely retained in the dendritic cytoplasm until synaptic activity drives their membrane expression (Lee et al., 2003; Mack et al., 2001; Passafaro et al., 2001; Shi et al., 2001; Zamanillo et al., 1999). Conversely, the GluR2 subunit is constitutively cycled between intracellular compartments and the synapse, and likely exerts primary control over the degradation of the AMPAR complex once it has accumulated intracellularly (Lee et al., 2004; Passafaro et al., 2001). These differences in trafficking are related to heterogeneity in the C-terminus of GluR1 and GluR2 subunits. When coexpressed as GluR1/2 heteromers, the GluR1 subunit determines synaptic delivery. These heteromers may be the primary contributors to the increase in synaptic AMPAR content following LTP induction in hippocampal slices, although the contribution of GluR1 homomers cannot be excluded (Bagal et al., 2005a; Shi et al., 2001).

During withdrawal from 1-week FZP treatment, rats exhibit anxiety along with increased hippocampal CA1 pyramidal neuron excitability and mEPSC amplitude. The \textit{in vitro} strengthening of CA1 neuron excitatory synaptic function and the expression of anxiety \textit{in vivo} is mitigated by pharmacological antagonism of AMPARs, underscoring the association of these neurophysiological and behavioral changes (Van Sickle and Tietz,
LTP is the most widely investigated model of activity-dependent synaptic plasticity in hippocampal CA1 region. This phenomenon is associated with the coordinate activation of CaMKII and PKA, direct PKA-mediated phosphorylation of the GluR1 subunit on Ser\textsuperscript{845}, and a subsequent increase in the synaptic expression of GluR1-AMPARs (Andrasfalvy and Magee, 2004; Esteban et al., 2003; Hayashi et al., 2000; Lee et al., 2000; Lee et al., 2003; Malinow and Malenka, 2002; Poncer, 2003). During FZP-withdrawal, we have detected comparable alterations in CA1 hippocampus, including increased AMPAR radioligand binding and increased Ser\textsuperscript{845} phosphorylation of the GluR1 subunit (Song and Tietz, 2004; Van Sickle and Tietz, 2002). The goal of the present study was to examine the cellular distribution and synaptic expression of GluR1 subunits on hippocampal CA1 neurons using confocal and electron microscopic techniques.
2. Materials and methods

2.1 Benzodiazepine Treatment

After a 2 day acclimation period during which 0.02% saccharin water was offered, juvenile male Sprague-Dawley rats (125-150 g; Harlan, Indianapolis, IN) were offered FZP (100 mg/kg x 3 days; 150 mg/kg x 4 days) in 0.02% saccharin water as the sole source of drinking water. Controls received saccharin water for 1 week. The target for the FZP group was an average dose of 100 mg/kg/day for the first three days, and 150 mg/kg/day for the following four days. Rats that did not achieve a weekly average of 100 mg/kg/day were excluded. After drug removal, rats were given saccharin water for an additional 2 days prior to tissue preparation. This dosing regimen reliably induces manifestations of both BZ tolerance and dependence (Tietz et al., 1999b; Van Sickle et al., 2004).

2.2 Tissue preparation

Experimental protocols involving the use of vertebrate animals were approved by the Medical University of Ohio Institutional Animal Care and Use Committee (IACUC) and conformed to the National Institutes of Health guidelines. All efforts were made to minimize animal distress. For confocal and electron microscopic studies, rats were anesthetized (i.p. 100 mg/kg pentobarbital), and perfused via the left ventricle with 100 ml 0.9% NaCl followed by 300 ml 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M
Blocks containing bilateral hippocampii were hemisected and postfixed for 1 hr (immunofluorescence) or 2 hr (electron microscopic studies), respectively.

2.3 Immunohistochemistry

2.3.1 Immunofluorescence

Free-floating sections (40 µm) were prepared with a frozen sliding microtome from blocks equilibrated in 15% sucrose in PBS. The sections were incubated 20 min in PBS containing 1% sodium borohydride, rinsed 4 x 5 min in PBS containing 0.1% Triton X-100 (pH 7.4, PBST), blocked in 10% normal horse serum for 30 min, then placed overnight at 4°C in PBS containing rabbit anti-GluR1 subunit (1:1000) and mouse anti-NeuN (1:1000). After rinsing, sections were exposed to donkey anti-rabbit IgG-488 and donkey anti-mouse IgG-Texas Red (1:200 and 1:50 in PBST, respectively), rinsed, mounted on glass slides coated with 0.5% gelatin/0.05% chrome alum and coverslipped with Vectashield (Vector Labs, Burlingham CA). Controls included the omission of the primary or secondary antibody in both single- and double-labeling experiments. These controls verified the lack of significant auto-fluorescence, the lack of secondary antibody interactions with naïve tissue, and the lack of secondary antibody interactions with primary antibodies of different species.

2.3.2 Freeze slamming, cryosubstitution and low temperature embedding
Coronal vibratome sections (500 µm) were exposed to 1% sodium borohydride in PB for 60 min, rinsed 6 x 30 min, placed in 4% glucose overnight (4°C), equilibrated in glycerol (10, 20, and 30% in 0.01 M PBS, pH 7.4), then slammed to a precooled copper mirror (-190°C, Leica EM CPC). For cryosubstitution and low temperature embedding, tissues were transferred to Leica EM AFS and treated as follows: 12 hrs in 1% uranyl acetate in methanol (-80°C), 4 x 30 min absolute methanol (this and following at -50°C), 2 hrs each in 50%, 75%, 100%, 100% lowicryl HM20, and 2 x 8 hours in 100% lowicryl. Sections were then transferred to glass slides coated with formen-trenmittel (Electron Microscopy Sciences, Ft. Washington, PA), and polymerized with UV light (48 hrs at -50°C, 72 hrs at 0°C, 48 hrs at 20°C). Blocks containing CA1 area of hippocampus were excised, ultrathin sectioned at 90 nm (silver/gold sections) and collected on nickel grids.

2.3.3 Post-embedding immunohistochemistry

Grids were wet in nanopure dH2O, etched in sodium ethanolate 3 sec, rinsed multiple times in nanopure dH2O, then incubated for 10 min in 1% normal horse serum, 2% bovine serum albumin, 0.1% cold fish gelatin in 50 mM Tris-HCl containing 0.1% Triton X-100 (pH 7.6, TBNT). Grids were then incubated 1 hr in rabbit anti-GluR1 subunit (1:50), rinsed 3 x 5 min in TBNT and exposed to goat anti-rabbit IgG conjugated to 10 nm gold particles (EMGAR10, BBI, United Kingdom) diluted 1:50 in TBNT (pH 8.2). After
rinsing (3 x 5 min in nanopure dH$_2$O), grids were contrasted with uranyl acetate and lead citrate (5 and 2 min, respectively).

2.4 Data Analysis

2.4.1 Confocal Microscopy

Using an Olympus Fluoview laser scanning confocal microscope, images were collected from CA1 cell bodies 7 µm from the tissue surface. Only cells in which the nucleolus was visible were used in the analysis. Areas containing cytoplasmic GluR1 subunit reactivity were isolated by outline of the cell periphery delineated by the GluR1 subunit staining, and the nuclear periphery delineated by NeuN reactivity. Within these areas clusters from individual cells were analyzed. The mean cluster intensity from individual neurons within a section was measured (Image Pro v4.1, MediaCybernetics) and compared by an observer blind to experimental group. Minimum detection threshold for individual clusters was set to 10X nuclear background intensity. Mean cluster intensity from control and FZP-withdrawn groups was compared by ANOVA with post-hoc analysis by least significant difference (LSD) test.

2.4.2 Electron Microscopy

Reacted ultrathin sections were scanned in a Phillips 201 EM electron microscope, and postsynaptic densities (PSDs) in the CA1 SO and SR were photographed. Digital images of asymmetric synapses in these regions were
captured at a final magnification of 56,000 (Gatan BioScan Camera) by an observer blind to the experimental groups. Fifty images from each of 6 rats were analyzed with Image Pro-Plus (v. 5.0 Media Cybernetics) and the number of particles within 20 nm (~antibody size) of the PSD were counted and recorded as particles per linear \( \mu \text{m} \) of PSD.

Background labeling in each image was calculated using randomly placed bars (25) of average length and thickness for the sampled PSD populations. PSDs were considered positively labeled when the linear particle density was greater than 2.5 standard deviations above the average noise. The length, width, and immunogold labeling per linear \( \mu \text{m} \) of PSD was compared between control and FZP-withdrawn rats by ANOVA followed by a LSD post-hoc test. The proportion of synapses with immunogold labeling was also compared using a Student's t-test. Given that pyramidal cells outnumber interneurons in CA1 hippocampus by approximately 20:1, and contain far more excitatory synapses per cell (Bernard and Wheal, 1994; Megias et al., 2001), the presumed contribution of excitatory synapses from interneurons in this analysis was negligible.
3. Results

The rabbit anti-GluR1 subunit serum used herein was raised against the cytoplasmic tail of the GluR1 subunit and affinity purified (Petralia and Wenthold, 1992). Its specificity has been verified, and the staining pattern at the light and electron microscopic level has been reported (Petralia and Wenthold, 1992; Wenthold et al., 1996). A low power micrograph depicting a sagittal image of the rat brain stained with the GluR1 subunit antiserum demonstrates a pattern reflecting that previously reported (Petralia and Wenthold, 1992), that is strong GluR1 subunit-immunoreactivity (GluR1-IR) in the olfactory bulb, hippocampus and cerebellar cortex, and relatively sparse staining in the midbrain and brainstem (Figure 1a). Notably, there is robust staining in the CA1 dendritic regions SO and SR (Figure 1b), representing the high density of excitatory synapses in these areas (Megias et al., 2001).

3.1 GluR1 subunit immunofluorescence and confocal microscopy

Redistribution of GluR1 subunit-containing receptors from the cytoplasm to the synapse occurs during development and during activity dependent increases in synaptic strength (Mammen et al., 1997a; Passafaro et al., 2001). First, we wished to determine whether or not the changes we observe in glutamatergic function during FZP-withdrawal are accompanied by changes in the abundance of cytoplasmic GluR1 subunits. Using sections dual stained for NeuN and GluR1 subunits, we analyzed cytoplasmic GluR1
subunit cluster intensity in CA1 pyramidal cells of control and FZP withdrawn rats (see methods). Our analysis suggested a small (5%) non-significant reduction in cytoplasmic GluR1 subunit cluster intensity during FZP-withdrawal (Figure 2, Table 1). Thus, our findings were not consistent with a net loss of cytoplasmic GluR1 subunit containing receptors from the cytoplasmic compartment during FZP-withdrawal.

3.2 Synaptic GluR1 subunit-IR in dendritic regions of rat hippocampal CA1

In earlier studies, we identified increased mEPSC amplitude in hippocampal CA1 pyramidal cells (Van Sickle et al., 2002), therefore we examined the synaptic expression of GluR1 subunit immunoreactivity in this region. Given the relative paucity of excitatory synapses on pyramidal cell somata (Megias et al., 2001), and that dendritic shaft regions show the highest GluR1 subunit expression and most robust changes in AMPAR binding (Van Sickle and Tietz, 2002), we chose to focus on proximal regions of basal and apical dendritic areas, SO and SR, respectively. Areas within approximately 100 µm of the stratum pyramidale (SP) border in SO and SR were scanned, and asymmetric junctions representing excitatory synapses (Gray, 1959; Megias et al., 2001) were photographed. Background labeling (see methods) was similar among the samples, and represented less than 4% of total GluR1 subunit immunolabeling.

GluR1 subunit staining is robust in the SO of rat hippocampus (Figure 1). At the ultrastructural level the label is predominantly associated with the
PSDs, where localized high concentrations of GluR1 subunit provide an antigen concentration amenable to labeling with post-embedding IHC. In the SO, labeled synapses contained from 1-4 particles distributed throughout the length of the PSD in both control and FZP-withdrawn animals (Figure 3). The number of particles per linear µm PSD was somewhat greater in FZP-withdrawn than control rats, although this difference was not statistically significant (CON 2.8 ± 0.4, FZP 3.6 ± 0.2, p = 0.1, Table 2). Likewise, there was no significant change in the proportion of synapses that were GluR1 subunit-immunoreactive (CON 41.7 ± 2.2%, FZP 51.3 ± 5.0%, p = 0.10). There was also no difference in synapse length or width between control and FZP-withdrawn rats in this brain region (Table 2).

The SR also displays rich GluR1 subunit staining (Figure 1). Labeled synapses contained from 1-4 particles, and the labeling was apparent at the periphery and center of the PSD in both control and FZP-withdrawn rats (Figure 4). In this region of the hippocampus, FZP-withdrawal was accompanied by a significant increase in the number of particles per linear µm PSD (CON 2.4 ± 0.3, FZP 3.8 ± 0.4, p = 0.02). There was also a significant increase in the proportion of synapses with GluR1 labeling (CON 38.2 ± 3.8%, FZP 50.8 ± 2.4%, p = 0.01). There was no difference in synapse length or width in the SR of FZP-withdrawn rats (Table 2).
4. Discussion

We have previously reported an increase in AMPAR-mediated mEPSC amplitude in rat CA1 hippocampal pyramidal cells 2 days after cessation of 1-week FZP treatment, an effect that coincides with behavioral manifestations of withdrawal (Van Sickle and Tietz, 2002; Van Sickle et al., 2004). Using high magnification electron microscopy, we now report a significant 35% increase in synaptic AMPAR GluR1 subunit expression in the apical dendrites (SR) of CA1 neurons, providing a substrate for the observed increases in hippocampal glutamatergic neurotransmission during FZP-withdrawal. The basal dendritic region (SO) of CA1 did not exhibit a statistically significant increase in synaptic GluR1 subunit expression. Although the present analysis may not have had sufficient power to detect a change in the SO region, the lack of regulation of synaptic GluR1 expression in SO during FZP-withdrawal may be related the differences in native AMPAR subpopulations between these dendritic regions. Bagal et al. (2005b) recently provided electrophysiological evidence for the presence of GluR1 homomers in apical, but not basal CA1 neuron dendrites. The presence of a GluR1 subunit in the AMPAR complex is required for activity-induced increases in synaptic expression (Shi et al., 2001; Zamanillo et al., 1999). The present findings indicate an increase in the proportion of GluR1 subunit-containing synapses. Whether or not this represents incorporation into silent synapses is presently unclear, an issue that can be resolved with the analysis of whole synapses by
serial sections and detailed electrophysiological studies (Nusser et al., 1998b; Takumi et al., 1999). Moreover, as with activity dependent plasticity, whether GluR1 homomers or GluR1/2 heteromers are incorporated at CA1 pyramidal neuron synapses during benzodiazepine withdrawal will require further investigation (Bagal et al., 2005a; Shi et al., 1999).

The cytoplasm contains a store of GluR1-containing AMPARs, that distribute to the synapse upon induction of LTP concomitant with activation of CaMKII and PKA (Malinow and Malenka, 2002). Although we detected a significant increase in the synaptic expression of the GluR1 subunit during FZP-withdrawal, there was not a coordinate decrease in cytoplasmic GluR1 subunit levels in this study. One possibility is that the methodology utilized may not have been sensitive enough to detect a decrease in intracellular stores of the GluR1 subunit. Alternately, while early increases in synaptic number may come at the expense of cytoplasmic GluR1 subunits, sustained incorporation of synaptic AMPARs likely coincides with increased receptor expression and decreased receptor degradation, events that may maintain cytoplasmic stores within normal limits (Malinow and Malenka, 2002; Nayak et al., 1998).

4.1 GluR1 subunit regulation in alterations of excitatory synaptic strength

The most widely studied example of synaptic plasticity is the stimulus-induced potentiation of the Schaffer collateral – CA1 neuron glutamatergic pathway (Bear and Malenka, 1994; Malenka and Bear, 2004).
In the adult hippocampus, up to 25% of synapses do not contain AMPARs and are termed “silent synapses”. Upon LTP induction there is a decrease in the number of synaptic failures, indicating a conversion of silent to non-silent synapses, an effect that depends on increasing synaptic expression of GluR1-containing AMPARs (Malinow and Malenka, 2002; Poncer, 2003; Shi et al., 1999). Genetic knockout studies corroborate the importance of the GluR1 subunit: LTP cannot be induced in mice lacking the GluR1 subunit, but can be induced in mice lacking GluR2 and GluR3 subunits (Jia et al., 1996; Meng et al., 2003; Zamanillo et al., 1999). Direct phosphorylation of GluR1 subunit by PKA “primes” these receptors and permits their translocation from intracellular pools to the extrasynaptic membrane (Esteban et al., 2003; Oh et al., 2005; Passafaro et al., 2001). Subsequently, in an event likely CaMKII-dependent, GluR1-containing AMPARs are recruited to synaptic sites (Hayashi et al., 2000; Ju et al., 2004; Passafaro et al., 2001; Shi et al., 2001; Shi et al., 1999). Direct phosphorylation of GluR1 subunits by PKA and CaMKII can also increase single channel function and contribute to forms of activity-dependent plasticity (Banke et al., 2000; Derkach et al., 1999; Luthi et al., 2004). Thus, there is significant evidence that GluR1-containing AMPARs are imperative for and responsible for the activity-dependent increases in CA1 pyramidal neuron excitatory synaptic strength.

The neurophysiological changes that occur during prolonged exposure to drugs of abuse are often compared to those occurring in LTP, on the assumption that neurons have a finite repertoire of adaptations and that drug-
induced and activity-dependent plasticity share similar mechanisms (Nestler, 2002). The increase in synaptic strength we observe in CA1 hippocampus during FZP-withdrawal using electrophysiological approaches coincides with an increase in GluR1 subunit expression at asymmetric synapses. Given that the cytoplasmic loop of the GluR1 subunit is associated with multiple scaffolding proteins and proximate to PKA anchoring proteins in rat CA1 synapses, phosphorylation of GluR1 subunits may moderate the increase in synaptic GluR1 subunit levels and related increase in excitatory synaptic strength (Colledge et al., 2000; Lilly et al., 2005). Consistent with this hypothesis, PKA-mediated GluR1-Ser\textsuperscript{845} phosphorylation 12 hours and 1 day after FZP treatment cessation precedes the increase in CA1 neuron mEPSC amplitude in from 1-day and 2-day FZP-withdrawn rats. Since GluR1 pSer\textsuperscript{845} levels return to normal and membrane associated PKA activity is reduced at 2-days in the setting of a persistent increase in mEPSC amplitude, it is conceivable that phosphorylation and dephosphorylation of GluR1-containing AMPARs signals the onset and recovery of changes in excitatory tone and associated manifestations of withdrawal (Lilly et al., 2003; Song and Tietz, 2004; Van Sickle et al., 2004).

4.2 GluR1 subunit regulation during drug withdrawal

A model of benzodiazepine dependence has been proposed in which excitatory components are upregulated following prolonged BZ-mediated GABAergic enhancement. Upon abrupt withdrawal of the benzodiazepines,
the increase in excitatory tone may persist and contribute to the expression of a withdrawal syndrome that includes anxiety (Allison and Pratt, 2003; Stephens, 1995). In many of these paradigms, including withdrawal from 1-week FZP exposure, AMPAR antagonists ameliorate both the alterations in GluR1 subunit expression and drug withdrawal behaviors (Jackson et al., 2000; Nestler, 2002; Van Sickle et al., 2004). Collectively these data suggest that increased glutamatergic tone is important for the acquisition or expression of drug withdrawal behaviors that occurs upon discontinuation of a variety of drugs of abuse.

4.3 Conclusion

An increase in synaptic GluR1 subunit expression was detected during FZP-withdrawal at asymmetric synapses on CA1 neuron apical dendrites. This is in agreement with previous work that indicated increased AMPAR radioligand binding and GluR1 subunit expression at the light microscopic level (Izzo et al., 2001; Tietz et al., 2003; Van Sickle and Tietz, 2002; Van Sickle et al., 2004). It will be important to determine how specific AMPAR subpopulations in hippocampal CA1 neurons are regulated during FZP-withdrawal, and the relative contribution of GluR1 homomers and GluR1/2 heteromers to the increased glutamatergic strength in this brain region. Nevertheless, these findings provide mechanistic insight into the increased mEPSC amplitude and hyperexcitability of CA1 pyramidal neurons associated with drug discontinuation. They also lend further support to the notion that
adaptive strategies characteristic of drug withdrawal and other forms of activity-dependent plasticity are highly conserved in the CNS.
Acknowledgements

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Figure 1. GluR1 subunit immunostaining in the rat brain. (a) Saggital section of rat brain depicting GluR1 subunit-IR. Note the relatively intense staining within layers of the olfactory tubercle, hippocampus, and cerebellum, while the midbrain and hindbrain are relatively devoid of GluR1 subunit-IR. (b) Higher power image the hippocampus. GluR1 subunit-IR is present at high levels throughout all principal cell dendritic layers in the hippocampus and dentate gyrus, including stratum oriens and stratum radiatum of CA1 (SO and SR, respectively). This pattern closely resembles that reported previously (Petralia and Wenthold, 1992). Scale bars = 5 mm (A), 1 mm (B).
**Figure 2.** Representative images of GluR1 subunit immunofluorescence in CA1 hippocampus from control and 2-day FZP-withdrawn rats. (a). Low power micrograph of GluR1 subunit staining in CA1 of hippocampus. Notice the rich dendritic distribution of GluR1 subunit in SO and SR. (b) Higher power micrographs depicting the nuclear marker NeuN (red, left panel of B and C), overlapped with GluR1 subunit (green, right panel of B and C). Mean cytoplasmic GluR1 subunit cluster intensity was measured 7 µm from the surface of the section, at the level of the nucleolus (n) as described in the methods. Cluster intensity was marginally decreased (5%) in FZP-withdrawn rats (see Table 1), though this was not statistically significant. Scale bars 100 µm (a), 37.5 µm (b, c).
<table>
<thead>
<tr>
<th>Treatment</th>
<th># Cells (# Clusters)</th>
<th>Mean Cluster Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>99 (5228)</td>
<td>158.2 ± 3.9</td>
</tr>
<tr>
<td>FZP</td>
<td>112 (4942)</td>
<td>149.7 ± 5.2</td>
</tr>
</tbody>
</table>

Values represent mean of the n=3 rats/group ± S.E.M.
Groups compared by ANOVA and post-hoc LSD test, *p<0.05
Figure 3. Representative electron micrographs depicting synapses in rat SO from control (A, B) and FZP-withdrawn (C, D) rats. Ultrathin sections were reacted with anti-GluR1 subunit antibodies and 10 nm immunogold (see methods). There was no significant difference in the density of particles per µm of asymmetric density. Moreover, synapse size was similar between control and FZP-withdrawn rats (Table 2). Scale bars equal 0.2 µm.
Figure 4. Representative electron micrographs depicting synapses in rat SR from control (A, B) and FZP-withdrawn (C, D) rats. Ultrathin sections were reacted with anti-GluR1 subunit antibodies and 10 nm immunogold (see methods). There was a significant increase in the number of particles per linear m of PSD in this brain region, however, synapse size was similar between control and FZP-withdrawn rats (Table 2). Scale bars equal 0.2 m.
Table 2

Synaptic GluR1 subunit immunoreactivity in hippocampal CA1

<table>
<thead>
<tr>
<th>Treatment (No. Synapses)</th>
<th>Length (nm)</th>
<th>Width (nm)</th>
<th>Particles/µm length</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON (n = 191)</td>
<td>202 ± 16</td>
<td>48 ± 4</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>FZP (n = 188)</td>
<td>228 ± 6</td>
<td>51 ± 3</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>*p value</td>
<td>0.17</td>
<td>0.50</td>
<td>0.12</td>
</tr>
<tr>
<td>SR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON (n = 208)</td>
<td>204 ± 5</td>
<td>48 ± 2</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>FZP (n = 181)</td>
<td>228 ± 16</td>
<td>48 ± 1</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>*p value</td>
<td>0.19</td>
<td>0.88</td>
<td><strong>0.02</strong></td>
</tr>
</tbody>
</table>

Values represent means of the n=3 rats/group ± S.E.M.
Groups compared by ANOVA and post-hoc LSD test, *p<0.05
SUMMARY

The present studies convey several novel findings that collectively implicate a role for PKA in the inhibitory and excitatory synaptic alterations that occur in hippocampal CA1 following chronic FZP treatment. They provide the first direct evidence of a spatially-restricted decrease in neuronal membrane-associated PKA activity, in conjunction with decreased expression of PKA-RIIβ, the regulatory subunit responsible for targeting the PKA holoenzyme to neuronal membranes (Lilly et al., 2003). Moreover, there is now unequivocal evidence of AKAP150, the PKA-RIIβ scaffolding molecule, adjacent to excitatory (but not inhibitory) synapses in hippocampal CA1, an arrangement that would allow PKA to locally affect the maintenance and the plasticity of excitatory synaptic function in this region (Lilly et al., 2005). Finally, these studies indicate that the increased excitatory neurotransmission in CA1 hippocampus after chronic FZP-treatment is associated with an increased density of synaptic GluR1, underscoring the similarities between these alterations and those occurring in other models of activity-dependent excitatory synaptic plasticity, in which protein kinase activity is essential (Lilly et al., 2006).

Decreased PKA activity and PKA-RIIβ protein in the membrane fraction of CA1 hippocampal neurons occurs 2d after cessation of chronic FZP treatment, and may contribute to the changes in inhibitory and excitatory neurotransmission that are manifest at this time. This is supported by the similarities in the spatial restriction of the effects, that is, the PKA changes
and the changes in ionotropic receptor function occur in CA1, and are not evident in the dentate gyrus. Each of these changes also requires chronic FZP treatment, and are not apparent after a single exposure of FZP (Lilly et al., 2003; Van Sickle and Tietz, 2002; Zeng and Tietz, 1999). Support for a kinase-mediated event also is provided by the time course of ionotrophic receptor changes. For example, \( \text{GABA}_A \) receptor dysfunction and benzodiazepine sub-sensitivity can be reversed within hours after exposure to a benzodiazepine site antagonist, and antagonist-mediated reversal of the AMPA receptor changes occurs with a similar time-course, each of which is consistent with phosphorylation-dependent mechanisms as potential intermediaries (Tietz et al., 1999b; Van Sickle et al., 2004). Apart from these general similarities, PKA has well-described effects on inhibitory and excitatory neurotransmitter systems, and its effects are consistent with some of the alterations observed 2 d after cessation of chronic FZP treatment.

Benzodiazepine anticonvulsant tolerance \textit{in vivo} is apparent 2 d after cessation of chronic FZP treatment and accompanied by \textit{in vitro} benzodiazepine sub-sensitivity and GABAergic dysfunction in hippocampal CA1 pyramidal neurons, phenomena that likely reflect persistent GABA/benzodiazepine allosteric uncoupling, and decrease in \( \alpha_1 \) subunit protein, respectively (Chen et al., 1999; Chen and Tietz, 1995; Xie and Tietz, 1992; Zeng and Tietz, 1999). A candidate mechanism for the GABA/benzodiazepine uncoupling and the decrease in \( \alpha_1 \) subunit protein has not been identified. However, the decrease in local PKA activity and PKA-
RIlβ protein is functionally consistent with both (Lilly et al., 2003). Though the processes of GABA/benzodiazepine allosteric uncoupling and receptor internalization are experimentally dissociable, they are linked by the use-dependent regulation of GABAβ receptors, with uncoupling serving as the immediate prelude to receptor endocytosis (Barnes, 1996). In vitro studies have indicated that PKA inhibition uncouples the GABA/benzodiazepine binding sites on the GABAβ receptor, and elicits the subsequent internalization of the complex (Ali and Olsen, 2001). In fact, PKA-mediated β subunit phosphorylation protects the GABAβ receptor complex from constitutive endocytosis, and is necessary for the maintenance of mIPSC amplitude (Kittler et al., 2005). Thus, a decrease in PKA activity local to GABAβ receptors would conceivably uncouple the allosteric interaction between the GABA/benzodiazepine binding sites, and permit increased receptor internalization, manifesting physiologically as benzodiazepine subsensitivity and decreased mISPC amplitude, respectively. The nearly complete restoration of mIPSC amplitude in CA1 pyramidal neurons from FZP-treated rats resulting from acute PKA activation may reflect a rapid increase in synaptic receptor number. This underscores the ability of PKA to modulate synaptic GABAβ receptor function, and substantiates the importance of PKA activity in the early phases of use-dependent GABAβ receptor downregulation (Lilly et al., 2003; Otis et al., 1994; Poisbeau et al., 1997). A decrease in membrane-associated PKA activity would likewise suppress GABAβ receptor α1 subunit expression. Transcription of the α1
subunit gene is positively coupled to PKA via activation of the transcription factor CREB (Bateson, 2002; Gerner et al., 1997). The PKA-RIIβ subunit protein is critical in this regard, as this particular subunit is essential for transducing the local increases in cAMP that occur at the neuronal membrane into nuclear CREB activity (Colledge and Scott, 1999; Lilly et al., 2003; Paolillo et al., 1999). Thus, the decrease in membrane-associated PKA activity and PKA-RIIβ expression in hippocampal CA1 neurons 2 d after cessation of chronic FZP treatment is functionally consistent with (1) GABA/benzodiazepine uncoupling, (2) the decrease in synaptic receptor number and mIPSC amplitude, and (3) the decrease in α1 subunit protein expression (Chen and Tietz, 1995; Lilly et al., 2003; Tietz et al., 1999b; Zeng and Tietz, 1999).

The selective modification of synaptic and subsynaptic GABAA receptors and the rapidity with which restoration of GABAA receptor function can occur in this model both favor a population of PKA that is spatially proximate to inhibitory synapses. This notion is supported by the co-purification of GABAA receptor β subunits and enzymatically active PKA, a fact that led some investigators to propose that AKAP150, the linking protein largely responsible for targeting PKA-RIIβ to membrane components, was directly associated to GABAA receptors at inhibitory synapses (Brandon et al., 2003). This arrangement would provide local control over receptor coupling and internalization, and serve to transmit local changes in cAMP activity into nuclear CREB activation, with subsequent effects on GABAA receptor subunit
transcription (Liu et al., 2000; Paolillo et al., 1999; Veruki and Yeh, 1992, 1994). Despite in vitro evidence for the interaction between GABA<sub>A</sub> receptor β subunits and AKAP150, when the ultrastructural distribution of AKAP150 in CA1 hippocampus was evaluated, it was not found at or near inhibitory synapses in this brain region (Lilly et al., 2005). Thus, although PKA is likely spatially restricted near GABAergic synapses in hippocampal CA1, it is unlikely to occur via an AKAP150 scaffold. Whether other AKAPs or scaffolding molecules mediate this interaction, or if there is a direct interaction between GABA<sub>A</sub> receptor subunits and PKA remains unclear (Brandon et al., 2002, 2003; Fritschy and Brunig, 2003).

There is, however, a concentration of AKAP150 immunoreactivity adjacent to excitatory synapses in rat hippocampal CA1. This distribution reflects that reported in the human hippocampus, and corroborates a number of in vitro studies that have identified an interaction between AKAP150 and components of the excitatory post-synapse (Colledge et al., 2000; Diviani and Scott, 2001; Lilly et al., 2005; Sik et al., 2000). Moreover, this localization is functionally significant, as disrupting the interaction in AKAP150 and PKA results in an acute decrease in AMPA receptor currents, consistent with obligate PKA-mediated GluR1 Ser<sup>845</sup> phosphorylation of the AMPA receptor complex prior to membrane and synaptic incorporation (Esteban et al., 2003; Rosenmund et al., 1994; Shi et al., 2001). The selective localization of the AKAP150 near excitatory synapses in hippocampal CA1 provides a spatial relationship between the alterations in PKA activity and PKA-RIIβ protein that
occur 2 d after cessation of chronic FZP treatment, and the alterations in excitatory synapses that occur at the same time point.

A benzodiazepine withdrawal syndrome, manifests as increased anxiety in vivo and is apparent after cessation of 1 wk FZP treatment and accompanied by an increase in AMPA receptor-mediated synaptic neurotransmission in hippocampal CA1 neurons (Van Sickle and Tietz, 2002; Van Sickle et al., 2004). Postsynaptic mechanisms underlying the increase in mEPSC amplitude include an increase in the synaptic receptor number or an increase in the single channel conductance of synaptic channels, though the former has been more commonly implicated in the normal and induced variations of AMPA receptor function (Andrasfalvy and Magee, 2001; Esteban, 2003; Nusser et al., 1998b). Similarly, the increase in mEPSC amplitude during FZP withdrawal occurs by a significant increase in the density of synaptic GluR1 subunits in the apical dendritic region of hippocampal CA1, without a detectable change in the cytoplasmic levels of GluR1 subunit (Lilly et al., 2006). Consistent with earlier reports, these findings suggest that local GluR1 trafficking mechanisms, and not an overall increase in the expression of GluR1 subunits, underlies the increase in excitatory function at this time point (Van Sickle et al., 2002). They also provide an additional correlate between the mechanisms of increased excitatory synaptic strength that operate during withdrawal from chronic FZP treatment and those that underlie other forms of activity-dependent plasticity,
further validating this framework as reference for comparison of these changes.

The mechanisms that underlie the increase in synaptic GluR1 during FZP withdrawal can be inferred from those that underlie the changes in synaptic AMPA receptor expression during LTP and LTD (Nestler, 2002). PKA-mediated phosphorylation of GluR1-Ser^{845} is an obligate step for the translocation of GluR1-containing AMPA receptors to the neuronal membrane, and subsequent synaptic incorporation, while dephosphorylation of this residue precedes the synaptic removal of AMPA receptors, events that are coordinated by the AKAP150 scaffold (Ehlers, 2000; Esteban et al., 2003; Oh et al., 2005; Tavalin et al., 2002). Similarly, the increase in synaptic AMPA receptor GluR1 subunit expression during withdrawal from chronic FZP is associated with an early and transient increase in GluR1 pSer^{845}, which normalizes 2d after cessation of FZP treatment while synaptic AMPA receptor expression remains elevated (Lilly et al., 2006; Song and Tietz, 2004). Thus, if the temporally concordant decrease in membrane-associated PKA activity and PKA RIIβ protein expression measured at this time point reflects the population of AKAP150-tethered PKA molecules and impinges on AMPA receptor synaptic expression, the decrease in PKA activity may signal the impending recovery of normal synaptic AMPA receptor expression (Lilly et al., 2003, 2005, 2006).

A role for PKA in the alterations of GABAergic function in rat hippocampal CA1 following chronic FZP treatment draws support from the
clear similarities between the functional deficits apparent in this region during FZP withdrawal, and those induced in a variety of models with endogenously stimulated or exogenously applied PKA. However, the major PKA-RIIβ scaffolding protein is localized to excitatory synapses in CA1 hippocampus, and has not been found at inhibitory synapses. If local suppression of PKA activity serves to modify excitatory synaptic function in this model, then the changes identified 2d after cessation of chronic FZP treatment are likely mediated by substrates associated with the NMDA receptor and a prelude to the normalization of AMPA receptor function. Collectively, however, the possibility exists that decreases in membrane-associated PKA activity contributes to both the inhibitory and excitatory alterations in CA1 hippocampus after chronic FZP treatment, by AKAP150-independent and dependent mechanisms, respectively. The role of local PKA activity in regard to these synaptic alterations and as it pertains to the expression of benzodiazepine tolerance and withdrawal, may be resolved in the future by transgenic disruption of PKA-RIIβ or AKAP150, or by the pharmacological manipulation of their interaction.
CONCLUSIONS

1. Two days after cessation of 1 wk FZP treatment there is a decrease in total PKA activity associated with insoluble neuronal elements, accompanied by a decrease in PKA-RIIβ protein.

2. Two days after cessation of 1 wk FZP treatment there is not a change in neuronal cAMP levels, total cytoplasmic PKA activity, or the expression of PKA-R1α, PKA-R1Iα, PKA-Cβ.

3. The PKA-RIIβ scaffolding protein AKAP150 is present near, but not within, excitatory post-synapses in rat hippocampal CA1 neurons. The subsynaptic distribution of AKAP150 is corroborated by the lack of overlap between immunofluorescent markers of excitatory synapses and AKAP150.

4. AKAP150 is not associated with inhibitory synaptic profiles at the ultrastructural level or with markers of inhibitory synapses at the light microscopic level in rat hippocampal CA1.

5. Two days after cessation of 1 wk FZP treatment, there is a significant 35% increase in synaptic AMPA receptor GluR1 subunit content in the SR region, but not the SO region of hippocampal CA1. Synaptic size was not different between control and FZP-treated rats.

6. Control and FZP-treated rats do not exhibit different levels of cytoplasmic GluR1 subunits.


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ABSTRACT

Chronic FZP treatment induces anticonvulsant tolerance and the expression of a withdrawal syndrome upon drug discontinuation. These behavioral changes are accompanied by selective in vitro alterations in synaptic neurotransmission of the hippocampal CA1 region that, like in vivo manifestations of tolerance and withdrawal, can be pharmacologically reversed with a single exposure to specific receptor antagonists. A candidate mechanism for the coordinate changes in inhibitory and excitatory neurotransmission occurring 2d after cessation of 1-week FZP treatment, is a reduction in membrane-associated PKA activity and PKA-RIIβ protein, effects that are spatially and temporally consistent with inhibitory and excitatory synaptic alterations (Lilly et al., 2003). Indeed, the functional deficits apparent in GABA_A receptors apparent at this time point, which include GABA/benzodiazepine allosteric uncoupling, a decreased in mIPSC amplitude, and a decrease in α1 subunit protein expression, can be accomplished by local reductions in PKA activity. However, AKAP150, the major PKA-RIIβ scaffolding protein and determinant of membrane-associated PKA activity, is located proximate to excitatory, not inhibitory synapses in rat hippocampal CA1 (Lilly et al., 2005). At these synapses, there is an increase in the expression of GluR1-containing AMPA receptors 2d after cessation of FZP treatment, a finding that implies an alteration in local trafficking of these receptors, for which AKAP150-tethered PKA has been implicated (Lilly et al., 2006). A decrease in AKAP150-tethered PKA activity would favor the
dephosphorylation of the AMPA receptor GluR1 subunit on Ser$^{845}$, and the subsequent internalization of the receptor complex. This interpretation is consistent with the normalization of GluR1-pSer$^{845}$ at this time point and the subsequent normalization of AMPA receptor synaptic currents. Collectively these studies provide support for the hypothesis that alterations in membrane-associated PKA activity contribute to the acquisition and persistence of GABA$\text{A}$ receptor dysfunction, and the impending recovery of normal AMPA receptor function 2d after cessation of chronic FZP treatment.