

Benzodiazepine Withdrawal-Induced Glutamatergic Plasticity Involves Up-Regulation of GluR1-Containing α -Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptors in Hippocampal CA1 Neurons[§]

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ABSTRACT

Modification of glutamatergic synaptic function, a mechanism central to neuronal plasticity, may also mediate long-term drug effects, including dependence and addiction. Benzodiazepine withdrawal results in increased glutamatergic strength, but whether α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (AMPA) are functionally and structurally remodeled during benzodiazepine withdrawal is uncertain. Whole-cell recordings of rat hippocampal CA1 neurons, either acutely dissociated or in hippocampal slices, revealed that AMPAR function was enhanced up to 50% during flurazepam (FZP) withdrawal, without changes in whole-cell channel kinetic properties. Agonist-elicited AMPA currents showed a negative shift in rectification in the presence of spermine, suggesting augmented membrane incorporation of glutamate receptor (GluR) 2-lacking AMPARs. As GluR1-containing AMPARs are critical for activity-dependent alterations in excitatory strength, we sought to determine whether changes in GluR1 subunit distribution in CA1 neurons occurred during benzodiazepine withdrawal. Confocal image analysis revealed that FZP with-

drawal promoted GluR1 subunit incorporation into somatic and proximal dendritic membranes of CA1 neurons without GluR2 subunit alterations. Findings of immunoblot studies were consistent with immunofluorescent studies indicating increased GluR1, but not GluR2, subunit protein levels in cytosolic, crude membrane and postsynaptic density-enriched fractions from CA1 minislices. As with long-term potentiation (LTP), the FZP-withdrawal-induced GluR1 incorporation into CA1 neuron membranes may require the GluR1-trafficking protein, synapse-associated protein 97, which was also elevated in membrane-associated fractions. Together, our findings provide evidence that during FZP withdrawal, increased membrane incorporation of GluR1-containing AMPARs and associated up-regulation of AMPAR functions in hippocampal CA1 pyramidal neurons share fundamental similarities with the mechanisms underlying LTP. This implies that glutamatergic neuronal remodeling observed in LTP also subserves physiological adaptations to drug withdrawal.

Remodeling of glutamatergic neurotransmission has recently been associated with dependence on and addiction to

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various drugs of abuse (Nestler, 2005), including the benzodiazepines (Izzo et al., 2001; Van Sickle et al., 2004; Xiang and Tietz, 2007). Benzodiazepines are widely prescribed anxiolytics and hypnotics but have more limited clinical use as anticonvulsants due to the development of tolerance, largely mediated by dysfunction of GABA_A receptors, the site of benzodiazepine allosteric actions (Zeng and Tietz, 1999; Bateson, 2002). Abrupt benzodiazepine withdrawal causes anxiety and insomnia, which can lead to drug self-administration and contribute to benzodiazepine misuse and abuse

ABBREVIATIONS: AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AMPAR, AMPA receptor; mEPSCs, miniature excitatory postsynaptic currents; FZP, flurazepam; LTP, long-term potentiation; GluR, glutamate receptor; SAP97, synapse-associated protein 97; PIPES, 1,4-piperazinediethanesulfonic acid; TTX, tetrodotoxin; APV, DL-2-amino-5-phosphopentanoic acid; GYKI53655, 7H-1,3-dioxolo(4,5-H)(2,3)-benzodiazepine-7-carboxamide-8,9-dihydro-5-(4-aminophenyl)-8-dimethyl-mono-hydrochloride; NAS, 1-naphthylacetyl spermine; QX-314, N-(2,6-dimethylphenyl)carbamoylmethyl)triethylammonium bromide; CGP-35348, (3-aminopropyl)(diethoxymethyl) phosphinic acid; sp, stratum pyramidale; sr, stratum radiatum; PSD, postsynaptic density; CON, control; so, stratum oriens; PDZ, PSD-95, Dlg, and ZO-1; PKA, cAMP-dependent protein kinase A.

(Griffiths and Johnson, 2005). Recent evidence suggests that the benzodiazepine withdrawal syndrome is related to the regulation of the glutamatergic system (Izzo et al., 2001; Bateson, 2002; Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Xiang and Tietz, 2007). The amplitudes of α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPA)-mediated miniature excitatory postsynaptic currents (mEPSCs) and AMPAR-specific binding were increased in hippocampal CA1 pyramidal neurons after FZP withdrawal (Van Sickle and Tietz, 2002), associated with anxiety-like behavior in the elevated plus maze (Van Sickle et al., 2004; Xiang and Tietz, 2007). The strengthening of CA1 neuron excitatory synaptic function and the expression of anxiety were mitigated by prior pharmacological antagonism of AMPAR up-regulation, underscoring the association of these neurophysiological and behavioral changes (Van Sickle et al., 2004; Xiang and Tietz, 2007). However, the exact mechanisms by which glutamatergic neurotransmission is enhanced during benzodiazepine withdrawal are not well understood.

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

In rat hippocampus, three AMPAR subpopulations are expressed: GluR1 homomers and GluR1/2 and GluR2/3 heteromers (Wenthold et al., 1996). The AMPAR GluR1 subunit is essential for neuronal plasticity, because the presence of a GluR1 subunit alone was sufficient to sustain plasticity in GluR2/3 double knockout mice (Meng et al., 2003). GluR1 homomers that lack GluR2 subunits are permeable to calcium and susceptible to spermine blockade, thus showing inward rectification at positive holding potentials (Lerma et al., 1994). Recombinant AMPARs show a graded inward rectification proportional to the number of functionally expressed nonGluR2 to GluR2 subunits (Washburn et al., 1997). Glutamate-activated AMPAR currents in hippocampal CA1 pyramidal neurons with a heterogeneous complement of primarily GluR2-containing AMPARs (Wenthold et al., 1996) show predominantly outward rectification (Lerma et al., 1994).

To further explore the molecular mechanisms underlying enhanced glutamatergic strength in CA1 neurons during benzodiazepine withdrawal, we used whole-cell electrophysiological recordings, confocal immunofluorescent imaging and immunoblot approaches to examine synaptic and extrasynaptic AMPAR channel properties and AMPAR subunit composition and distribution, using our well established FZP withdrawal model (Van Sickle et al., 2004; Xiang and Tietz,

2007). We show that FZP withdrawal enhances GluR1 subunit expression and directs GluR1-containing AMPARs, including both GluR1 homomers and GluR1/2 heteromers, to extrasynaptic and synaptic sites on hippocampal CA1 pyramidal neurons. Moreover, this delivery may be promoted by synapse-associated protein 97 (SAP97), directing GluR1 subunit redistribution (Leonard et al., 1998; Sans et al., 2001). The findings implicate enhanced AMPAR function with associated CA1 neuron hyperexcitability during FZP withdrawal (Van Sickle et al., 2004; Xiang and Tietz, 2007) as a potential substrate of physical dependence, a mechanism that shares fundamental characteristics with other forms of neuronal plasticity.

Materials and Methods

Chronic FZP Treatment. All methods were approved by the University of Toledo College of Medicine (formerly the Medical University of Ohio), Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines. A standard 1-week FZP treatment, which reliably enhances AMPAR function, was performed as established previously (Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Xiang and Tietz, 2007). Male Sprague-Dawley rats (Harlan, Indianapolis, IN), postnatal days 36 to 42 at the time of study, were first adapted for 2 to 4 days to 0.02% saccharin vehicle to disguise the bitter taste of FZP. Rats were then offered FZP (provided by the National Institute on Drug Abuse Drug Supply Program) in the vehicle for 1 week (100 mg/kg for 3 days and 150 mg/kg for 4 days) as their sole source of drinking water, followed by 2 days of drug withdrawal. The drug concentration offered is incremented from 100 to 150 mg/kg to avoid further aversion to the drug. Daily water consumption was monitored to adjust drug concentration on the basis of the volume consumed. The actual dose achieved was calculated, and rats that did not achieve a criterion dose of a weekly average of 120 mg/kg/day were excluded. During drug withdrawal, rats received saccharin water. Control rats received saccharin vehicle in parallel. All electrophysiological and immunohistochemical studies were conducted with the observer blind to the experimental treatment. This dosing regimen reliably induces manifestations of both benzodiazepine tolerance and dependence (Tietz et al., 1999b; Zeng and Tietz, 1999; Van Sickle et al., 2004; Xiang and Tietz, 2007). Rats treated in this way show a progressive increase in AMPA mEPSC amplitude from day 1 (~15%) to day 2 (~30%) after benzodiazepine withdrawal. Both the increase in AMPAR function and the associated anxiety-like behavior are transient and return to control levels within 4 days after treatment cessation (Van Sickle et al., 2004).

FZP is prescribed as a hypnotic at doses 6 times lower than those for diazepam, reflecting differences in its clinical potency and bioavailability (Chouinard, 2004) and is a full agonist at recombinant GABA_A receptors with a relative potency ~10 times lower than that of diazepam (Downing et al., 2005). Thus, the concentration of FZP (100–150 mg/kg) offered to rats is appropriate to its relative potency. When expressed in diazepam equivalents, a 1-week FZP treatment results in brain levels of FZP and its active metabolites equivalent to those for 0.6 μ M diazepam, as determined by radioreceptor assay (Xie and Tietz, 1992), comparable to that reported for other typical chronic diazepam treatments (Izzo et al., 2001). Because of the short half-life of FZP in rat brain (<12 h), the FZP level is negligible in the hippocampus 2 days after FZP withdrawal (Xie and Tietz, 1992; Van Sickle et al., 2004).

Preparation of Hippocampal Tissue for Electrophysiology. For acutely isolated neurons, coronal half-brain sections (400 μ m) were prepared on a Vibroslice (Campden Instruments Ltd., Lafayette, IN) in ice-cold, pregassed (95% O₂/5% CO₂) Ca²⁺-free PIPES solution containing 120 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 25 mM

glucose, 20 mM PIPES (pH 7.4, 305 mOsm). For in vitro recordings, transverse hippocampal slices (400 μ m) were prepared on a Vibratome (Ted Pella, Redding, CA) as described previously (Van Sickel et al., 2004) in low Ca^{2+} -high Mg^{2+} dissection buffer and then maintained for ≥ 1 h in artificial cerebrospinal fluid (119 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.25 mM NaH_2PO_4 , 26 mM $NaHCO_3$, and 10 mM D-glucose; pH 7.4) before whole-cell recording at room temperature.

Acute Neuron Isolation for Electrophysiology. Coronal slices were maintained at room temperature for >1 h in gassed PIPES and then digested in 1.4 U/ml protease XIV (Sigma-Aldrich, St. Louis, MO) in PIPES buffer at 32°C for 40 min. After a wash in 0.1% bovine serum albumin and then in PIPES, the hippocampus was dissected on ice. CA1 minislices were notched on the dorsal and ventral edges, followed by trituration in 100 μ l of PIPES using a 22-gauge and then a 30-gauge pipette. The supernatant was removed and plated onto acid-washed coverslips. Cells were allowed to attach 15 min before use.

Whole-cell Recording in Isolated Neurons. AMPAR currents were recorded in dissociated neurons in external solution, including 154 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.35, 315 mOsm) with 200 μ M $CdCl_2$ and 0.5 μ M tetrodotoxin (TTX) (Sigma-Aldrich) to block voltage-gated Ca^{2+} and Na^+ channels. Neurons were selected by their relative size and pyramidal morphology as described previously (Tietz et al., 1999a). Elliptical shaped, very large, or bipolar neurons were excluded. The borosilicate micropipette (4–6 M Ω ; Sutter Instruments, Novato, CA) internal solution contained 115 mM $CsCH_3SO_3$, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 0.6 mM EGTA, 4 mM Na_2ATP , 0.4 mM $NaGTP$, 10 Na_2 phosphocreatine, and 50 units/ml creatine phosphokinase (pH 7.25, 295 mOsm). Bath application of 100 μ M DL-2-amino-5-phosphopentanoic acid (APV) (Sigma-Aldrich) was used to block N-methyl-D-aspartate receptor currents activated by glutamate. Whole-cell recordings with ultra-fast drug application were performed at 22°C on lifted, isolated CA1 pyramidal cells, similar to the procedure of Mayer et al. (Partin et al., 1996). Drugs were applied to lifted cells positioned near the adjacent opening of a three-barrel square-tip pipette, coupled to a fast-stepper device (Warner Instruments, Hamden, CT). Each barrel is ~ 7 times larger (~ 160 μ m) than the dissociated CA1 neuron, allowing for sufficient drug exposure. Transition time (20–80%, 0.09–0.47 ms) was estimated with the open-tip patch electrode response to a rapid solution change from normal (2.5 mM) to 100 mM external KCl at the end of the experiment. These application rates were shown previously to be sufficient for detection of whole-cell AMPAR currents and evaluation of AMPAR current kinetics (Partin et al., 1996).

The degree of inward rectification of currents elicited by glutamate or kainate application to acutely isolated neurons or by endogenous glutamate release onto CA1 neurons in hippocampal slices was used to evaluate the effect of benzodiazepine withdrawal on the proportion of GluR2-lacking to GluR2-containing AMPARs at extrasynaptic and synaptic sites (Washburn et al., 1997). In hippocampal neurons in heterologous expression systems, GluR2-containing AMPARs, due to the Q residue at the Q/R RNA editing site in the intramembrane domain, are resistant to spermine blockade and show linear current-voltage (I-V) relationships or outward rectification in a voltage- and activity-dependent manner. Conversely,

AMPA receptors lacking the GluR2 subunit are susceptible to spermine blockade and show inward rectification at positive holding potentials (Lerma et al., 1994). All of the widely used AMPAR agonists, including glutamate and AMPA, evoke strong desensitization of the receptor, with the exception of kainate (Patneau and Mayer, 1991). Importantly, kainate responses in CA1 neurons can also be completely blocked by the AMPA antagonist, GYKI 53655 (Seifert et al., 2000). Therefore, rectification studies were carried out during both ultra-rapid application of glutamate and prolonged exposure to kainate with spermine in the micropipette. To facilitate rapid cell dialysis, spermine (100 μ M; Sigma-Aldrich) was contained in the tip solution and the micropipette was then backfilled with the spermine-containing internal solution. Currents were elicited by 1 ms application of 2 mM glutamate ($V_H = -60$ and $+40$ mV) onto lifted cells or during a 6-s exposure to 300 μ M kainate during a voltage ramp ($V_H = -100$ through $+80$ mV, 1 mV/ms ramp) to avoid AMPAR desensitization. Voltage-ramp currents were recorded on the same cell before and during kainate application. The kainate-induced I-V curve was obtained by subtracting the former from the latter currents.

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

Currents were low-pass filtered at 5 kHz (Axoclamp 200A; Molecular Devices, Sunnyvale, CA) and digitized at 10 to 20 kHz (Digidata 1322; Molecular Devices). The series resistance was compensated at least $>85\%$. Whole-cell responses from isolated neurons (Table 1) were analyzed off-line from digitized current traces using Clampfit (Molecular Devices) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Peak current (picoamperes) was defined as the initial maximal negative deflection from the baseline value determined immediately before the onset of the drug response. To avoid current variances due to differences in cell size, current density (picoamperes/picofarads) was set as peak current amplitude (picoamperes) divided by membrane capacitance (picofarads) from the same cell. The rise and decay kinetics of the AMPA currents were measured after application of a 1-ms pulse of 2 mM glutamate, whereas a 180-ms application was delivered for the measurement of desensitization kinetics. The rise and decay times were determined from 10 to 90% of the AMPA current rising phase or decay phase, respectively. The time constant (τ) of desensitization was fit by a one-component exponential equation:

$$[f(t) = \sum_{i=1}^n A_i \exp(-t/\tau_i) + C] \quad (1)$$

Concentration-response curves were fit by nonlinear regression [sigmoidal dose-response (variable slope)]:

$$I = I_{\min} + (I_{\max} - I_{\min}) / (1 + 10^{-(\log EC_{50} - \log[\text{glutamate}]) \times \text{Hill slope}}) \quad (2)$$

TABLE 1

AMPA kinetics of 1-ms glutamate (2 mM) application

Whole-cell recordings were made on lifted CA1 pyramidal neurons with an ultra-fast drug application system. Current density was significantly increased during FZP withdrawal in the absence of alterations of AMPAR channel kinetics. Data are listed as means \pm S.E.M. (number of cells). C_m , R_a , I_{peak} , and current density were analyzed by a two-tailed unpaired Student's t test; 10–90% rise time and decay time were analyzed by a Mann-Whitney U test.

Group	C_m pF	R_a $M\Omega$	Rise Time ms	I_{peak} pA	Current Density pA/pF	Decay Time ms
CON	20.8 \pm 0.6 (18)	15.0 \pm 0.9 (18)	1.3 \pm 0.1 (18)	4185.4 \pm 479.0 (18)	200.0 \pm 21.5 (18)	16.2 \pm 1.1 (18)
FZP	19.6 \pm 0.5 (19)	13.4 \pm 0.7 (19)	1.3 \pm 0.1 (19)	5248.6 \pm 482.2 (19)	268.8 \pm 22.8 (19)*	17.3 \pm 1.0 (19)

C_m , membrane capacitance; R_a , access resistance; rise time, 10–90% rise time; I_{peak} , peak amplitude of current.

* Significant difference, $p < 0.05$.

The rectification index was defined as peak current amplitude at a holding potential of +40 mV divided by that at -60 mV. For analysis of voltage ramps during kainate application, the ratio of the ramp slope measured at approximately +40 (+35 to +45) and -60 (-65 to -55) mV was calculated. A linear I-V relationship yielded a ratio of 1, whereas ratios >1 or <1 were indicative of outward or inward rectification, respectively. Kainate-induced current density was also measured at $V_H = -60$ mV.

Whole-Cell Recording in Hippocampal Slices. For recording of action potential-independent, AMPAR-mediated mEPSCs, hippocampal slices were perfused (2.5 ml/min) at room temperature with gassed artificial cerebrospinal fluid. Patch pipettes (5–9 M Ω) were filled with internal solution containing 132.5 mM CsCH₃SO₃, 17.5 mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 8 mM NaCl, 2 mM Mg-ATP, 0.3 mM Na₃-GTP, 2 mM QX-314, and 0.1 mM spermine, pH 7.2, adjusted with CsOH. Resting membrane potential was measured immediately upon cell break-in and was invariant between groups (Table 2). Cells in which the holding current changed by more than 20% or the seal degraded were abandoned. Neurons were voltage-clamped (V_H -60 to +40 mV) in continuous mode using an Axoclamp 2A amplifier (Molecular Devices). Baseline mEPSC activity was recorded for at least 8 min with 1 μ M TTX, 25 μ M CGP-35348, 50 μ M picrotoxin, and 50 μ M APV. Current output, monitored online (pClamp 9.0 Software, Axon Instruments Inc.) was low-pass filtered (10 kHz), amplified 100 \times , d.c. offset, and then digitized at 20 kHz (Digidata 1320A; Molecular Devices) for later analysis.

mEPSC events were detected and averaged using MiniAnalysis software (Synaptosoft, Leonia, NJ). Peak current amplitude was derived from averaged events aligned by rise time (<5 ms). Peak mEPSC amplitude and current kinetics were estimated using a single exponential function:

$$y(t) = a \exp(-t/\tau) \quad (3)$$

The rectification index in CA1 neurons from hippocampal slices was defined as in isolated neurons.

Immunofluorescence Studies. For confocal imaging of surface GluR1 and GluR2 subunits and SAP97 antibody immunofluorescence, 5-mm hippocampal blocks were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight after decapitation. For dual labeling of surface N-GluR1/N-GluR2, non-permeabilizing conditions were used on free-floating coronal slices (40 μ m). After blocking with 10% donkey serum, signals were detected with a rabbit antibody against the amino terminus of the GluR1 subunit (N-GluR1 antibody, 1:5; Calbiochem, Darmstadt, Germany) and a mouse antibody directed against the amino terminus of the GluR2 subunit (N-GluR2 antibody, 1:250; Chemicon International, Temecula, CA), and then visualized with an Alexa 488-conjugated donkey anti-rabbit antibody for N-GluR1 (1:500; Invitrogen, Carlsbad, CA) and an Alexa 594-conjugated donkey anti-mouse secondary antibody for N-GluR2 (1:500; Invitrogen). For SAP97 analysis, tissue was permeabilized with 0.1% Tween 20. After blocking with 10% donkey serum, slices were incubated at first with a rabbit anti-SAP97 antibody (1:250; Affinity BioReagents, Golden,

CO), followed by incubation with an Alexa 488-conjugated donkey anti-rabbit antibody (1:500). Experimental controls to verify antibody specificity included antigen preabsorption, omission of primary antibodies, host-matched γ -globulin, or incubation with detergent. This penetration with detergent has been reliably applied for detecting total antibody signal, including that associated with cytoplasmic and membrane compartments. Omission of detergent was designed to assess surface signal. Sections were visualized and quantified using an Olympus BX51WI microscope (Olympus America Inc., Melville, NY), coupled to a Radiance 2000 laser scanning confocal system (Bio-Rad, Hercules, CA) with constant laser power, gain, iris, and offset settings between samples. All data collection and analysis were conducted with the observer being unaware of the experimental group.

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

Subcellular Fractionation and Immunoblotting. Fractionation methods were modified from those of Smith et al. (2006), to yield P2 and P3 fractions relatively enriched in PSDs, as confirmed by PSD-95 (Chemicon International) immunoblotting. All procedures were conducted at 0 to 4°C. Hippocampi were rapidly dissected from matched pairs of control and FZP-withdrawn rats and the CA1 region microdissected from 2- to 3-mm hippocampal slices was immediately submerged in ice-cold homogenization buffer [10 mM Tris, pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 μ M cyclosporine A, 0.5 μ M okadaic acid, and 1% protease inhibitor (Sigma-Aldrich)]. Homogenates were centrifuged at 960g for 10 min to remove large debris. The supernatant (S1) was then centrifuged at 10,000g for 30 min to obtain the crude membrane pellet (P2) and cytosol (S2). The PSD-enriched fraction (P3) was obtained by incubating P2 pellets in Triton X-100 homogenate buffer on ice for 20 min and then centrifuging at 32,000g for 1 h. Proteins in S2 were precipitated with acetone at -20°C for more than 2 h, centrifuged at 3000g for 30 min, and air dried. Final pellets were sonicated in resuspension buffer (10 mM Tris, pH 8, 1 mM EDTA, and 1% SDS). Protein concentrations were determined with a BCA protein assay kit (Pierce Chemical, Rockford, IL).

Ten or 15 μ g of protein per well was mixed with sample buffer [Laemmli sample buffer (Bio-Rad) plus 5% β -mercaptoethanol]

TABLE 2

Characteristics of mEPSCs

mEPSCs were recorded in CA1 pyramidal neurons in hippocampal slices at $V_H = -60$ mV and +40 mV. There was a significant increase in mEPSC peak current amplitude in FZP-withdrawn neurons at $V_H = -60$ mV. Peak current amplitude (I_{peak}) was derived from the averaged events with a rise time <5 ms. Data listed as means \pm S.E.M. (number of cells) were analyzed by a two-tailed unpaired Student's *t* test.

Group	RMP	$V_H = -60$ mV			$V_H = +40$ mV		
		I_{peak}	Rise Time	Decay (τ)	I_{peak}	Rise Time	Decay (τ)
	mV	pA	ms		pA	ms	
CON	67.3 \pm 0.8 (9)	7.0 \pm 0.3 (9)	2.8 \pm 0.2 (9)	15.0 \pm 1.0 (9)	5.3 \pm 0.2 (9)	3.4 \pm 0.4 (9)	12.5 \pm 3.0 (9)
FZP	64.8 \pm 1.3 (11)	8.3 \pm 0.3 (11)*	2.7 \pm 0.1 (11)	16.0 \pm 1.1 (11)	4.9 \pm 0.3 (11)	2.9 \pm 0.2 (11)	14.1 \pm 3.5 (11)

RMP, resting membrane potential.

* Significant difference, $p < 0.05$.

and running buffer (25 mM Tris base, 200 mM glycine, and 0.1% SDS) and then loaded on a 10% polyacrylamide gel. Protein was wet-transferred to a nitrocellulose membrane. Primary antibodies were incubated with membranes overnight at 4°C. The antibody signal was detected with horseradish peroxidase-coupled secondary antibodies (1:10,000/20,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), followed by enhanced chemiluminescence (Denville Scientific Inc., Metuchen, NJ). All P2 and S2 fractions were run under the same conditions simultaneously to obtain P2/S2 density ratios. P3 fractions were run separately. Primary antibodies used included anti-GluR1 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), anti-GluR2 (1:500 or 1:1000; Invitrogen), anti-SAP97 (1:4000; Nventa Biopharmaceuticals, San Diego, CA), and anti-actin (1:20,000; Chemicon International). Images of immunoblots were scanned, and immunoreactivity was quantified with UN-SCAN-IT (version 6.1; Silk Scientific Inc., Orem, UT). Each antibody signal was normalized to the corresponding actin signal.

Statistical Analyses. Data are reported as means \pm S.E.M., and the significance level was set at $p < 0.05$. Error bars represent the S.E.M. Deviations from normality and homogeneity of variance were determined by the Shapiro-Wilk test and F test, respectively. Statistical differences were determined by an unpaired two-tailed Student's t test or alternately by the Mann-Whitney U test when samples were not normally distributed.

Results

Synaptic and Extrasynaptic AMPAR Function Is Increased in Response to FZP Withdrawal. To investigate AMPAR channel properties, acutely isolated CA1 pyramidal neurons and hippocampal slices were prepared from control and 2-day FZP-withdrawn rats. AMPARs desensitize rapidly in the continued presence of agonist (Patneau and Mayer, 1991), limiting kinetic analysis. To avoid this problem, whole-cell recordings were first performed on lifted, isolated CA1 neurons (Fig. 1A), using ultra-rapid application of a saturating concentration (2 mM) of glutamate for 1 ms. Neurons from FZP-withdrawn rats showed enhanced AMPAR function and an increase in current density (picoamperes/picofarads), i.e., current amplitude normalized by cell size, compared with controls. In some studies, excised patches have been used to investigate AMPAR kinetics. However, outside-out patch recordings may be misleading because of sampling errors, as individual patches will contain only a small sample of membrane from the soma or primary dendrites and may contain either synaptic or extrasynaptic receptors. Therefore, whole-cell recordings rather than outside-out patch recordings were preferable to assess the physiological changes in both synaptic and extrasynaptic cell surface AMPAR populations on both soma and proximal dendrites. There were no significant alterations in membrane capacitance, access resistance, rise time, or decay time between control and FZP groups (Fig. 1, B and C; Table 1). These results suggest that the function of both extrasynaptic and synaptic AMPARs may be up-regulated in response to FZP withdrawal, although the contribution of synaptic AMPARs is uncertain.

To better address whether synaptic AMPAR function is augmented, whole-cell recordings were also performed in intact pyramidal neurons in hippocampal slices. Consistent with the former findings in isolated neurons, we also observed a significant increase in AMPAR mEPSC amplitude at $V_H = -60$ mV, without a change in current kinetics (Table

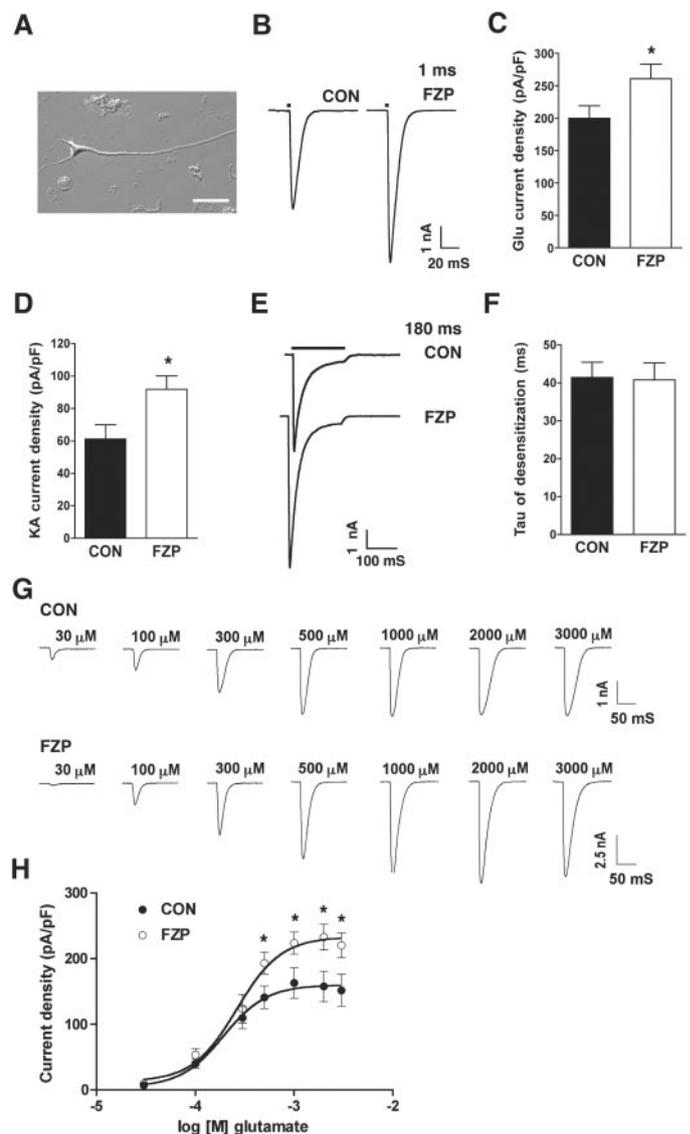


Fig. 1. AMPAR current density is increased in dissociated CA1 neurons after FZP withdrawal. Currents were recorded at $V_H = -60$ mV with $0.5 \mu\text{M}$ TTX, $200 \mu\text{M}$ CdCl₂, and $100 \mu\text{M}$ APV in isolated neurons from CON and 2-day FZP-withdrawn rats. A, photomicrograph of an acutely dissociated neuron. Scale bar: $40 \mu\text{m}$. B, evoked AMPAR-mediated currents after ultra-rapid, 1-ms application of 2 mM glutamate. C, glutamate (Glu)-induced current density (picoamperes/picofarads) was significantly increased after 2-day FZP withdrawal ($n = 19$ cells, 6 rats/group). D, currents evoked by the AMPAR agonist kainate (KA) ($300 \mu\text{M}$) during a 1 mV/ms voltage ramp as shown in Fig. 2E. KA-induced current density (picoamperes/picofarads) at $V_H = -60$ mV was significantly increased after 2-day FZP withdrawal (CON: $n = 14$ cells, 3 rats; FZP: $n = 21$ cells, 3 rats; $p < 0.02$). E, currents evoked by 180-ms application of 2 mM glutamate. F, no significant difference in τ of desensitization between CON and FZP groups (CON: $n = 15$ cells, 5 rats; FZP: $n = 16$ cells, 5 rats). G, representative currents elicited by a 10-ms application of increasing glutamate concentrations (30 – $3000 \mu\text{M}$). H, concentration-response curve representing average AMPAR current density (\bullet , CON; \circ , FZP). Current densities (picoamperes/picofarads) were significantly increased in the FZP group at glutamate concentrations from 500 to $3000 \mu\text{M}$ (CON: $n = 10$ cells, 7 rats; FZP: $n = 13$ cells, 7 rats). Data are represented by means \pm S.E.M.; Error bars in this and all other figures represent the S.E.M. *, $p < 0.05$ by unpaired Student's t test.

2). These results are also compatible with previous mEPSC recordings in CA1 neurons in hippocampal slices from FZP-withdrawn rats (Van Sickle and Tietz, 2002; Van Sickle et al., 2004; Xiang and Tietz, 2007). Collectively, these findings

suggest that FZP withdrawal facilitates both extrasynaptic and synaptic AMPAR function.

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

To address changes in glutamate affinity and efficacy at AMPARs during FZP withdrawal, glutamate concentration-response studies were conducted on lifted, isolated CA1 neurons. At saturating concentrations, from 500 to 3000 μ M, neurons from the FZP-withdrawn group exhibited significantly increased AMPAR current density (Fig. 1, G and H). Analysis of individual fits of concentration-response data revealed that average maximal current density (I_{max}) was significantly augmented during FZP withdrawal (CON 161.7 ± 24.2 pA/pF, FZP 233.4 ± 19.3 pA/pF; $p < 0.05$). A statistical comparison of $\log EC_{50}$ values (CON -3.74 ± 0.08 M, FZP -3.58 ± 0.07 M; $p > 0.05$) indicated no change in glutamate EC_{50} (CON 210 ± 36 μ M, FZP 282 ± 36 μ M; $p > 0.05$), suggesting that there was no change in glutamate affinity. Because the increased AMPAR current density was observed at saturating glutamate concentrations, an increase in the number of functional AMPARs at the cell surface rather than altered glutamate affinity or efficacy is chiefly responsible for the enhanced AMPAR function.

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

synaptic GluR2-lacking AMPARs during FZP withdrawal, which probably represent GluR1 homomers.

Because kainate is the only AMPAR agonist with a minimal capacity to induce desensitization and because at this age (postnatal days 36-42) more than 95% of kainate-induced currents in CA1 neurons are due to AMPAR activation (Seifert et al., 2000), we applied kainate on CA1 neurons to further characterize the I-V properties of altered AMPA currents. There was a clearly observable (Fig. 2, E and F) negative shift in the slope ratios derived from individual pyramidal neuron responses to a voltage ramp (-100 to +80 mV) during kainate application (CON slope ratio 1.44 ± 0.08 , $n = 14$; FZP slope ratio 1.15 ± 0.03 , $n = 21$; $p < 0.001$). Eleven of 14 (71%) control neurons were outwardly rectifying (slope ratio of 1.2-2.1), whereas the remainder (3 of 14) had rectification indices between 1.1 and 1.2. In contrast, 4 of 21 FZP-withdrawn neuron responses were inwardly rectifying with slope ratios < 1.0 (0.83-0.95), whereas 16 had rectification indices between 1.0 and 1.2. One FZP-withdrawn cell exhibited outward rectification with a slope ratio of 1.39.

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

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

To detect whether GluR1 and/or GluR2 subunit surface incorporation was altered in response to FZP withdrawal, dual-immunofluorescence confocal imaging studies of anti-N-terminal GluR1 and GluR2 antibodies were performed in the somatic and dendritic regions of the CA1 area (Fig. 3A) under nonpermeabilizing conditions, the most practical available approach to label surface AMPAR subunits. Experimental conditions were validated by γ -globulin (Fig. 3B) and perme-

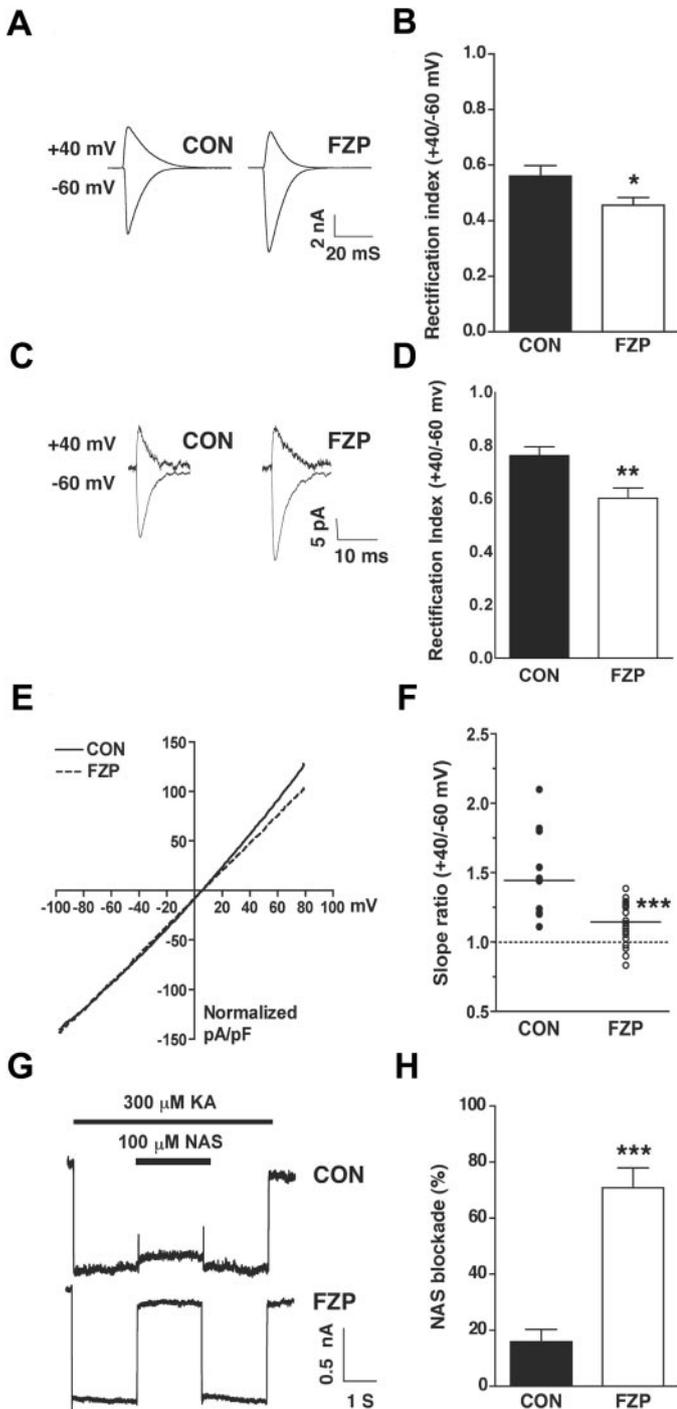


Fig. 2. A shift toward inward rectification and spermine blockade of agonist-evoked currents in CA1 neurons after FZP withdrawal. **A**, AMPAR-mediated currents were recorded at -60 and +40 mV holding potentials in isolated neurons after a 1-ms application of 2 mM glutamate with 0.5 μ M TTX, 200 μ M CdCl₂, and 100 μ M APV in the bath. Spermine (100 μ M) was included in the micropipette to replace endogenous spermine lost during dialysis. **B**, the rectification index (+40/-60 mV) for glutamate was significantly decreased after 2-day FZP withdrawal (CON: 18 cells, 6 rats; FZP: 20 cells, 6 rats). *, $p < 0.05$ by unpaired Student's *t* test. **C**, representative averaged AMPAR-mediated mEPSCs recorded at -60 and +40 mV holding potentials in hippocampal slices in the presence of 1 μ M TTX, 50 μ M APV, 50 μ M picrotoxin, and 25 μ M CGP35348 and with spermine in the micropipette. **D**, the mEPSC rectification index (+40/-60 mV) was significantly decreased after 2-day FZP withdrawal (CON: 9 cells, 8 rats; FZP: 11 cells, 6 rats). **, $p < 0.05$ by unpaired Student's *t* test. **E**, representative pair of voltage ramps at holding potentials from -100 to +80 mV generated at 1 mV/ms during 300 μ M

ablization controls (Supplemental Fig. 1). A marked increase in the GluR1 signal as well as GluR1/2 colocalization was observed in CA1 neuron somata (sp, Fig. 3C) and apical dendrites (sr, Fig. 3D) but not basal dendritic regions [stratum oriens (so); data not shown]. The increased GluR1 signal in sr, but not in so, agrees with recent preliminary studies that showed a differential distribution of GluR1 homomers in apical versus basal dendrites (Bagal et al., 2005). No differences were detected in surface GluR2 signal in somatic or dendritic areas (Fig. 3, C and D, center), suggesting that a change in GluR2 subunit surface expression is not involved in AMPAR potentiation during FZP withdrawal. Quantification of signal intensity (Fig. 3, E and F) revealed that surface GluR1 signal was augmented more than GluR1/2 colocalization in sp (mean density increase: 64 versus 26%) and sr (mean density increase: 71 versus 45%), indicating that GluR1 subunits were inserted in somatic or apical dendritic membranes during FZP withdrawal as GluR1 homomers and GluR1/2 heteromers. Given the absence of alterations in surface GluR2 labeling, the observation that GluR1/2 colocalization was also enhanced suggests that membrane-inserted AMPARs might be reassembled from the GluR2/3 AMPAR subpopulation into GluR1/2 heteromers. Nevertheless, other possibilities such as synaptic delivery of homomeric GluR1 AMPARs adjacent to existing GluR2-containing receptors cannot be excluded.

To corroborate immunofluorescence studies, immunoblot analyses of subcellular CA1 neuron fractions were performed. Three subcellular fractions were collected from hippocampal CA1 minislices and used in this study, including S2 (cytosol), P2 (crude membrane), and P3 (PSD-enriched) fractions. An enrichment of the PSD-95 signal was demonstrated in the P3 versus the P2 fraction (Fig. 4). There were significant increases in GluR1 immunoreactivity in cytosolic and crude membrane fractions in response to FZP withdrawal (Fig. 5A), suggesting that total GluR1 protein expression was augmented and may contribute to the elevated surface GluR1 incorporation. Moreover, a 57% increase in the amount of GluR1 in the PSD-enriched, P3 fraction was found in membranes from FZP-withdrawn compared with control rats (Fig. 5A), which supports the electrophysiological findings and suggests that more GluR1 homomers may be inserted in synaptic membrane. In contrast with the increased GluR1 signal density, no alterations in the amount of GluR2 protein were found in any fraction after FZP withdrawal (Fig. 5B), in agreement with the lack of change in GluR2 immunofluorescence labeling. Meanwhile, the P2/S2 ratio of both GluR1 and GluR2 subunits was unaltered in CA1 minislices from the FZP versus the control group (GluR1 P2/S2: CON $1.09 \pm$

kainate (KA) application. The current density from the FZP neuron was normalized to the CON neuron. At positive holding potentials, the CON neuron showed outward rectification (slope ratio = 1.46), whereas the FZP neuron rectification was nearly linear (slope ratio = 1.08). **F**, distribution of slope ratios from individual CON (●) and FZP neurons (○). The slope ratios (+35 to +45 mV/-65 to -55 mV) derived from the voltage ramp during KA application were significantly decreased after 2-day FZP withdrawal (CON: 14 cells, 3 rats; FZP: 21 cells, 3 rats). Horizontal bar represents the mean. ***, $p < 0.001$ by unpaired Student's *t* test. **G**, representative currents elicited at -80 mV by 6-s application of 300 μ M KA and 2-s coapplication of 100 μ M NAS without intracellular spermine. **H**, cells from the FZP group exhibited significantly increased NAS blockade compared with the CON group (CON: 16 cells, 3 rats; FZP: 13 cells, 3 rats). ***, $p < 0.001$ by Mann-Whitney *U* test.

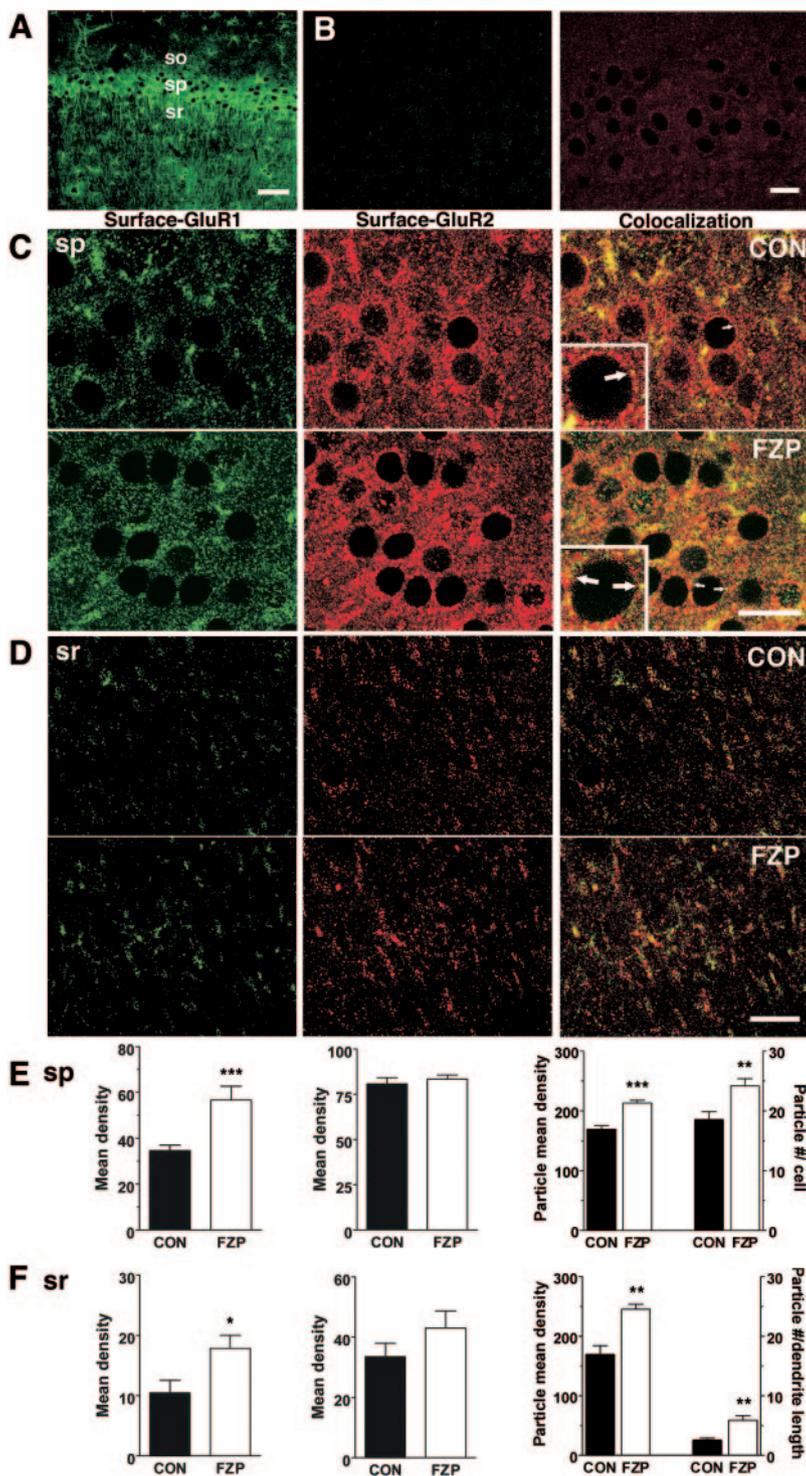


Fig. 3. Surface GluR1, but not GluR2, subunit immunofluorescence is increased in CA1 neurons after FZP withdrawal. GluR1 and GluR2 surface expressions were detected with anti-N-terminal antibodies. A, surface GluR1 subunit staining in hippocampal CA1 area. Scale bar: 50 μ m. B, γ -globulin controls for surface GluR1 (center) and GluR2 (right). Scale bar: 20 μ m. C, representative photomicrographs of GluR1 (left) and GluR2 (center) and colocalization of GluR1/2 staining (right) in sp from the CON (top) and FZP (bottom) groups. Scale bar: 20 μ m. Arrows within inset images (enlarged 200%) depict examples of colocalized signal at the membrane, quantified in E as a function of cell circumference. D, representative photomicrographs of GluR1 (left) and GluR2 (center) and colocalization of GluR1/2 staining (right) in the first 100 μ m of the apical dendritic area (sr) immediately adjacent to sp, from the CON (top) and FZP (bottom) groups. Scale bar: 20 μ m. E, quantification of GluR1 (left) and GluR2 (center) and colocalization of GluR1/2 (right) immunofluorescence signals in sp. The FZP group showed a significant increase in the surface GluR1 signal (mean density) compared with the CON group by a Mann-Whitney *U* test. No significant differences were found in the GluR2 signal between groups. Quantification of the GluR1/2 colocalization signal between groups showed a significant increase in particle mean signal density and particle number/cell in the FZP group by an unpaired Student's *t* test. F, quantification of GluR1 (left) and GluR2 (center) and colocalization of the GluR1/2 (right) immunofluorescence signal in sr. The FZP group showed a significant increase in the surface GluR1 signal (mean density) by an unpaired Student's *t* test. No significant differences were found in the GluR2 signals between groups. Quantification of the GluR1/2 colocalization signal between groups showed a significant increase in particle mean signal density and particle number/dendrite length by an unpaired Student's *t* test. CON: $n = 19$ to 27 cells, 4 rats; FZP: $n = 19$ to 28 cells, 4 rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

0.11, FZP 1.14 ± 0.18 ; GluR2 P2/S2: CON 1.58 ± 0.30 , FZP 2.23 ± 0.58 ; $p > 0.05$). Collectively, our immunochemical data suggest that enhanced AMPAR GluR1 subunit expression but not redistribution contributes to AMPAR membrane incorporation and subsequent receptor potentiation during FZP withdrawal. Taken together, the findings suggest that enhanced GluR1 subunit expression and subsequent membrane delivery, rather than elevated GluR2 degradation, may result in increased GluR1 homomer incorporation into extrasynaptic and synaptic membranes, because neither total

GluR2 protein level nor its P2/S2 ratio was changed after FZP withdrawal.

SAP97 May Promote GluR1 Membrane Delivery in Response to FZP Withdrawal. The increase in GluR1-containing cell surface AMPARs observed with FZP withdrawal is similar to that observed with LTP, and thus we sought to determine whether other similar mechanisms might be involved. In LTP, GluR1-containing AMPAR trafficking depends on interactions between the C terminus of the GluR1 subunit and type I PSD-95, Dlg, and ZO-1 (PDZ)

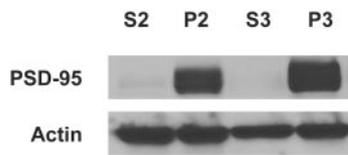


Fig. 4. PSD-95 distribution in subcellular compartments from CA1 minislices. The PSD-95 signal was enriched in the P3 fraction, compared with the crude membrane (P2) fraction. The density ratio of PSD-95/actin was 1.35 in P2 and 1.92 in P3. The signal ratio was negligible in the S2 (0.03) and S3 (0.07) fractions.

domain-containing proteins, such as SAP97 (Leonard et al., 1998). SAP97, a 97-kDa postsynaptic PDZ protein, demonstrated to specifically associate with GluR1 but not with other AMPAR subunits (Leonard et al., 1998), was shown to act in the early secretory pathway of membrane-targeted GluR1-containing AMPARs (Sans et al., 2001). To investigate whether SAP97 may be involved in the control of GluR1-containing AMPAR trafficking during FZP withdrawal, immunofluorescence studies with an anti-SAP97 antibody were performed after membrane permeabilization. Cytoplasmic SAP97 clusters were more dense than those observed in dendrites, and a significantly increased SAP97 signal (20%) was apparent in sp but not sr of the FZP-withdrawn group

(Fig. 6A). There was no change in the SAP97 signal in basal dendrites (so) during FZP withdrawal (data not shown).

The SAP97 protein level was further investigated by subcellular fractionation and immunoblotting. As seen in Fig. 6B, a significant up-regulation of SAP97 expression was found in CA1 neuron P2 and P3 membrane components from FZP rats, with no apparent regulation in S2. Interestingly, the P2/S2 density ratio of anti-SAP97 labeled protein was significantly elevated (~45%) during FZP withdrawal. As S2 SAP97 was unaltered, the increased in SAP97 protein associated with crude membranes (P2) and those enriched in asymmetric synapses (P3) may indicate a possible role for SAP97 in facilitating GluR1-containing AMPAR trafficking to extrasynaptic and synaptic membranes during benzodiazepine withdrawal.

Discussion

AMPA-mediated current potentiation and a switch in AMPAR subunit composition at the postsynaptic membrane are critical to activity-dependent changes in synaptic strength. The nature of regulated AMPAR trafficking in various forms of synaptic plasticity is well characterized (Shi et

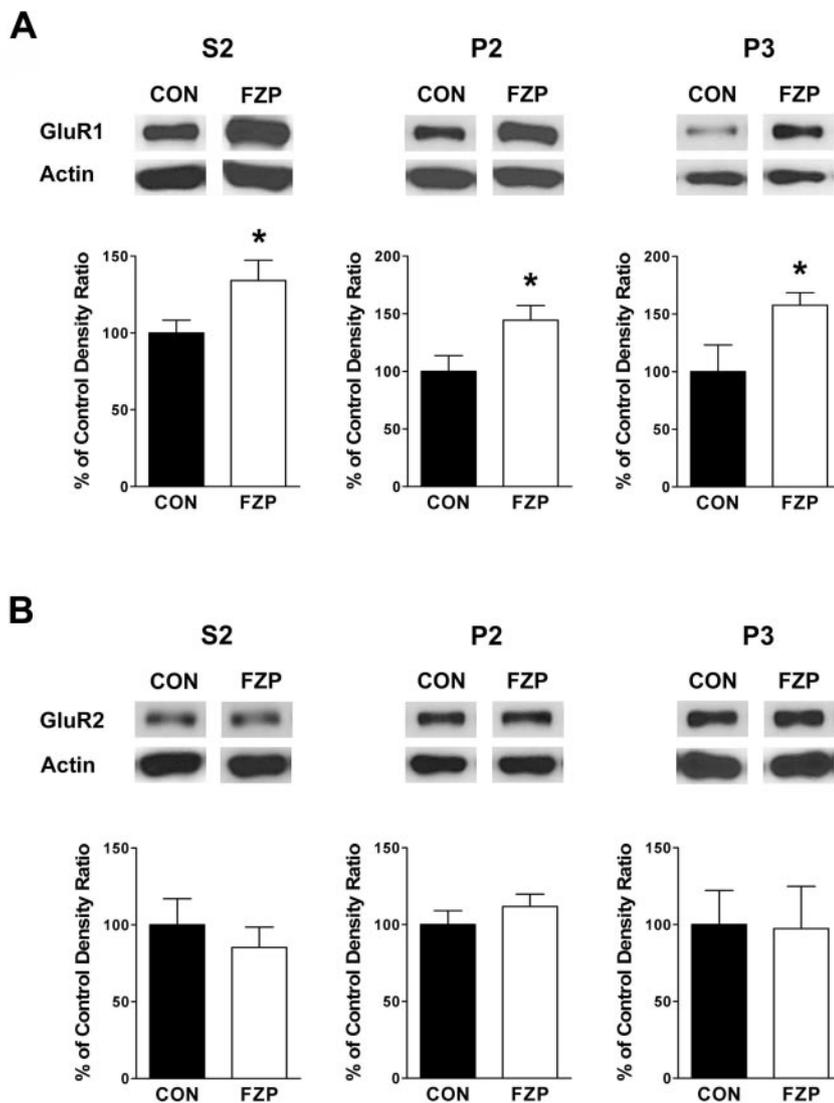


Fig. 5. Total GluR1, rather than GluR2, protein levels were enhanced in CA1 neurons after FZP withdrawal. CA1 minislices from pairs of CON and FZP-withdrawn groups were fractionated to obtain S2 (cytosolic), P2 (crude membrane), and P3 (PSD-enriched) fractions. GluR1 and GluR2 signal densities were normalized to the respective actin signal. A, total GluR1 signal density was significantly increased in S2, P2, and P3 fractions derived from FZP-withdrawn rats. Representative blots are illustrated in the top panels; quantitative analyses of signal density are shown in the histograms below (*, $p < 0.05$, $n = 7$ /group). GluR1 subunit protein did not redistribute during 2-day FZP withdrawal with a P2/S2 ratio $105 \pm 17\%$ of control ($p > 0.05$). S2 and P2: $15 \mu\text{g}$ protein/well; P3: $10 \mu\text{g}$ protein/well. B, GluR2 signal density did not change in any fraction in response to FZP withdrawal ($p > 0.05$, $n = 7$ /group). Although there was a slight increase in the GluR2 P2/S2 ratio in FZP rats ($41 \pm 37\%$), no significant difference was found between groups. All data were analyzed by Student's *t* test. All fractions loaded with $15 \mu\text{g}$ of protein/well.

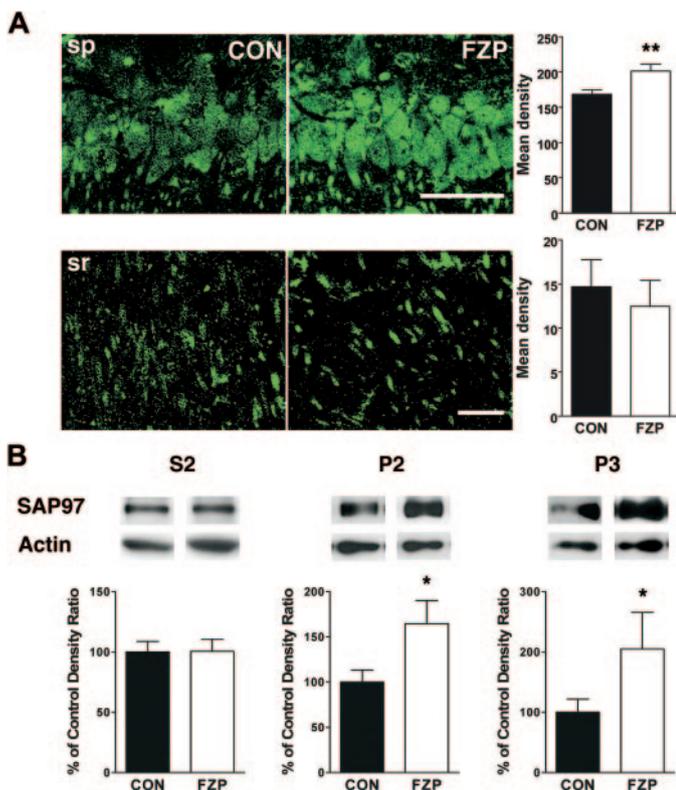


Fig. 6. SAP97 signal increased in CA1 neurons after FZP withdrawal. SAP97 immunoreactivity was detected with confocal imaging (A) and immunoblotting (B). A, representative images of the SAP97 signal from CON and FZP-withdrawn groups (sp, top; sr, bottom). Scale bar: 50 or 20 μ m for sp or sr, respectively. Quantifications of the SAP97 signal is shown on the right. Compared with the CON group, the FZP group showed a significant increase in sp SAP97 signal (mean density) by a Mann-Whitney *U* test. No significant differences in sr SAP97 signal were found between groups. CON and FZP: $n = 19$ cells, 4 rats/group. *, $p < 0.05$. B, SAP97 signal density was normalized to the respective actin signal. There were significant increases in SAP97 signal density in the crude membrane (P2) and PSD-enriched (P3) fractions from CA1 minislices of FZP-withdrawn rats compared with the CON group. Representative immunoblots are illustrated in the top panel; quantitative analyses of signal density is shown in the histograms below. Redistribution of SAP97 was also observed in the FZP-withdrawn group with a P2/S2 ratio of $144 \pm 15\%$ of the CON group. *, $p < 0.05$, $n = 6$ to 7 pairs of rats. The P3 SAP97 signal was analyzed by a Mann-Whitney *U* test; other fractions were analyzed by a Student's *t* test. S2 and P2, 15 μ g of protein/well; P3, 10 μ g of protein/well.

al., 1999), whereas its role in mediating long-term drug effects, in particular FZP withdrawal-induced glutamatergic remodeling, is unclear. In our previous studies, anxiety-like behavior was associated with increased CA1 neuron AMPAR function in FZP-withdrawn rats (Van Sickel et al., 2004, Xiang and Tietz, 2007). The current electrophysiological studies demonstrate that extrasynaptic and synaptic AMPAR currents were both significantly enhanced during benzodiazepine withdrawal with no change in AMPAR channel properties. The relative shift toward inward rectification of AMPAR-mediated currents in the presence of polyamines indicates a substantial contribution of GluR1-lacking AMPARs to the increased AMPAR potentiation during FZP-withdrawal. Immunofluorescence studies and immunoblot analysis revealed increases in extrasynaptic and synaptic GluR1 signals with an increase in the total amount of GluR1 protein, collectively suggesting that FZP withdrawal promotes the expression of GluR1 subunits, as well as the de-

livery of GluR1 homomers into somatic and apical dendritic membranes and synapses. Concomitant with the increased level of GluR1 in P2 and P3 fractions, FZP withdrawal also promotes SAP97 expression, which may favor GluR1 subunit release from the endoplasmic reticulum and drive GluR1 subunits into membranes/synapses. Moreover, the significant increases in membrane association of SAP97 and a shift of SAP97 from the S2 to the P2 fraction further supports the notion that SAP97 redistribution may be an important step for GluR1 membrane/synapse delivery during FZP withdrawal, as with other forms of activity-dependent plasticity. This is the first demonstration that FZP withdrawal promotes AMPAR membrane/synapse trafficking and subunit remodeling, leading to potentiation of AMPAR function, a mechanism that is also central to LTP (Zamanillo et al., 1999; Shi et al., 2001; Malinow and Malenka, 2002). Moreover, the findings suggest that the mechanisms underlying activity-dependent hippocampal glutamatergic plasticity may be highly conserved and subserve the physiological adaptations that occur during drug withdrawal as well as other adaptive behaviors.

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

AMPA Membrane Delivery Increases Glutamatergic Strength in Response to FZP Withdrawal. Considerable evidence indicates that native AMPAR subunits have different trafficking patterns; GluR1-containing AMPARs are inserted into membranes in an activity-dependent manner, determined by interactions between GluR1 and type I PDZ proteins (Leonard et al., 1998; Sans et al., 2001), whereas GluR2-containing AMPARs are continuously cycled in and out of membranes, replacing existing receptors (Shi et al., 2001; Malinow and Malenka, 2002). These subunit-specific properties govern membrane delivery of AMPARs in response to neuronal activity. As in LTP, GluR1 membrane delivery requires the interaction between the PDZ binding domain of GluR1 and SAP97, the only PDZ protein known to

interact specifically with the GluR1 but not other AMPAR subunits (Leonard et al., 1998). Our immunofluorescence studies demonstrated that surface GluR1 signal was augmented in sp, associated with increased SAP97 expression in the cell body, suggesting that SAP97 may be involved in facilitating release of GluR1 subunits from the endoplasmic reticulum and their further delivery to membranes/synapses. Nevertheless, immunofluorescence studies lack the sensitivity required to detect synaptic SAP97 incorporation, and an increase in dendritic SAP97 signal was not detected in confocal imaging studies. In subfractionation studies, enhanced GluR1 levels were accompanied by up-regulation of SAP97 in P2 and P3 fractions. An increase in the SAP97 P2/S2 ratio raises the possibility that SAP97 may redistribute from the cytosol to the membrane or that locally synthesized SAP97 near the membrane might be enhanced. The membrane/synaptic relocalization of SAP97 was suggested to enable its participation in the regulation of AMPAR trafficking (Wu et al., 2002) and GluR-Ser⁸⁴⁵ phosphorylation (Colledge et al., 2000). Thus, coupled with immunofluorescence studies, subfractionation studies suggest that similar mechanisms may be operative during FZP withdrawal, resulting in potentiation of AMPAR function and the associated anxiety-like behavior (Van Sickel et al., 2004; Xiang and Tietz, 2007).

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

Other candidates implicated in “priming” AMPAR trafficking, such as cAMP-dependent protein kinase A (PKA)-mediated phosphorylation, may also be involved and account for the discontinuity between the increase in sr GluR1, without a change in sr SAP97 levels. It has been reported that direct phosphorylation of the GluR1 subunit by PKA permits

AMPA translocation from intracellular pools to the extrasynaptic membrane (Esteban et al., 2003; Oh et al., 2006) and that PKA-mediated phosphorylation may also be associated with GluR1 synaptic remodeling (Oh et al., 2006). Moreover, SAP97 could target the A kinase-anchoring protein/PKA complex to GluR1 subunits via a PDZ domain interaction to facilitate phosphorylation of AMPAR GluR1 subunits by PKA (Colledge et al., 2000). Thus, SAP97 and PKA signaling pathways may be cooperatively involved in FZP-withdrawal-induced AMPAR remodeling. Detailed studies on A kinase-anchoring protein/PKA complex distribution and activity in response to FZP withdrawal will be helpful. On the other hand, new evidence from our laboratory suggests that Ca²⁺/calmodulin-dependent protein kinase II signaling pathways, linked to LTP induction and maintenance (Soderling and Derkach, 2000), also play a role in enhanced AMPAR function during FZP withdrawal (Shen and Tietz, 2005).

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

Switch in AMPAR Composition and Its Role in FZP Withdrawal. Previous studies have indicated that the presence of the AMPAR GluR1 subunit alone was sufficient to sustain plasticity, because LTP could still be established in GluR2/3 double knockout mice (Meng et al., 2003). In contrast, a critical role for the AMPAR GluR2 subunit was also found in some studies (Oh and Derkach, 2005). A switch in AMPAR composition has been observed in other forms of plasticity, such as developmental strengthening of excitatory synapses, and after synaptic activation (Liu and Cull-Candy, 2000). Thus, it is important to clarify whether a switch in AMPAR composition is involved in glutamatergic remodeling during FZP withdrawal. Acutely dissociated hippocampal neurons, most but not all with a pyramidal shape, were previously classified into three types: type I (RI \approx 1.61, 20.5%), outward rectifying; type II (RI \approx 0.5, 23%), inwardly rectifying; and type III (RI \approx 1.13, 56.4%), linear I-V relationship. The degree of kainate-induced AMPAR-mediated current rectification and Ca²⁺ permeability was inversely correlated (Lerma et al., 1994). The distribution of rectification indices in control pyramidal neurons in the present study is consistent with that found by Lerma et al. (1994). The degree of rectification in CA1 neurons from FZP-withdrawn rats suggests that there was a relative shift in composition of the AMPAR subunit population: more GluR2-lacking AMPARs, possibly GluR1 homomers, were inserted into the membrane, resulting in AMPAR current potentiation. It appears that the removal of membrane-associated GluR2 subunits was not responsible for the shift in rectification, as there was no evidence of an alteration in GluR2 subunit expression at any

level examined. Meanwhile, surface GluR1 signal and GluR1/2 colocalization were significantly increased in the sp and sr areas during FZP withdrawal. These findings suggest increased delivery of GluR1 homomers to the membrane and also a possible switch in AMPAR composition from GluR2/3 to GluR1/2 heteromers. Immunofluorescence analyses revealed a detectable increase in GluR1 subunit expression in apical (sr) dendritic areas, rather than in basal dendrites (so). Additional electron microscopic studies focusing on GluR1 expression in apical and basal dendritic regions, as well as the relative proportion of GluR1 and GluR2 or GluR3 subunits will be beneficial to further elucidate changes in the synaptic composition of AMPARs during benzodiazepine withdrawal.

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

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

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