

Increased AMPA Receptor GluR1 Subunit Incorporation in Rat Hippocampal CA1 Synapses During Benzodiazepine Withdrawal

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ABSTRACT

Prolonged benzodiazepine treatment leads to tolerance and increases the risk of dependence. Flurazepam (FZP) withdrawal is associated with increased anxiety correlated with increased alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor (AMPA)-mediated synaptic function and AMPAR binding in CA1 pyramidal neurons. Enhanced AMPAR synaptic strength is also associated with a shift toward inward rectification of synaptic currents and increased expression of GluR1, but not GluR2, subunits, suggesting augmented membrane incorporation of GluR1-containing, GluR2-lacking AMPARs. To test this hypothesis, the postsynaptic incorporation of GluR1 and GluR2 subunits in CA1 neurons after FZP withdrawal was examined by postembedding immunogold quantitative electron microscopy. The percentage of GluR1 positively labeled stratum radiatum (SR) synapses was significantly increased in FZP-withdrawn rats ($88.2\% \pm 2.2\%$) compared with controls ($74.4\% \pm 1.9\%$). In addition, GluR1 immunogold density was significantly increased by 30% in SR synapses in CA1 neurons from FZP-withdrawn rats compared with control rats (FZP: 14.1 ± 0.3 gold particles/ μm ; CON: 10.8 ± 0.4 gold particles/ μm). In contrast, GluR2 immunogold density was not significantly different between groups. Taken together with recent functional data from our laboratory, the current study suggests that the enhanced glutamatergic strength at CA1 neuron synapses during benzodiazepine withdrawal is mediated by increased incorporation of GluR1-containing AMPARs. Mechanisms underlying synaptic plasticity in this model of drug dependence are therefore fundamentally similar to those that operate during activity-dependent plasticity. *J. Comp. Neurol.* 511:832–846, 2008. © 2008 Wiley-Liss, Inc.

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Fast excitatory neurotransmission in the mammalian central nervous system (CNS) is mediated predominantly by alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPA). Mature ionotropic AMPARs are expressed throughout the brain and are highly clustered at postsynaptic sites, where they respond rapidly to synaptically released glutamate. AMPARs are tetramers composed of combinations of four subunits: GluR1–4 (Petralia and Wenthold, 1992; Wenthold et al., 1996). GluR1, GluR3, and GluR4 subunits form calcium-permeable inwardly rectifying channels, whereas GluR2 subunits form calcium-impermeable channels with linear or outward rectification (Jonas and Burnashev, 1995; Washburn et al., 1997). Distinct populations of GluR1/2 and GluR2/3 containing heterooligomeric complexes are present in the mature hippocampus (Wenthold et al., 1996). Activity-dependent changes in synaptic strength such as long-term potentiation (LTP) are thought to be primarily due to trafficking and insertion of GluR1-containing AMPARs into excitatory synapses, which are subsequently replaced by a constitutively recycled pool of GluR2/3 receptors (Hayashi et al., 2000; Shi et al., 2001; Malinow and Malenka, 2002; Lee et al., 2003; Nicoll, 2003; Plant et al., 2006; Brown et al., 2007; Greger and Esteban, 2007; Elias and Nicoll, 2007).

Similar mechanisms underlying activity-dependent glutamatergic synaptic plasticity may also play important roles in neural and behavioral adaptations during development of dependence and addiction resulting from chronic exposure to drugs of abuse (Nestler, 2002; Kauer and Malenka, 2007). For example, chronic cocaine increases the insertion of GluR1-containing AMPARs in dopaminergic synapses in the ventral tegmental area (VTA) and nucleus accumbens of rodents (Carlezon et al., 1997; Boudreau et al., 2007). Enhanced glutamatergic strength is also involved in the development of dependence on alcohol or morphine (Molleman and Little, 1995; Sanchis-Segura et al., 2006). Chronic morphine treatment raises AMPAR GluR1 subunit levels in VTA, and morphine-induced tolerance and dependence is reduced in mice deficient in AMPAR GluR1 subunits (Fitzgerald et al., 1996; Vekovischeva et al., 2001).

Benzodiazepines, including diazepam and flurazepam (FZP), are widely used sedative-hypnotics, anxiolytics, and anticonvulsants that exert their clinical effects by allosteric augmentation of γ -aminobutyric acid (GABA)-gated chloride currents through central GABA type A receptors (GABAR; Wafford, 2005). Despite their acute efficacy, long-term exposure to these drugs results in tolerance and dependence, which increase their potential for abuse and limit their clinical utility (Griffiths and Johnson, 2005). Although development of tolerance has been associated primarily with GABAR dysfunction (Bateson, 2002; Wafford, 2005), it has been long known that anxiety, seizures, and other signs of benzodiazepine dependence can be suppressed by glutamate antagonists (Steppuhn and Turski, 1993). The hippocampus plays an important role in the expression of anxiety and seizures and is an important site of acute benzodiazepine anxiolytic and anticonvulsant actions (Ashton et al., 1988; McNaughton and Gray, 2000; Engin and Treit, 2007). Moreover, recent evidence from our laboratory and others suggests that enhanced glutamatergic transmission in the hippocampus is associated with benzodiazepine withdrawal phenomena

(Izzo et al., 2001; Van Sickle et al., 2004; Allison and Pratt, 2006; Xiang and Tietz, 2007).

LTP of hippocampal CA1 synapses is usually associated with learning (Whitlock et al., 2006), but similar changes in excitability within the neural circuits that subserve LTP might also have more undesirable effects. For example, a subset of CA1 neurons displaying strong LTP was shown to underlie hippocampal seizure susceptibility and initiate epileptiform activity (Chang et al., 2007). As noted above, LTP-like mechanisms have been related to synaptic plasticity in other brain regions after chronic drug use. Thus, LTP-like synaptic plasticity in the CA1 region might contribute to increasing hippocampal glutamate neurotransmission during development of benzodiazepine withdrawal-anxiety.

Previous studies showed that both AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) and AMPAR binding were enhanced in hippocampal CA1 pyramidal neurons following 1-week FZP administration, concomitant with the appearance of CA1 neuron hyperexcitability and anxiety-like behavior in FZP-withdrawn rats (Van Sickle et al., 2004). Moreover, anxiety-like behavior in this model was prevented by pretreatment with an AMPAR antagonist (Xiang and Tietz, 2007). Enhanced glutamatergic strength was also manifested by the increased amplitude of glutamate-evoked whole-cell currents recorded in acutely isolated hippocampal CA1 pyramidal neurons. The AMPAR currents also showed a shift toward inward rectification in both dissociated neurons and hippocampal slices, suggesting postsynaptic incorporation of AMPARs lacking the GluR2 subunit. The changes in AMPAR function during withdrawal from prolonged FZP exposure were associated with increased GluR1 expression detected using immunoblots and with immunofluorescence (Song et al., 2007). These findings provide strong evidence that enhanced AMPAR function in rat hippocampal CA1 neurons during benzodiazepine withdrawal share similarities with mechanisms underlying activity-dependent synaptic plasticity, such as LTP.

The goal of the present study was to investigate whether AMPAR GluR1 subunits are specifically incorporated into hippocampal CA1 synapses during benzodiazepine withdrawal in an LTP-like fashion. Electron microscopy postembedding immunogold labeling demonstrated increased density of GluR1 subunits at synapses during benzodiazepine withdrawal, without significant changes in GluR2 subunit synaptic content. These findings provide further evidence that adaptations associated with dependence on drugs of abuse are similar to mechanisms underlying activity-dependent synaptic plasticity.

MATERIALS AND METHODS

Chronic benzodiazepine treatment

Experimental protocols involving the use of vertebrate animals were approved by the University of Toledo College of Medicine (formerly the Medical University of Ohio) Institutional Animal Care and Use Committee (IACUC) and conformed to the National Institutes of Health guidelines. All efforts were made to minimize animal distress.

After a 2–3-day acclimation period during which 0.02% saccharin water was available, juvenile male Sprague-Dawley rats (Harlan, Indianapolis, IN) were offered FZP in 0.02% saccharin water as the sole source of drinking

water for 1 week. The target for the FZP group was an average dose of 100 mg/kg/day for the first 3 days and 150 mg/kg/day for the following 4 days. Animals that did not achieve a weekly average of 120 mg/kg/day were excluded. After drug removal, animals were given saccharin water for an additional 2 days prior to euthanasia and tissue preparation for analysis. At the time of death, the animals were between 35 and 40 postnatal days of age. Controls received only saccharin water in parallel to the experimental group during acclimation, 1-week treatment, and withdrawal period.

The concentration of FZP (100–150 mg/kg) offered to rats was appropriate to its relative potency and oral bioavailability (Chouinard, 2004). One-week FZP treatment results in a brain concentration of FZP and its active metabolites of 1.2 μ M, equivalent to 0.6 μ M diazepam (Xie and Tietz, 1992). Because of the short half-life of FZP in rat brain (<12 hr), the level of FZP and its active metabolites is negligible in the hippocampus 2 days after FZP withdrawal (Xie and Tietz, 1992; Van Sickle et al., 2004). This dosing regimen has been shown to induce manifestations of both benzodiazepine tolerance and dependence reliably (Tietz et al., 1999; Van Sickle et al., 2004). Rats treated in this way show a progressive increase in AMPA mEPSC amplitude from day 1 (~15–30%) to day 2 (~30–50%) after benzodiazepine withdrawal. The increases in both AMPAR function and the associated anxiety-like behavior are transitory, and they return to control levels within 4 days after treatment cessation (Van Sickle et al., 2004). Seizure activity is not observed at any time during withdrawal from 1-week FZP treatment.

Transcardial perfusion and tissue preparation

For electron microscopic studies, rats were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg). A vascular rinse solution (pH 7.4) containing (in mM) 136 NaCl, 3.5 KCl, and 6 mM NaHCO₃ was bubbled with 95% O₂/5% CO₂ for 15 minutes. After bubbling and prior to perfusion, heparin (5 units/ml) was added to the vascular rinse. Rats were rapidly perfused via the aorta with 50 ml of the equilibrated vascular rinse solution, followed by perfusion with 300–350 ml fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Blocks containing bilateral hippocampi were hemisected and postfixed in the same solution for 4 hr.

Freeze slamming, cryosubstitution, and low-temperature embedding

Coronal vibratome sections (500 μ m) were exposed to 1% sodium borohydride in PB for 60 minutes, rinsed for 6 \times 30 minutes, 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, Bannockburn, IL). For cryosubstitution and low-temperature embedding, tissues were transferred to Leica EM AFS and treated as follows: 12 hr in 1% uranyl acetate in methanol (–80°C); 4 \times 30 minutes in absolute methanol (this and the following at –50°C); 2 hr each in 50%, 75%, 100% lowicryl HM20; and 2 \times 8 hr in 100% lowicryl. Sections were then flat-embedded using lowicryl between glass coverslips coated with formen-trenmittel (Electron Microscopy Sciences, Fort Washington, PA) and polymerized with UV light (48 hr at –50°C,

72 hr at 0°C, 48 hr at +20°C). The CA1 area of the hippocampus was excised and glued to EM blocks. Ultrathin sections were obtained (65–70 nm thickness; silver/gold sections) and collected on formvar-coated 200-mesh nickel grids.

Antibody characteristics

Anti-GluR1. A rabbit anti-GluR1 subunit antibody, kindly donated by Dr. R. Wenthold, NIDCD, was used in initial studies. The Wenthold antibody was obtained from host rabbit by injecting a BSA-conjugated synthetic peptide (SHSSGMPLGATGL) corresponding to amino acid residues 877–889 of the carboxy terminus (C-terminus) of the rat GluR1 subunit. With this antibody, immunoblots of GluR1 transfected cells and solubilized rat brain membranes showed a single band corresponding to a molecular weight of ~108 kDa (Wenthold et al., 1992). The Wenthold rabbit polyclonal anti-GluR1 antibody has been further characterized by light and electron microscopy (Petralia and Wenthold, 1992). Further studies were performed using a commercially available rabbit polyclonal anti-GluR1 antibody (Chemicon International, Temecula, CA; catalog No. AB1504, lot No. 0508008855). The Chemicon anti-GluR1 antibody was obtained by injecting the same C-terminus peptide of rat GluR1 (SHSSGMPLGATGL) conjugated to KLH into host rabbit (manufacturer's technical information). This antibody has been extensively characterized by immunoblot and light and electron microscopic techniques and used in several studies reporting the distributions of glutamate receptors in diverse regions of the mammalian brain (Joshi et al., 2004; King et al., 2006; Kim et al., 2006). Immunoblot analysis of this antibody in the rat brain showed a single band of ~110 kDa comigrating with GluR1 subunit expressed in transfected cells (manufacturer's technical information). In addition, the specificity of this antibody in CA1 synapses has been tested in GluR1 knockout mice by using immunocytochemical techniques (Zamanillo et al., 1999).

Anti-GluR2. The Chemicon anti-GluR2 antibody used in this study (rabbit polyclonal anti-GluR2 antibody; Chemicon International; catalog No. AB1768, lot No. 0702052022) was obtained by injecting the host rabbit with a synthetic immunogenic peptide (VAKNPQN-INPSSSQNS) corresponding to amino acid residues 827–842 of rat GluR2 C-terminus conjugated to BSA. Immunoblot analysis of rat brain showed a single band of ~108 kDa, and immunocytochemical analysis of transfected cells showed no cross-reactivity with GluR1, GluR3, or GluR4 subunits (Petralia et al., 1997). The specificity of this antibody was further tested in tissue from a GluR2 knockout (KO) mouse. Ultrathin sections from the hippocampal region from a KO and a matching wild-type (WT) littermate were kindly donated by Drs. Ronald Petralia and Ya-Xian Wang (Sans et al., 2003). Sections from KO and WT were immunolabeled in parallel using conditions identical to those described below. In addition, some WT sections were processed omitting the primary antibody. Immunolabeling of synapses in the GluR2 KO was negligible, with only a few synapses labeled in the KO, usually with only one gold particle. Similar labeling was observed in WT tissue after omitting the primary antibody (see Results), suggesting that the labeling originated from a low-level of nonspecific absorption of secondary antibodies and was clearly different in density and incidence from the specific PSD labeling analyzed.

Postembedding immunogold labeling

Grids were wetted in Tris-buffered saline with 0.1% Triton X-100 (TBST), pH 7.6, and then incubated for 30 minutes in 1:10 normal goat serum (Sigma-Aldrich, St. Louis, MO; catalog No. 9023) containing 0.1% Na-borohydride and 50 mM glycine. In initial experiments, grids were etched for 3 seconds in sodium ethanolate. A saturating concentration of NaOH was dissolved in 70% ethanol at least 24 hr before use. The solution was used for several days until its transparency was lost. Sections were then washed in TBST and incubated for 2 hr at room temperature in rabbit anti-GluR1 subunit antibody (1:50; R. Wenthold). Although etching increased immunolabeling, it also clearly affected ultrastructural definition and contrast. With a more concentrated solution (1:10) of the commercial rabbit polyclonal anti-GluR1 subunit antibody, the labeling efficiency at CA1 asymmetrical synapses was increased even without etching, so this step was omitted. Separate grids were also incubated without etching in a rabbit polyclonal anti-GluR2 subunit antibody (1:50; Chemicon). After 2 hr of incubation in the respective primary antibodies at room temperature, grids were transferred to 4°C for overnight incubation. Grids were rinsed for 3 × 5 minutes in TBST pH 7.6, then TBST pH 8.2, and incubated 2 hr in goat anti-rabbit IgG conjugated to 10-nm gold particles (EMGAR10; BBI, United Kingdom) diluted 1:25 in TBST pH 8.2. After rinsing for 3 × 5 minutes in TBST (pH 8.2) and filtered (0.22 μm) distilled water (dH₂O), grids were contrasted with 5% uranyl acetate in filtered dH₂O and lead citrate, for 5 and 4 minutes, respectively.

Data analysis: electron microscopy

Reacted ultrathin sections were analyzed in a Philips 201 EM electron microscope at 70 kV, and the PSDs in the stratum oriens and radiatum (SO and SR) of CA1 were imaged. Given that pyramidal cells outnumber interneurons in the CA1 region of the hippocampus by approximately 20:1 and contain far more excitatory synapses per cell (Megias et al., 2001), the presumed contribution of excitatory synapses from interneurons was considered negligible in this analysis. The analysis was performed in single cross-sections of synapses rather than in synapses reconstructed through serial sections. This allowed the analysis of relatively larger samples of synapses per rat (~30–70 synapses per animal sampled in one to three different blocks).

Areas within approximately 100 μm of the stratum pyramidale (SP) border in SO and SR of the CA1 region of the hippocampus were scanned, and digital images of asymmetric junctions representing excitatory synapses (Megias et al., 2001) were acquired at a magnification of ×36,000 (Gatan BioScan camera) by an observer blind to the experimental groups. An image was acquired for every asymmetric synapse encountered, unlabeled or immunogold labeled, in random surveys of the target region. All synapses demonstrating a clear synaptic cleft were included in the sample. Thus, synapses included in the analyses were all cut in a similar plane approximately perpendicular to the synapse, which avoids problems associated with differences in immunogold access, profile size, and identification of obliquely or tangentially cut PSDs. In initial studies, the GluR1 antibody provided by R. Wenthold was used. Approximately 50 images were

obtained from immunoreacted sections (1:50 dilution) derived from matched pairs of control and FZP-withdrawn rats (n = 3 per group) and analyzed with Image Pro Plus software (v. 5.0; Media Cybernetics, Silver Springs, MD). Some images had more than one synapse, so the number of synapses analyzed varied from 48 to 79 in different animals within a region. In this first experimental series, changes in GluR1 immunoreactivity between the SR and the SO regions were compared. Subsequently, GluR1 and GluR2 immunoreactivity was compared specifically in the SR region, which showed the largest changes in GluR1, as expected. For this second experimental series, 30–70 images were obtained from matched pairs of a different set of control and experimental animals. Five control and five FZP-withdrawn rats were analyzed with the commercially available anti-GluR1 antibody (Chemicon; dilution 1:10). For the study with the anti-GluR2 antibody (Chemicon; dilution 1:50), tissues from the same set of animals (control, n = 4 rats; FZP-withdrawn, n = 3 rats) were compared. In these experiments, a larger proportion of synapses in control animals was labeled with Chemicon's anti-GluR1 antibody diluted 1:10 than with Wenthold's anti-GluR1 antibody diluted 1:50. Despite different sensitivities in detecting synaptic GluR1, an increase in immunolabeling was found in the SR region of FZP-withdrawn animals compared with their matched controls with both anti-GluR1 antibodies in the two different experimental series.

The numbers of gold particles inside the PSD, or at a maximum 20 nm from the surrounding edge of the PSD (see Fig. 1A–C), including those falling in the synaptic cleft, were counted in all synapses sampled and defined as postsynaptic labeling. Gold particles that were outside the 20-nm surrounding edge of the PSD and fell on the presynaptic membrane were not counted. Labeling precision with 10-nm colloidal gold conjugates of complete IgGs, using indirect immunolabeling methods, has been estimated to be within 20–30 nm of the epitopes (see Hainfeld, 1987; Herman et al., 1996; Matsubara et al., 1996). Immunogold densities were estimated as particles per linear micrometer of PSD. Perisynaptic labeling was defined as immunogold labeling within 100 nm lateral to either side of the PSD along the plasma membrane, excluding the 20-nm edge zone, where colloidal gold particles were considered associated with the PSD itself (Fig. 1A–C).

Statistical analyses

The number of synapses labeled in FZP-withdrawn neurons was compared by Mann-Whitney U test with the expected value obtained from control hippocampii and expressed as a percentage of labeled synapses for comparison between groups, which contained different numbers of synapses in each sample. Density of labeling was estimated as the number of gold particles divided by PSD length in micrometers and compared by Student's *t*-test between FZP-withdrawn and control groups. Measures of PSD size were compared between groups via ANOVA with post hoc analysis by Bonferroni's multiple-comparisons test. A regression analysis was used to evaluate the relationship between the number of GluR1- or GluR2-immunogold particles and PSD length within the aggregate of synapses from each control and experimental animal. The differences in lengths between control and FZP-withdrawn immunonegative and immunopositive

PSDs were compared for both GluR1- and GluR2-labeled synapses by one-way ANOVA with post hoc analysis by Bonferroni's multiple-comparisons test. The relative frequency distribution of different numbers of GluR1- or GluR2-immunogold gold particles in synapses of FZP-withdrawn vs. control neurons was compared by χ^2 analysis. Comparison of numbers of particles (0 to ≥ 9) between groups was by Mann-Whitney U test. All data are reported as mean \pm SEM. The significance level was set at $P < 0.05$.

To determine more precisely the distribution of gold particles within synapses, the position of gold particles was measured relative to the postsynaptic membrane in a representative subset of GluR1- and GluR2-labeled synapses in control and experimental groups. The distance between the center of each gold particle and the outer leaflet of the postsynaptic membrane was measured with Image Pro-Plus software (v. 5.0; Media Cybernetics), grouped into 4-nm bins, and plotted against the number of gold particles.

EM micrographs in Figures 1, 2 and 4 were composed in Photoshop 5.0 (Adobe Systems, San Jose, CA) or Corel-Draw 12 (Corel, Fremont, CA) from digitized images, with minor adjustments to brightness and contrast. Camera scratches and dust particles in Figure 1 (presented as full field of view) were digitally removed. Data were statistically analyzed and Figures 3–8 generated in Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

RESULTS

To determine possible changes in the number of GluR1 and GluR2 AMPA receptor subunits during FZP withdrawal, immunoreactivity for these subunits was examined in synapses on the dendritic regions of CA1 hippocampal neurons. These hippocampal regions show the highest changes in dendritic AMPAR binding during FZP withdrawal (Van Sickle and Tietz, 2002). Moreover, diazepam withdrawal anxiety was also associated with a significant enhancement of immunolabeling in hippocampal CA1 pyramidal neuron dendritic fields (Izzo et al., 2001).

Specificity of immunolabeling, regions of analysis, definition of immunolabeled synapses

GluR1 and GluR2 immunoreactivity was highly localized to synaptic sites, with little immunoreactivity in the extrasynaptic membrane or on the neuropil (Fig. 1A,B). Synaptic labeling was analyzed in the PSD and surrounding 20-nm region (see Materials and Methods), whereas perisynaptic labeling was quantified in the plasma membrane within a 100-nm distance from the PSD (excluding the 20-nm juxta-PSD region; Fig. 1B,C). GluR1 antibodies included one donated by R. Wenthold (diluted 1:50) and one available commercially (diluted 1:10; Chemicon). The GluR2 antibody used was the one commercially available (diluted 1:50; Chemicon).

The level of nonspecific labeling was determined by placing 25 test rectangles of average length and width (0.22 μm and 0.045 μm , respectively) on each image (see Fig. 1D). The size of the test rectangles was empirically determined to match the average PSD size determined in our initial studies. The probabilities of one, two, three, or more particles falling on top of a region of size similar to a

PSD by random chance was then calculated for each image and averaged to provide an estimate for each immunoreaction and animal (Fig. 1E). Only gold particles whose center was within the rectangle were counted. Gold particles within rectangles that randomly fell on PSDs were included. Test rectangles that fell on top of regions with only resin (no tissue) and areas that were obvious artifacts (holes in lowicryl, precipitates) or the lumen of capillaries were not included in the analyses. In the 45–55 images used in GluR1 analyses for each control or FZP-withdrawn animal, 1,231 \pm 60 and 1,277 \pm 38 test rectangles were analyzed, respectively. Fewer images were analyzed for the GluR2 antibody, but nevertheless these included analysis of 775 \pm 59 and 763 \pm 48 test rectangles in control and FZP-withdrawn animals. A similar analysis was performed in images obtained from WT and GluR2 KO mouse tissue sections immunolabeled with the GluR2 antibody or omitting the primary antibody (2,108, 2,179, and 918 rectangles in 87, 90, and 38 images, respectively). The same secondary antibodies were used for GluR1 and GluR2 immunolabeling.

The probability of finding one or more particles in test rectangles was less than 3% in either SR or SO using the Wenthold anti-GluR1 antibody diluted 1:50 and less than 5% using the commercial anti-GluR1 and anti-GluR2 antibodies diluted 1:10 and 1:50, respectively (Fig. 1E; only SR was tested). In sections from WT mice immunolabeled with GluR2 antibodies, the probability of finding one or more particles was 1% and dropped to 0.4% in KO sections, suggesting that some of this immunolabeling might represent specific labeling of nonsynaptic pools of GluR2 subunits. After omitting the primary antibody in WT tissue, the probability of labeling in test rectangles was similar to that in KO tissue (0.3%), suggesting that the true level of background labeling in the neuropil is less than half of the low levels estimated with test rectangles.

It is possible, however, that PSDs are sites of preferential antibody absorption and contain levels of nonspecific labeling higher than those observed in test rectangles placed randomly in the neuropil. To estimate the extent of nonspecific PSD labeling, the number of immunolabeled PSDs was determined in sections from GluR2 KO and WT mice, after omitting the primary antibody. In both cases, the percentage of synapses containing at least one gold particle was similar (9% in KO tissue, $n = 100$ synapses sampled; 8% in WT tissue after omitting the primary antibody, $n = 50$ synapses sampled), and less than WT synapses immunolabeled with GluR2 antibodies (35%, $n = 100$ synapses). These results confirm the specificity of the commercial GluR2 antibody used. The specificity of the commercial GluR1 antibody has previously been confirmed in GluR1 KO tissue (Zamanillo et al., 1999). In addition, the data indicate that the error of considering one gold particle as positive labeling is small and always less than 10%. Therefore, synapses containing just one gold particle were defined as immunopositive.

AMPA GluR1 subunit immunoreactivity is increased in hippocampal CA1 synapses during FZP withdrawal

Preliminary analyses compared the proximal regions of basal and apical dendrites located in SO and SR, respectively, using the GluR1 antibody donated by R. Wenthold (diluted 1:50). There was a significant increase in the

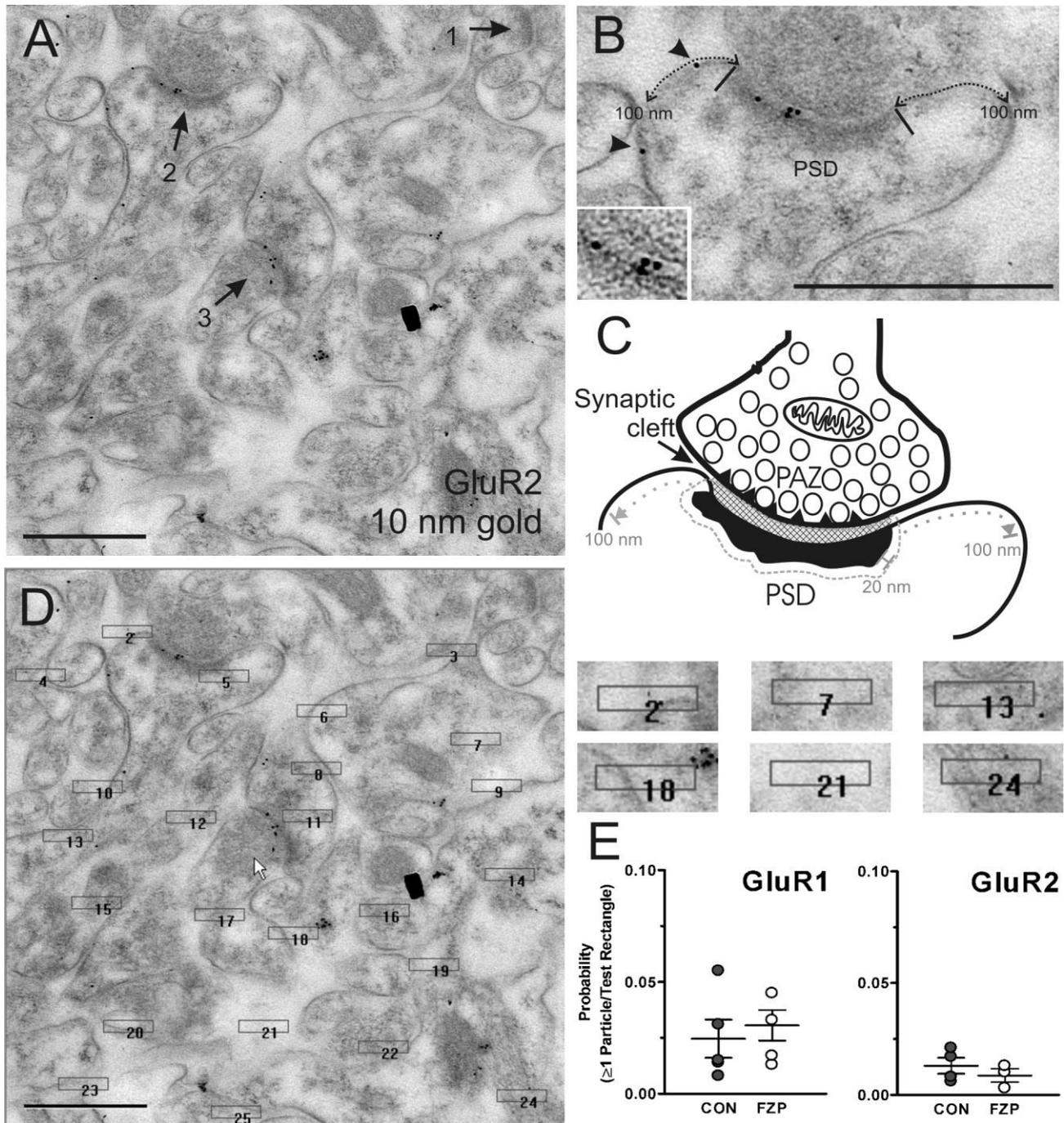


Fig. 1. Location of immunogold labeling. **A:** Full-field image of a digital electron micrograph of the CA1 SR region used in the analysis and containing three synapses (arrows 1–3). Two of them contain GluR2 immunogold labeling (10-nm particles). All the synapses analyzed are clearly asymmetric, and many can be identified on spine heads. **B:** Magnified view of synapse 2 showing the lateral edges of the PSD region (black bars), extrasynaptic GluR2 immunolabeling (arrowheads), and the approximately 100-nm plasma membrane regions that were considered as “perisynaptic” in these cross-section analyses (dotted line). The inset shows labeling at high contrast to define better the pre- and postsynaptic membranes. **C:** Diagram of a synapse similar to the one shown in B, marking the presynaptic active zone (PAZ; triangles inserted in the membrane of the presynaptic bouton), opposed by a synaptic cleft (generally more electron-dense than the

extrasynaptic intercellular space) and the PSD region. Immunogold particles related to the PSD were counted if they were located within a 20-nm boundary region surrounding the edges of the PSD. This region included all the synaptic cleft area. Particles were considered extrasynaptic if clearly located on the membrane and within 100 nm of the PSD (excluding the 20-nm drop zone associated with the PSD). **D:** Test rectangles on top of the image shown in A. The immunogold content in these rectangles was used to calculate the probability that one or more particles were randomly associated with a region similar to the PSD. Test rectangles were $0.22 \times 0.045 \mu\text{m}$ in size and randomly placed on the images. **E:** The probability that one or more gold particles were related to a test rectangle was less than 5% in both GluR1- and GluR2-labeled synapses in both control and FZP-withdrawn rats. Scale bars = $0.5 \mu\text{m}$.

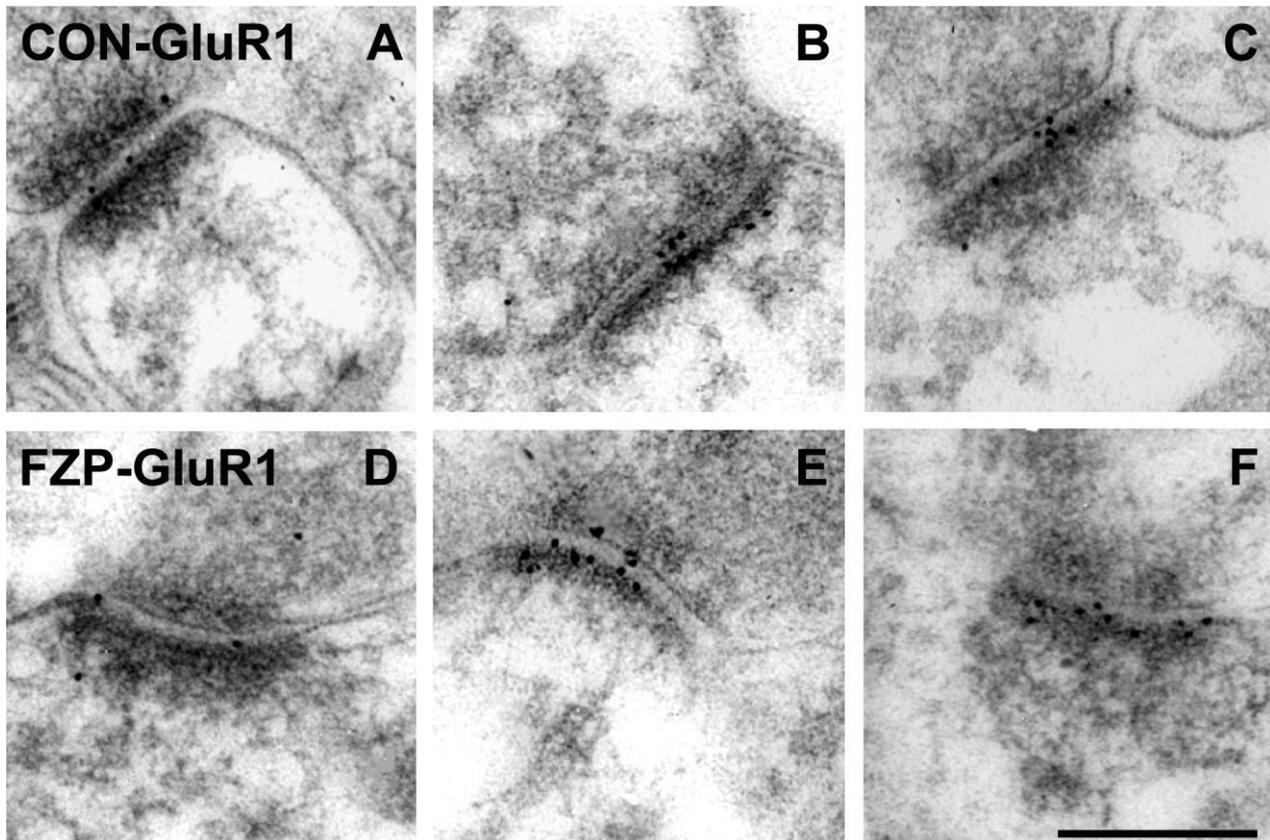


Fig. 2. Electron micrographs of AMPAR GluR1 subunit immunogold labeling in hippocampal CA1 SR from control and FZP-withdrawn rats. **A–C**: Representative images of GluR1-labeled (Chemicon) asymmetric synapses from control rats. Immunogold particles (10 nm) are located primarily within the postsynaptic density (PSD) and extend into the synaptic cleft. **D–F**: Representative images

of GluR1-labeled asymmetric synapses from FZP-withdrawn rats also show immunogold labeling within the PSD and synaptic cleft. GluR1 immunogold labeling was significantly increased in FZP-withdrawn CA1 synapses compared with controls (see Table 1 and Fig. 3). All images are at the same magnification. Scale bar = 0.25 μm .

number of particles per synapse in both the SO (CON: 0.59 ± 0.01 ; FZP: 0.77 ± 0.03 ; $P = 0.01$, Student's *t*-test) and SR regions (CON: 0.51 ± 0.05 ; FZP: 0.83 ± 0.03 ; $P < 0.01$). However, the percentage of unlabeled synapses in controls was very high (~60–65% in SO and SR regions) in these immunolabeled preparations. Many of these non-immunoreactive synapses likely represent false negatives and therefore hampered accurate estimation of changes in the number of labeled synapses between control and experimental tissues.

To expand on these findings, additional studies were focused in the SR region in additional matched pairs of control and FZP-withdrawn rats ($n = 5$ rats/group). In the latter studies, a commercial GluR1 polyclonal antibody was used at a lower dilution (1:10; Chemicon). In these preparations, there was a higher degree of labeling, without a significant increase in nonspecific labeling, and the percentage of unlabeled synapses was <30%. Figure 2 illustrates representative examples of synapses labeled with the anti-GluR1 antibody (Chemicon) in control (Fig. 2A–C) and FZP-withdrawn rats (Fig. 2D–F). The percentage of synapses demonstrating GluR1 AMPAR immunogold labeling (Fig. 3A) was significantly increased in the SR region in FZP-withdrawn rats ($88.2\% \pm 2.2\%$, $n = 5$

rats, 51–59 synapses per animal) compared with controls ($74.4\% \pm 1.9\%$, $n = 5$ rats, 46–67 synapses per animal; $P = 0.002$, Student's *t*-test). In the collection of immunolabeled synapses (i.e., containing at least one gold particle), immunogold density was also significantly higher in FZP-withdrawn rats (14.1 ± 0.3 particles/ μm , $n = 5$ rats) compared with controls (10.8 ± 0.4 particles/ μm , $n = 5$ rats; $P < 0.001$; Fig. 3B). Thus, both the percentage of labeled synapses (18.5% change) and the GluR1 immunogold labeling density (30.5% change) increased significantly in the CA1 SR region of the hippocampus in FZP-withdrawn rats compared with controls.

PSDs in excitatory synapses of rat hippocampal CA1 SR region increase in size in response to FZP withdrawal

Long-lasting changes in synaptic strength during activity-dependent forms of plasticity may require structural modifications such as increases in size, morphology, and number of synapses (Harris et al., 2003; Lang et al., 2004). To determine whether gross morphological changes occur during FZP withdrawal, we measured the length, thickness, and area of the PSD of CA1 excitatory synapses

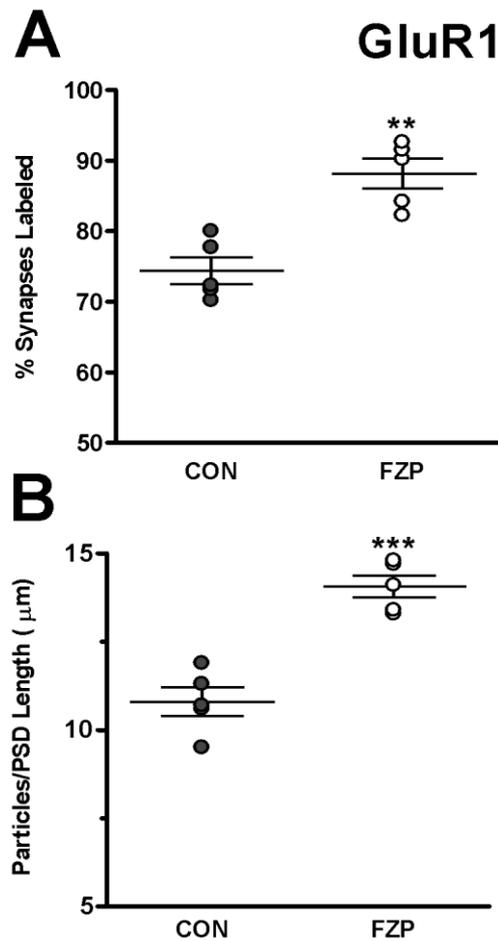


Fig. 3. FZP-withdrawal increases AMPAR GluR1 subunit incorporation in hippocampal CA1 asymmetric synapses. **A:** The percentage of synapses with immunogold labeling for GluR1 was significantly higher (** $P < 0.01$) in synapses from FZP-withdrawn rats compared with controls. **B:** GluR1 immunogold density estimated by the number of gold particles per micrometer of length of synapse was also significantly increased in synapses from FZP-withdrawn rats compared with controls ($n = 5$ rats/group, *** $P < 0.001$). Error bars indicate SEMs.

in the SR region of the hippocampus. The average PSD length (\pm SEM) from all control synapses (immunopositive and immunonegative) reacted with the anti-GluR1 antibody (Chemicon) was $0.239 \pm 0.009 \mu\text{m}$ ($n = 5$ rats, PSDs sampled = 263) compared with immunopositive and immunonegative synapses from FZP-withdrawn rats ($0.270 \pm 0.008 \mu\text{m}$, $n = 5$ rats, PSDs sampled = 277; $F = 476.8$, $df = 5$, $P = 0.001$), indicating a significant elongation of the synapse. PSD thickness and total PSD cross-sectional area were not significantly different between FZP-withdrawn and control animals (see Table 1), but there was nevertheless a tendency for PSD cross-sectional areas to be larger in FZP-withdrawn animals. Average PSD areas were $0.014 \pm 0.001 \mu\text{m}^2$ in control synapses compared with $0.017 \pm 0.001 \mu\text{m}^2$ in experimental groups ($n = 5$ rats/group; $P = 0.051$, Student's t -test; $n = 52.6 \pm 3.9$ synapses analyzed per animal in control and 55.4 ± 3.9 in FZP-withdrawn rats). The findings overall suggest that the PSD in excitatory synapses in the SR of the CA1 region of the hippocampus increased modestly in size in association with the incorporation of GluR1-containing AMPARs during FZP withdrawal.

GluR2 subunits do not significantly increase in hippocampal CA1 synapses during FZP withdrawal

Sections obtained from the same rats were used for GluR2 immunogold labeling (CON: $n = 4$; FZP: $n = 3$). Positively labeled synapses were also identified as those with PSDs associated with one or more gold particles, as explained above. Figures 1 and 4 illustrate representative examples of synapses labeled with the anti-GluR2 polyclonal antibody (1:50; Chemicon) in control (Figs. 1, 4A–C) and FZP-withdrawn (Fig. 4D–F) rats. As with GluR1 antibodies, GluR2 immunogold labeling was present throughout the length of the PSD.

In contrast to GluR1, the percentage of synapses with GluR2 AMPAR labeling was not significantly different between the control ($76.7\% \pm 2.8\%$) and the FZP-withdrawn ($82.3\% \pm 6.5\%$) groups (Fig. 5A). In contrast to GluR1 immunogold labeling, GluR2 immunogold density did not increase in the FZP-withdrawn groups (8.7 ± 1.2 particles/ μm) compared with controls (10.5 ± 1.3 particles/ μm ; Fig. 5B). The data, however, showed a small trend toward an increase in the number of GluR2-

TABLE 1. GluR1 Subunit Immunogold Labeling

Rat No.	Synapses sampled	Labeled (%) ≥ 1 Particle	Particles/PSD length (μm)	PSD length (μm)	PSD thickness (μm)	PSD area (μm^2)
Control						
1	67	70.2	10.6	0.231	0.061	0.014
2	46	71.7	10.7	0.228	0.070	0.017
3	54	77.7	11.3	0.222	0.058	0.013
4	50	80.0	11.9	0.272	0.047	0.012
5	46	71.7	9.5	0.242	0.058	0.015
Mean \pm SEM	Total 263	74.4 ± 1.9	10.8 ± 0.4	0.239 ± 0.009	0.059 ± 0.004	0.014 ± 0.001
FZP-Withdrawn						
1	56	82.2	14.1	0.286	0.056	0.016
2	57	84.2	13.3	0.241	0.064	0.015
3	54	92.6	14.7	0.278	0.062	0.018
4	59	91.5	14.8	0.263	0.059	0.016
5	51	90.2	13.4	0.281	0.067	0.019
Mean \pm SEM	Total 277	88.2 ± 2.2	14.1 ± 0.3	0.270 ± 0.008	0.062 ± 0.002	0.017 ± 0.001
P value		0.008	0.0002	0.01	0.52	0.051

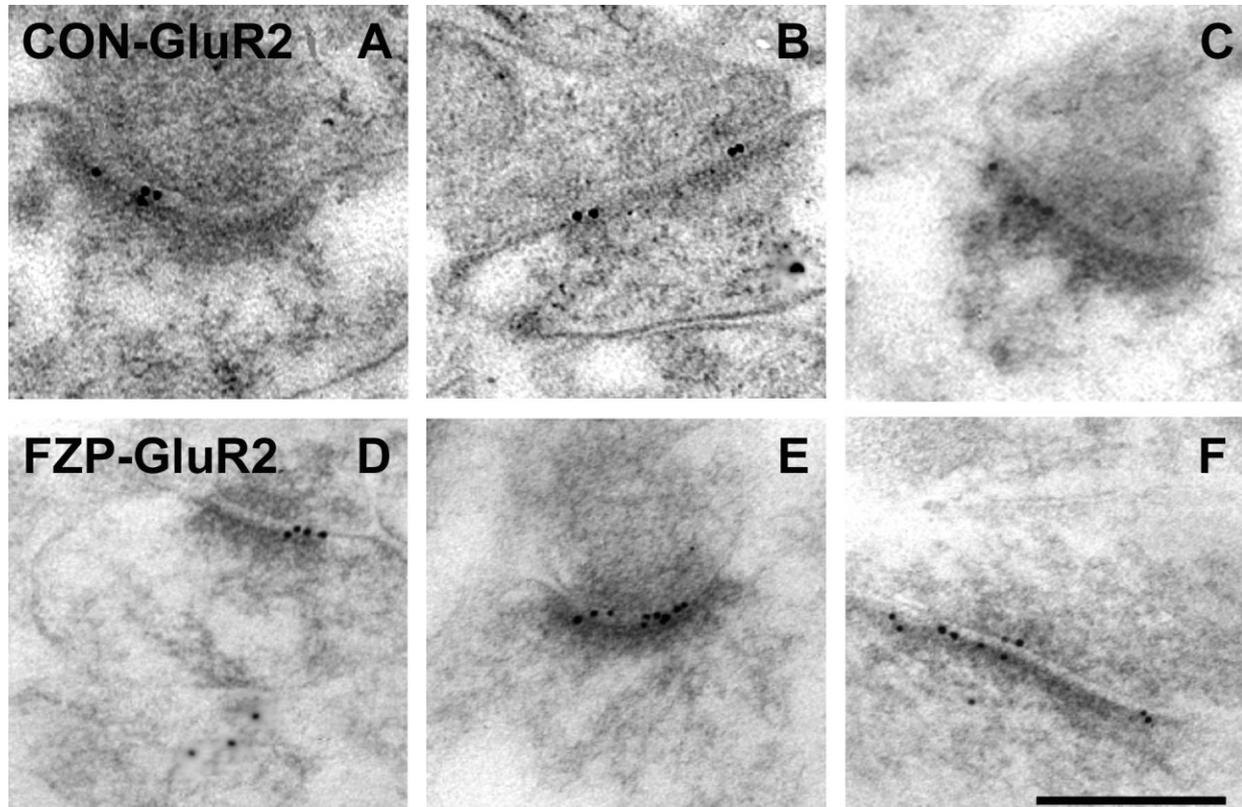


Fig. 4. Electron micrographs of AMPAR GluR2 subunit immunogold labeling in hippocampal CA1 SR from control and FZP-withdrawn rats. **A–C:** Representative images of GluR2-labeled (Chemicon) asymmetric synapses from control rats show immunogold particles (10 nm) mainly in the postsynaptic density and extending

into the synaptic cleft. **D–F:** GluR2 immunogold labeling was unchanged between control and FZP-withdrawn CA1 neuron synapses (see Table 1 and Fig. 5). All images are at the same magnification. Scale bar = 0.25 μm .

immunolabeled synapses between control and FZP-withdrawn rats that might have attained statistical significance with a larger sample size. The percentages of synapses labeled in the control groups with either anti-GluR1 or GluR2 antibodies were similar ($74.4\% \pm 1.9\%$ and $76.7\% \pm 2.8\%$ respectively). These proportions are similar to those reported previously in normal rat hippocampus (Petralia et al., 1999).

Unexpectedly, there were also no significant differences in the size of GluR2-labeled synapses (see Table 2), suggesting that the samples of GluR2- and GluR1-labeled synapses might somehow be different. Given that both subunits were found in a large proportion of synapses, the differences could arise if a subpopulation of larger PSDs containing only GluR1 subunits and/or a subpopulation of smaller PSDs containing only GluR2 subunits responded differently during FZP withdrawal. Dual-labeling experiments will be necessary to analyze synapses with different proportions of each of these subunits. However, the GluR1 and GluR2 antibodies used in this study were developed in rabbit, and our attempts to obtain dual labeling using two different methods to avoid cross-talk between primary antibody labeling produced inconsistent results. Similarly, reliable labeling with a mouse monoclonal GluR2 antibody was not obtained.

Relationship between PSD size and GluR1 or GluR2 immunogold labeling

Studies using serial section analyses have demonstrated that PSD size is positively correlated with the number of AMPAR (GluR1, GluR2/3, and GluR4) immunogold particles (Nusser et al., 1998; Takumi et al., 1999). In our samples, scatterplots of immunogold particle numbers plotted as a function of PSD length in GluR1- and GluR2-immunolabeled synaptic profiles analyzed in single cross-sections showed no significant correlation between PSD length and immunogold content (data not shown). However, these scatterplot analyses suggested possible differences in the size of AMPAR-immunonegative PSDs compared with either GluR1- or GluR2-immunopositive PSDs. Namely, the average length of immunonegative PSDs in tissues reacted with the GluR1 antibody was not significantly different between control ($0.208 \pm 0.005 \mu\text{m}$) and FZP-withdrawn tissues ($0.230 \pm 0.005 \mu\text{m}$; Fig. 6A). However, GluR1-immunopositive PSDs were significantly larger than their immunonegative counterparts and longer in the FZP-withdrawn group compared with control (CON: $0.250 \pm 0.010 \mu\text{m}$; FZP: $0.274 \pm 0.010 \mu\text{m}$; $P < 0.01$). Similarly, in sections reacted with the GluR2 antibody, the lengths of immunonegative PSDs in control and FZP-withdrawn animals were not significantly different

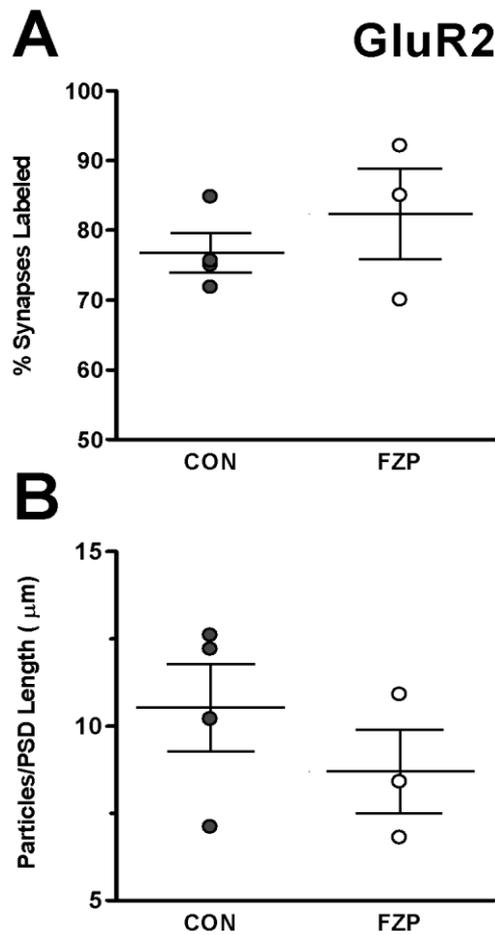


Fig. 5. FZP withdrawal has no effect on AMPAR GluR2 subunit incorporation in hippocampal CA1 asymmetric synapses. In contrast to changes observed in GluR1 immunogold labeling during FZP withdrawal, no significant ($P > 0.05$) changes in either percentage of synapses labeled (A) or mean GluR2 immunogold density (B) were observed between the two groups ($n = 4$ control and 3 FZP-withdrawn rats/group). Error bars indicate SEMs.

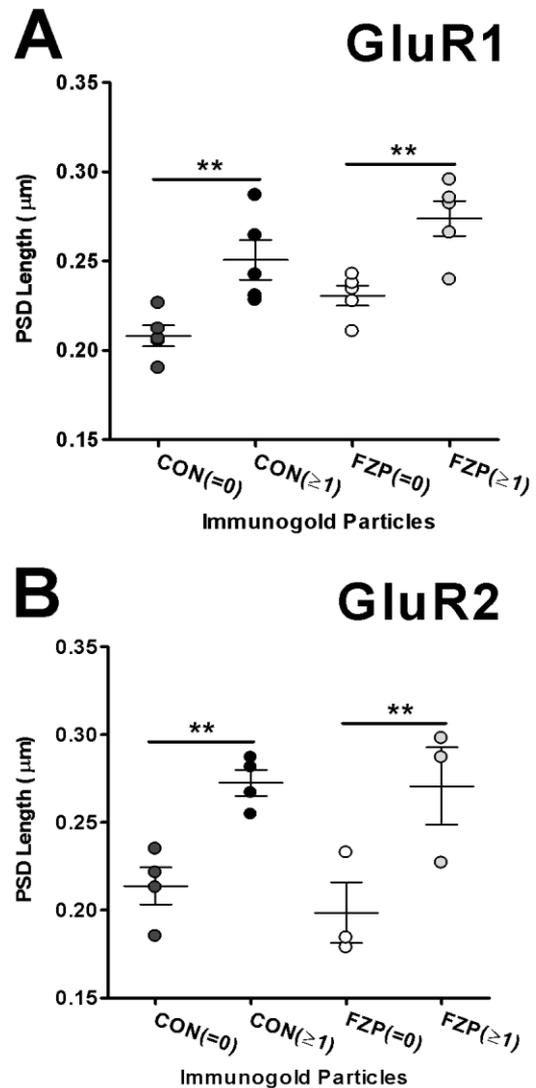


Fig. 6. Analysis of AMPA-immunonegative and -immunopositive synapse size. **A:** Analysis of PSD lengths in GluR1-labeled sections shows that AMPAR-immunonegative synapses ($=0$) are smaller than immunopositive synapses (≥ 1) in both control and FZP-withdrawn tissues ($n = 5$ rats/group). **B:** PSD lengths of immunonegative synapses from sections reacted with the GluR2 antibody were also significantly smaller than immunopositive synapses (** $P < 0.01$).

TABLE 2. GluR2 Subunit Immunogold Labeling

Rat No.	Synapses sampled	Labeled (%) ≥ 1 particle	Particles/PSD length (μm)	PSD length (μm)	PSD thickness (μm)	PSD area (μm^2)
Control						
1	44	75.0	12.2	0.253	0.056	0.013
2	44	75.0	10.2	0.246	0.050	0.013
3	33	84.8	12.6	0.282	0.058	0.017
4	39	71.8	7.1	0.254	0.062	0.016
Mean \pm SEM	Total 160	76.7 \pm 2.8	10.5 \pm 1.3	0.259 \pm 0.008	0.057 \pm 0.003	0.015 \pm 0.001
FZP-withdrawn						
1	40	70.0	8.4	0.278	0.092	0.024
2	41	85.0	10.9	0.221	0.043	0.010
3	38	92.1	6.8	0.279	0.056	0.017
Mean \pm SEM	Total 119	82.3 \pm 6.5	8.7 \pm 1.2	0.259 \pm 0.019	0.064 \pm 0.015	0.017 \pm 0.004
P value		0.70	0.35	0.98	0.59	0.62

(CON: $0.214 \pm 0.001 \mu\text{m}$; FZP: $0.199 \pm 0.002 \mu\text{m}$), whereas immunopositive PSDs were significantly larger but did not show differences between control and FZP-withdrawn groups (CON: $0.272 \pm 0.007 \mu\text{m}$; FZP: $0.270 \pm 0.020 \mu\text{m}$; Fig. 6B). The present data obtained from large samples of synaptic profiles analyzed in single cross-sections are therefore consistent with previous serial section analyses showing that AMPAR-immunonegative synapses are smaller than immunopositive synapses. Smaller immunonegative synapses have previously been interpreted as putatively "silent," with negligible or low AMPAR content (Takumi et al., 1999).

Perisynaptic labeling of AMPAR GluR1 and GluR2 subunits in hippocampal CA1 synapses during FZP withdrawal

Perisynaptic labeling with the GluR1 antibody, defined as labeling 100 nm lateral to the PSD (see Fig. 1), was also observed and represented 10.7% and 6.9% of total GluR1 gold particles sampled at sites defined as postsynaptic labeling in control and FZP-withdrawn rats, respectively. Perisynaptic labeling in synapses labeled with the GluR2 antibody represented 2.4% and 3.9% of GluR2 gold particles sampled at postsynaptic sites from control and FZP-withdrawn tissues, respectively. GluR1 perisynaptic labeling was always low and, if anything, decreased in FZP-withdrawn animals.

Frequency distributions of GluR1 and GluR2 subunit labeling

To determine whether some synapses express especially high or low levels of GluR1- or GluR2-containing receptors, the frequency distribution of immunogold particles in GluR1- and GluR2-labeled synapses was analyzed. A significant overall increase in the numbers of gold particles was observed in synapses from FZP-withdrawn rats compared with controls ($\chi^2 = 59.4$, $df = 9$, $P = 0.000$). In sections analyzed for GluR1 particles, relatively fewer synapses from FZP-withdrawn rats lacked immunoreactivity compared with control synapses (Fig. 7A). Thus, the observed fraction of immunonegative synapses lacking GluR1 particles was reduced by approximately twofold in FZP-withdrawn rats (0.12 ± 0.02) compared with controls (0.26 ± 0.02 ; $P = 0.008$, Mann-Whitney U test). The fraction of synapses expressing seven and eight gold particles in FZP-withdrawn rats was significantly increased compared with controls ($P = 0.008$). In sections analyzed for GluR2 particles, an overall change in distribution of gold particles across synapses between controls and FZP-withdrawn rats was observed ($\chi^2 = 77.8$, $df = 9$, $P = 0.000$); however, the observed fraction of synapses lacking GluR2 particles (0 particles) was not significantly different between groups (CON: 0.24 ± 0.03 ; FZP: 0.18 ± 0.07 ; $P = 0.86$, Mann-Whitney U test; Fig. 7B). There were also no significant differences in the fraction of labeled synapses with a given number of gold particles (1 to ≥ 9) between control and FZP-withdrawn groups ($P > 0.05$, Mann-Whitney U test).

GluR1 and GluR2 AMPAR subunit immunogold labelings show similar distributions in PSDs of synapses from control and FZP-withdrawn rats

Quantitative analysis of GluR1 and GluR2 immunogold particle location in PSDs was performed to determine

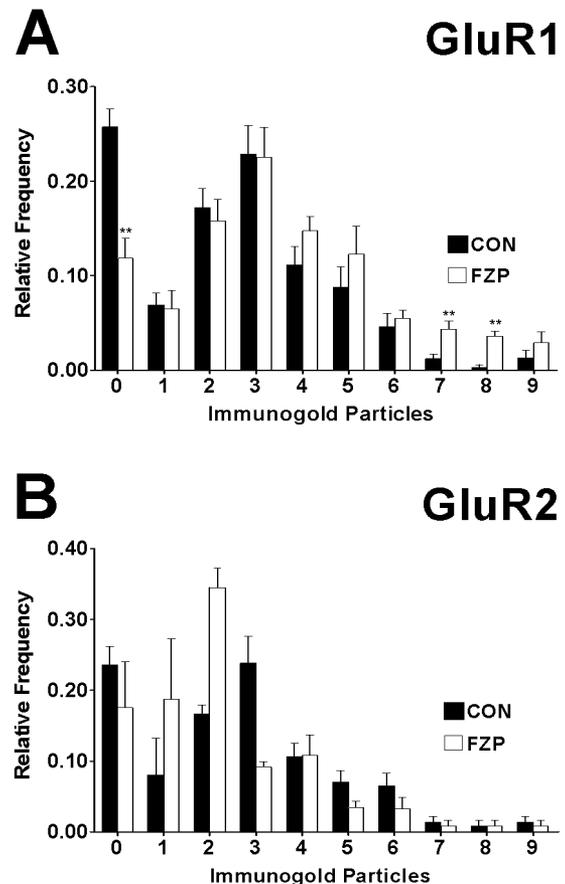


Fig. 7. Distribution histograms of synapses containing different numbers of GluR1 or GluR2 immunogold particles. **A:** Histogram bars represent average relative frequencies of synapses without GluR1 gold particles (0 particles) or containing from one to nine gold particles in control and FZP-withdrawn animals. The distributions of GluR1 particles in synapses from control (solid bars) and FZP-withdrawn rats (open bars) were compared by χ^2 analysis, and a significant overall increase in the number of synapses with a higher content of gold particles was detected in FZP-withdrawn rats compared with controls ($\chi^2 = 59.4$, $df = 9$, $P = 0.000$). The fraction of synapses lacking GluR1 immunogold labeling was significantly lower in asymmetric synapses from FZP-withdrawn rats compared with control rats (** $P < 0.01$, Mann-Whitney U test). A significant increase in the fraction of synapses expressing seven or eight gold particles was also observed after FZP withdrawal (** $P < 0.01$, Mann-Whitney U test). **B:** The distributions of GluR2 immunogold particle content were also significantly different in CA1 synapses between control and FZP-withdrawn animals ($\chi^2 = 77.8$, $df = 9$, $P = 0.000$). However, the observed fraction of synapses lacking GluR2 particles (0 particles) was not significantly different between groups ($P = 0.86$, Mann-Whitney U test), nor were there differences in any fraction of labeled synapses with a given number of gold particles (1 to ≥ 9 ; $P > 0.05$, Mann-Whitney U test).

more precisely the distribution of synaptic labeling in relation to the postsynaptic membrane for each subunit and whether the distribution changed during FZP withdrawal. Labeling for both receptor subunits was highly localized within the PSD of the synapse. GluR1 immunolabeling distribution was analyzed in 67 synapses from a control and 56 synapses from an FZP-withdrawn rat, each representative of its group. Positions from the center of

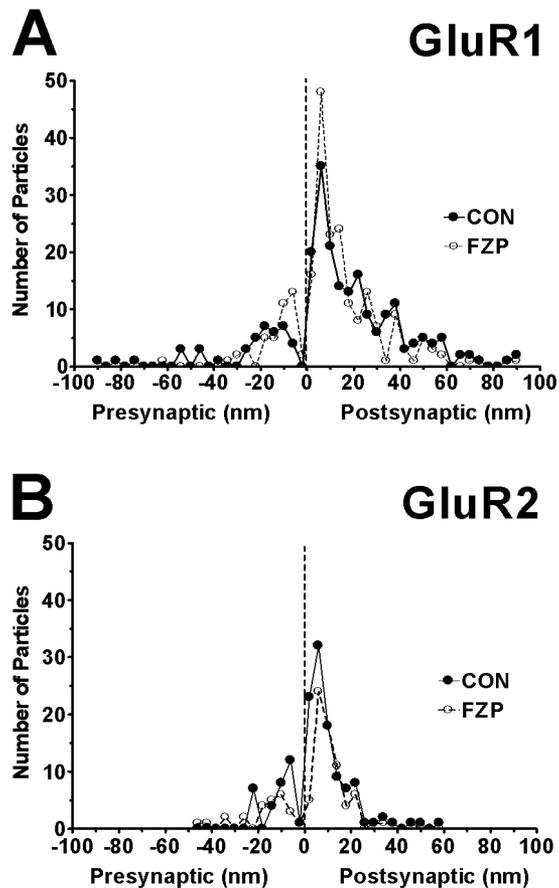


Fig. 8. Spatial distribution of all GluR1 (A) and GluR2 (B) immunogold particles found within 100 nm of the postsynaptic membrane on either the extracellular or the cytoplasmic side. Asymmetric synapses with clearly visible and well-defined PSDs and synaptic clefts were digitally imaged at a final magnification of $\times 36,000$. The distances between centers of each 10-nm gold particle to the outer leaflet of postsynaptic membrane were measured and grouped into 4-nm-wide bins (0 on the abscissa represents the external face of the postsynaptic membrane). Negative values indicate gold particles located at a distance from the postsynaptic membrane and in the direction of the presynaptic bouton and synaptic cleft (average width ~ 20 nm). Positive values indicate immunogold labeling on the cytoplasmic side. GluR1 and GluR2 immunogold labeling for both subunit antibodies peaked 6 nm inside the postsynaptic membrane (resolution ± 2 nm), with GluR1 labeling extending more distally toward the cytoplasmic side than GluR2 labeling. The majority of both GluR1 and GluR2 immunolabeling was located within the PSD region (width ~ 45 nm). Very few immunogold particles were detected extracellularly, beyond the synaptic cleft (values less than -20 nm). Thus, both the GluR1 and the GluR2 immunolabeling analyzed was very tightly related to the extent of the PSD. Data were obtained from 67 (CON) and 56 (FZP) synapses in GluR1-labeled tissues and from 44 (CON) and 40 (FZP) synapses in GluR2-labeled tissues.

each gold particle to the outer leaflet of the postsynaptic membrane were measured with a resolution of ± 2 nm (the pixel size in the $\times 36,000$ and $1,024 \times 1,024$ digital images) and binned in 4-nm increments (0 = outer edge of postsynaptic membrane; positive values represent the intracellular side and negative values the synaptic cleft side). As illustrated in Figure 8A, in control synapses, most GluR1-immunogold particles (81.3%) were located on

the postsynaptic side (i.e., positional value ≥ 0 in Fig. 8) and 52.9% within the first 25 nm (i.e., approximately half of the average width of the PSD), with a peak density +6 nm from the outer edge of the plasma membrane. This was similar in FZP-withdrawn animals.

Data for GluR2 labeling were obtained from 44 synapses from a representative control rat and 40 synapses from an FZP-withdrawn rat. The spatial distribution of GluR2 immunolabeling was also similar to that of GluR1 (peak +6 nm, 76.6% located on the postsynaptic side, 70.8% in the first 25 nm) and was not different in FZP-withdrawn animals (Fig. 8B). In conclusion, both GluR1 and GluR2 immunoreactivity analyzed in the PSD were strongly associated with the postsynaptic membrane in both control and FZP-withdrawn animals.

DISCUSSION

The present data provide ultrastructural evidence of a significant increase in AMPAR GluR1-mediated glutamatergic neurotransmission in excitatory synapses on the apical dendrites (SR) of hippocampal CA1 neurons during benzodiazepine withdrawal. The electron microscopy data agree with our and others' previous biochemical and functional studies demonstrating enhanced AMPAR-mediated mEPSC amplitude, AMPAR radioligand binding, and GluR1 subunit expression at the light microscopic level (Izzo et al., 2001; Van Sickle and Tietz, 2002; Van Sickle et al., 2004); a shift toward inward rectification of synaptic AMPAR currents (Song et al., 2007); and an increase in GluR1, but not GluR2, subunit protein expression (Song et al., 2007), all of which are indicative of postsynaptic incorporation of GluR1-containing, but GluR2-lacking, AMPARs and coincide with anxiety, a manifestation of withdrawal from chronic benzodiazepine treatment (Van Sickle et al., 2004; Xiang and Tietz, 2007).

Neurophysiological changes occurring during prolonged exposure to drugs of abuse have often been compared with those occurring in activity-dependent forms of plasticity such as LTP (Kauer and Malenka, 2007). A widely studied example of synaptic plasticity is the stimulus-induced potentiation of the Shaffer collateral-CA1 neuron glutamatergic pathway (Malenka and Bear, 2004). Numerous studies suggest an important role for enhanced expression of GluR1-containing AMPARs in LTP (Jia et al., 1996; Zamanillo et al., 1999), albeit transient (Hayashi et al., 2000; Plant et al., 2006; but see Jensen et al., 2003; Adesnik and Nicoll, 2007). Upon LTP induction, there is a decrease in the number of synaptic failures, indicating a conversion of "silent" synapses (not containing AMPAR) to "nonsilent" synapses, thought to depend on enhanced synaptic expression of GluR1-containing AMPARs (Malinow and Malenka, 2002; Poncer, 2003). This enhancement is accompanied by an increase in synapse size as Shaffer collateral AMPAR-immunopositive synapses are larger relative to putatively silent, AMPAR-immunonegative synapses (Nusser et al., 1998; Takumi et al., 1999).

The major findings of this study with postembedding immunogold were a significant increase in the density of synaptic GluR1 immunolabeling (expressed as particles per micrometer), a significant increase in percentage of synaptic profiles immunopositive for GluR1, and a small increase in the length of GluR1-immunopositive PSDs in FZP-withdrawn rats. Synaptic incorporation of the GluR2 subunit remained unchanged. Enhanced incorporation of

GluR1 subunits occurred in synapses of both apical and basal dendrites of CA1 neurons, but a greater change was observed in GluR1 immunolabeling in SR (63% increase in labeling density) compared with SO (37% increase).

Labeling for both GluR1 and GluR2 subunits was predominant in the PSD and largely concentrated within 20 nm of the postsynaptic plasma membrane, with a clear peak 6 nm intracellularly from the outer surface of the plasma membrane. This localization is consistent with previous reports suggesting a postsynaptic membrane localization of AMPAR subunits. This localization also agrees with the C-terminal location of AMPAR GluR1 and GluR2 subunits on the cytoplasmic aspect of the postsynaptic plasma membrane at a distance from the membrane that coincides with the location of C-terminal-interacting PSD proteins and the C-terminus of the N-methyl-D-aspartate receptor (Khazaria and Weinberg, 1999; Sassoe-Pognetto and Ottersen, 2000; Valtchanoff et al., 2000). The widths of labeling distributions obtained for each subunit are also consistent with the resolution of colloidal gold antibody probes, expected to locate epitopes within a 20–30-nm radius (Hainfeld, 1987; Herman et al., 1996; Matsubara et al., 1996). Interestingly, GluR1 immunolabeling occupied a larger intracellular extension in the PSD than GluR2 immunolabeling. A plausible explanation for this difference is that GluR2 C-terminal epitopes are located closer to the plasma membrane than GluR1 C-terminal epitopes. Indeed, the C-terminus of GluR2 spans only 50 amino acids (a.a. 813–862) from the membrane, and the peptide targeted by our antibodies occupies a midregion (a.a. 827–842; Petralia et al., 1997) compared with the more distal GluR1 C-terminal epitope targeted. The GluR1 C-terminus includes 81 amino acids (a.a. 809–889), and the peptide sequence recognized by the antibody is located at the very end of the tail (a.a. 877–889; Wenthold et al., 1992). In any case, the labeling distributions obtained in both experimental groups are identical for each probe, indicating that the changes observed in FZP-withdrawn animals reflect increased occupancy of the postsynaptic membrane by GluR1 subunits.

The above-described changes at excitatory glutamatergic synapses occurring during benzodiazepine withdrawal were notably similar to those observed after LTP induction. However, in contrast to transient insertion of GluR2-lacking AMPARs observed in some forms of LTP, which are then gradually replaced by GluR2-containing AMPARs (Plant et al., 2006), our studies coupled with previous electrophysiological reports suggest that prolonged administration of FZP and withdrawal induces a longer-lasting (at least 48 hr) change in AMPAR subunit composition, reflecting in all likelihood preferential synaptic incorporation of GluR1 subunit-containing receptors (Izzo et al., 2001; Song et al., 2007).

One proposed model of benzodiazepine dependence suggests that excitatory components are up-regulated in an effort to maintain homeostasis in the face of benzodiazepine-mediated enhancement of GABAergic inhibition. In this scenario, increased excitatory tone persists upon abrupt withdrawal of the benzodiazepines and contributes to the expression of a withdrawal syndrome that includes anxiety (Allison and Pratt, 2006). Persistent increases in GluR1 subunit levels occur in the amygdala, nucleus accumbens, and VTA following repeated cocaine administration (Fitzgerald et al., 1996; Lu et al., 2003), and mice deficient in the GluR1 subunit show blunted

morphine withdrawal (Vekovischeva et al., 2001). By using an immunohistochemical approach, Izzo et al. (2001) showed up-regulation of GluR1-subunit protein in dendritic regions of hippocampal pyramidal cells in rats withdrawn from diazepam, also coincident with the appearance of withdrawal anxiety. In many of these paradigms, including withdrawal from 1-week FZP exposure, AMPAR antagonists ameliorate both the alterations in GluR1 subunit expression and the drug withdrawal behaviors, including anxiety (Nestler, 2002; Van Sickle et al., 2004; Xiang et al., 2007). Collectively, these data suggest that increased synaptic incorporation of GluR1-containing AMPARs is important for the acquisition or expression of drug-withdrawal behaviors that occurs upon discontinuation of a variety of drugs of abuse. To our knowledge, the present electron microscopic study is the first to demonstrate enhanced incorporation of GluR1-containing receptors into excitatory synapses in the hippocampus during benzodiazepine withdrawal using a postembedding immunogold approach. This study and previous reports suggest the possibility that blockade of AMPAR-mediated excitatory transmission may be a viable approach for prevention and reduction of signs of benzodiazepine dependence (Steppuhn and Turski, 1993) and development of novel antianxiety treatments (Xiang et al., 2007).

In conclusion, the electron microscopic findings reported here provide insight into the mechanism of the enhanced AMPAR glutamatergic transmission associated with drug-withdrawal anxiety. 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 and GluR2/3 heteromers to the increased glutamatergic strength in this brain region. The findings also underscore the notion that the adaptive strategies characteristic of drug withdrawal are similar to activity-dependent plasticity and are highly conserved in the CNS.

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