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Role of protein kinase A in $GABA_A$ receptor dysfunction in CA1 pyramidal cells following chronic benzodiazepine treatment

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Abstract

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

Keywords: flurazepam, $GABA_A$ receptor, hippocampus, PKA, tolerance, VIP.

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$GABA_A$ receptors (GABARs) are pentameric ion channels, comprised of subunits from seven subunit families [α(1–6), β(1–3), γ(1–3), δ(1), ϵ(1), θ(1), π(1); Sieghart 1995; Whiting *et al*. 1999]. In the adult CNS, GABA binding results in Cl^- influx which hyperpolarizes the post-synaptic neuron decreasing neuronal excitability. Because GABARs are the major fast inhibitory neurotransmitter receptors in the CNS, they are targets for a variety of pharmacological agents (Sieghart 1995). For example, benzodiazepines (BZs) positively modulate GABARs by increasing GABA affinity and thus the frequency of channel opening (Lavoie and Twyman 1996). Consequently, BZs have been used clinically as anxiolytics, sedative-hypnotics and anticonvulsants. Their usefulness as anticonvulsants, however, is limited by the development of functional tolerance (e.g. Gonsalves and Gallager 1988; Rosenberg 1995; Tietz *et al*. 1999b; Izzo *et al*. 2001).

Using a 1-week flurazepam (FZP) treatment, we have identified a number of *in vitro* correlates of BZ anticonvulsant tolerance *in vivo*, which are prominent in the rat hippocampal CA1 area and less apparent in the dentate gyrus (DG). In this model, BZ anticonvulsant tolerance *in vivo* is accompanied by alterations in CA1 pyramidal cells including: loss of zolpidem-induced prolongation of miniature

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Abbreviations used: AKAP, A-kinase anchoring protein; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AX-314, lidocane *N*-ethyl bormide quaternary salt; BSA, bovine serum albumin; BZ, benzodiazepine; CREB, cAMP-responsive element binding protein; DG, dentate gyrus; FZP, flurazepam; GABAR, $GABA_A$ receptor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish-peroxidase; mIPSC, miniature inhibitory post-synaptic current; PKA, protein kinase A; SDS, sodium dodecyl sulfate; VIP, vasoactive intextinal polypeptide.

inhibitory post-synaptic current (mIPSC) decay, uncoupling of BZ/GABA binding sites, and downregulation of GABAR subunit proteins (Chen *et al*. 1995, 1999; Zeng and Tietz 1999). These measures are accompanied by a reduced mIPSC amplitude at hippocampal CA1 pyramidal neurons (Poisbeau *et al*. 1997; Zeng and Tietz 1999), evidence of GABAR dysfunction after chronic BZ exposure. Such changes have implications not only for subsequent BZ effectiveness, but also for normal GABAergic function in CA1 pyramidal cells, and fall within the context of use-dependent GABAR regulation (Barnes 1996). Notably, exposure to the BZ antagonist flumazenil, which reverses anticonvulsant tolerance *in vivo* (Gonsalves and Gallager 1988), co-ordinately reverses *in vitro* tolerance to zolpidem, GABAR subunit protein downregulation, and the reduction in mIPSC amplitude (Tietz *et al*. 1999b), underscoring the association between these phenomena.

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

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

PKA is a tetrameric serine/threonine kinase and in the CNS is comprised of two regulatory (RIα, RIIα, RIβ, or RIIβ) and two catalytic (Cα or Cβ) subunits (summarized in Doskeland *et al*. 1993). The association of the various regulatory isoforms with A-kinase anchoring proteins (AKAPs) targets PKA to discrete cellular compartments and confers spatial specificity (Colledge and Scott 1999; Diviani and Scott 2001). Activation of G protein-linked receptors positively coupled to adenylate cyclase increases cellular cAMP levels, and activates PKA. One such class of receptors includes the vasoactive intestinal peptide (VIP) receptors (VPAC1, VPAC2, and PAC1), which are present throughout most of the adult CNS, and reach appreciable density in the hippocampus (Harmar and Lutz 1994; Harmar *et al*. 1998; Brenneman *et al*. 2000). Moreover, hippocampal VIP receptors likely predominate at GABAergic synapses on CA1 neurons, as GABA and VIP co-localize in a subclass of interneurons that selectively impinge on the spine-free somata and proximal dendrites of pyramidal cells (Ascady *et al*. 1996).

Anticonvulsant tolerance *in vivo* is accompanied by changes in GABAR structure and function that are predominately evident in CA1 area of hippocampus and not DG (Poisbeau *et al*. 1997; Chen *et al*. 1999; Tietz *et al*. 1999a). Likewise, GABARs on CA1, but not DG, neurons are sensitive to regulation by PKA (Poisbeau *et al*. 1999). The present study was designed to determine whether changes in CA1 neuron GABAergic function following 1-week flurazepam treatment: (i) are associated with selective alterations in PKA activity and (ii) can be regulated by manipulation of the PKA system in these neurons. To this end, we evaluated endogenous hippocampal PKA activity and PKA isoform expression levels within cellular fractions containing either membrane-associated receptors or soluble substrates. We also evaluated the effect of PKA activation on GABAR mIPSC amplitude in CA1 pyramidal cells from control and FZP-treated rats.

Materials and methods

Benzodiazepine treatment

Experimental protocols involving the use of vertebrate animals were approved by the Medical College of Ohio Institutional Animal Care and Use Committee (IACUC), and conformed to National Institutes of Health guidelines.

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

Hippocampal slice preparation

Rats were decapitated and transverse dorsal hippocampal slices (500 μm) were cut on a vibratome (Pelco 101, Ted Pella, Inc., Redding, CA, USA) in ice-cold pre-gassed (95% O_2/5% CO_2) buffer containing: 120 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM D-glucose (288 mOsm, pH 7.2–7.4).

PKA activity assays

Hippocampal slices were prepared as described and areas CA1 and DG were microdissected and homogenized 10 times by hand in a 0.5-mL Pyrex™ Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh, PA, USA) containing 200 μL extraction buffer [25 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mg/mL aprotonin, 1 mg/mL leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. After centrifugation (14 000 *g*, 5 min at 4°C) the supernatant (soluble) was collected and the pellet (insoluble) was resuspended in extraction buffer containing 0.1% Triton X-100. Kinase activity in these fractions was assessed in the absence (basal) or presence (total) of 5 μM cAMP. Assays were carried out on 5 μL of sample at 37°C for 5 min PKA activity was measured by [γ^{32}-p]ATP incorporation into biotinylated kemptide, subsequently immobilized on avidin-coated filters (Promega, Madison, WI, USA). Negative control (5 μL dH_2O in place of homogenate) values were subtracted from each sample and activity was standardized to protein content determined by BCA protein assay (Pierce, Rockford, IL, USA). Data were expressed as pmol adenosine triphosphate (ATP) transferred/min/μg protein. Values for control and treated rats were analyzed using a paired *t*-test with a significance level of $p \leq 0.05$.

Determination of cAMP levels

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

Determination of mRNA levels by semi-quantitative RT–PCR

Total RNA was extracted from isolated whole rat hippocampi using the Trizol™ method (Invitrogen, Carlsbad, CA, USA). The RNA was DNase-treated and first-strand DNA was synthesized using oligo-dT primers (Invitrogen). PCR reactions contained: 1 μg cDNA, 0.1 μM forward and reverse primers, 1 × PCR buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$, and 2.5 units of Platinum *Taq* polymerase (Invitrogen) in a total volume of 50 μL. PCR primers, annealing temperature, and cycle number are listed in Table 1. Cycle parameters included an initial denaturation step (95°C for 2 min) followed by 30-s intervals of denaturation (95°C), annealing (Table 1), and extension (72°C). Pilot studies were performed with each set of primers to assess linearity of product formation with respect to cycle number, and care was subsequently exercised to ensure all products were within linear range at the terminal cycle. Samples were resolved on a 1.5% agarose gel containing 0.008% ethidium bromide. Bands were visualized at 610 nm on a Typhoon Imager (Amersham Biosciences, Piscataway, NJ, USA). Densities, normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) content, were compared with UnScanIt-gel software (Silk Scientific Co, Orem, UT, USA). Data for control and treated rats were analyzed using Student's *t*-test with a significance level of $p \leq 0.05$.

Immunoblotting

Slices were prepared as for PKA activity assays but after crude fractionation the supernatant was collected and the pellet resuspended in 100 μL of extraction buffer. A BCA protein assay was carried out prior to storage at − 70°C. Twenty to 40 μg protein from each CA1 and DG was resolved on a 10% sodium dodecyl sulfate (SDS) gel (Protean III, Bio-Rad, Hercules, CA, USA) at 150 V. The

Table 1 Primers and annealing conditions for RT–PCR studies

Target	Nucleotides	Sequence	Tm	Size	Source
VPAC2	619-638	F: 5′-CCAGCTCAGGTACACTGCGC-3′	60	479	Grimaldi and Cavallaro 1999
	1079-1098	R: 5′-CAAACACCATGTAGTGGACG-3′			
PAC1	1074-1093	F: 5′-CATCCTTGTACAGAAGCTGC-3′	60	472	Grimaldi and Cavallaro 1999
	1527-1546	R: 5′-GGTGCTTGAAGTCCATAGTG-3′			
PKA RIα	56-75	F: 5′-CAGCAGAACAATCTAGAGGA-3′	55	345	Giegerich *et al.* 1997
	401-420	R: 5′-CTTCCTCAGTGTAAACCTCA-3′			
PKA RIIα	895-914	F: 5′-CACTAGAGATGTCAGAACGA-3′	55	476	Giegerich *et al.* 1997
	1352-1371	R: 5′-CTGACTGGAAGGATTTGGTA-3′			
PKA RIIβ	128-148	F: 5′-CCTCATCTACAGAGATCTCA-3′	55	382	Dwivedi *et al.* 2002
	490-510	R: 5′-GCCACGGTTTGCATACTGACC-3′			
PKA Cβ	540-559	F: 5′-CCTCATCTACAGAGATCTCA-3′	55	321	Giegerich *et al.* 1997
	842-861	R: 5′-GATCCATTCGGTAGTAGCAA-3′			
β-Actin	76-97	F: 5′-GCTCGTCGTCGACAACGGCCTC-3′	55, 60	350	Pierson *et al.* 2002
	407-428	R: 5′-CAAACATGATCTGGGTCATCTTCTC-3′			
G3PDH	72-92	F: 5′-ATGGTGAAGGTCGGTGTGAAC-3′	55	437	Lai *et al.* 2000
	490-509	R: 5′-GCTGACAATCTTGAGGGAGT-3′			

gel was transferred (95 V for 3.5 h at 4°C) to nitrocellulose and blocked for 60 min at room temperature in TBST (50 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% milk + 1% bovien serum albumin (BSA; RIIβ) or 5% milk alone (Cβ). The membrane was immunoblotted for PKA RIIβ (1 : 5000, Chemicon, Temecula, CA, USA) or PKA Cβ (1 : 1000, Santa Cruz, Santa Cruz, CA, USA) diluted in blocking solution. After rinsing in TBST (6 × 5 min) membranes were incubated with anti-rabbit–horseradish-peroxidase (HRP; 1 : 100 000, Santa Cruz) diluted in blocking solution, rinsed (6 × 5 min) and developed for chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Actin immunoblotting (mouse antiactin 1 : 2000; Santa Cruz) was used as an internal standard for protein loading, and was either performed concurrently or after stripping and reprobing the nitrocellulose (0.5 M NaCl, 0.5 M acetic acid × 5 min, 1 N NaOH × 2 min). Care was exercised to ensure the chemiluminescent signal was linear with respect to loaded protein. Data for control and treated rats are expressed as proportion of actin and were analyzed using Student's *t*-test with a significance level of $p \leq 0.05$.

mIPSC recording

Slices were prepared as described above and maintained at room temperature in gassed buffer for 1–3 h prior to recording. GABAR-mediated action potential-independent mIPSCs were recorded in CA1 pyramidal neurons in the presence of: 1 μM tetrodotoxin (Na^+ channel blocker), 50 μM DL-2 amino-5 phosphonovaleric acid and 10 μM 6,7-dinitroquinoxaline-2,3-dione (glutamate receptor antagonists) using whole-cell voltage clamp techniques as described previously (Zeng and Tietz 1999). Patch pipettes (4–7 MΩ), were pulled from borosilicate capillaries (non-filamented, 1.5 mm A, Sutter Instruments, Novato, CA, USA) with a Flaming-Brown electrode puller (p-97, Sutter), and filled with internal solution: 130 mM CsCl, 1.0 mM EGTA, 0.5 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM lidocaine *N*-ethyl bromide quaternary salt (QX-314), 2.0 mM ATP, and 10.0 mM HEPES (pH 7.2). Cs^+ and QX-314 were included to eliminate $GABA_B$ receptor-mediated events and spontaneous firing of CA1 pyramidal neurons, respectively. Neurons were voltage-clamped (− 70 mV) in continuous mode with an Axoclamp 2 A amplifier (Axon Instruments, Foster City, CA, USA). The current output was low-pass-filtered (10 kHz), offset and amplified 10 000-fold. The signal was continuously monitored on-line (PClamp 6.0 Software, Axon Instruments), digitized (Digidata 1200, Axon Instruments) and stored on VCR tape for later analysis. Recorded events above the level of background (− 2.0 pA) with a duration of ≥ 3 ms were detected and averaged with Strathclyde CDR and SCAN software (J. Dempster, University of Strathclyde, Glasgow, UK) or MiniAnalysis software (Synaptosoft, Decatur, GA, USA).

Effects of 8-bromo cAMP and VIP on mIPSC amplitude

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

Results

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

Two days following 1-week FZP treatment, hippocampal tissue was prepared as described in Methods. PKA activity was assessed in the absence and presence of 5 μM cAMP, to reflect basal and maximal PKA activity, respectively. Pilot studies indicated that basal and stimulated values could be reduced 99% and 95%, respectively, by co-incubation of sample with protein kinase inhibitor (10 μM, data not shown). Positive control samples (10 ng PKA catalytic subunit) were, at a minimum, sixfold greater than the highest value in each experiment (data not shown). In the soluble CA1 fraction there was a significant ($p \leq 0.05$) increase in basal PKA activity (CON: 0.88 ± 0.25, FZP 1.75 ± 0.40 pmol ATP/min/μg protein); however, cAMP-activated PKA activity was not altered

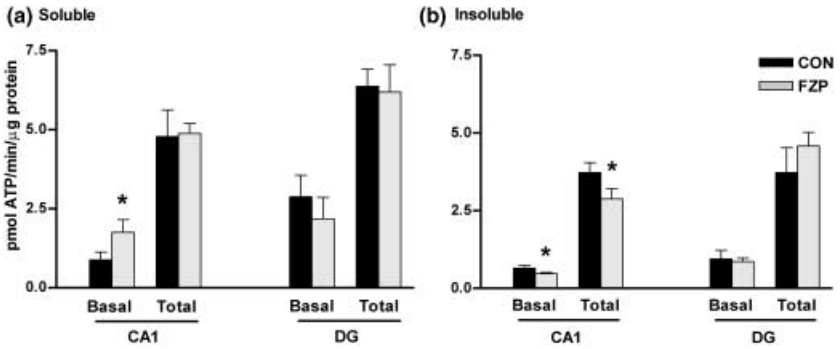

Fig. 1 Chronic FZP treatment alters hippocampal PKA activity. (a) The soluble fraction of CA1 exhibits greater basal PKA activity following 1-week FZP treatment (199% of control) but there is no effect on total, i.e. cAMP-stimulated PKA activity in CA1 area of hippocampus. Moreover, soluble PKA activity in the DG was not significantly affected. (b) The insoluble fraction of hippocampal CA1 exhibits less basal and less total PKA activity (72% and 77% of control, respectively) following 1-week FZP treatment. There is no statistically significant effect of FZP treatment on PKA activity in the DG. $n = 6$ rats/group. ■, CON; □, FZP. Asterisks denote significant differences between control and FZP-treated groups, $^*p \leq 0.05$.

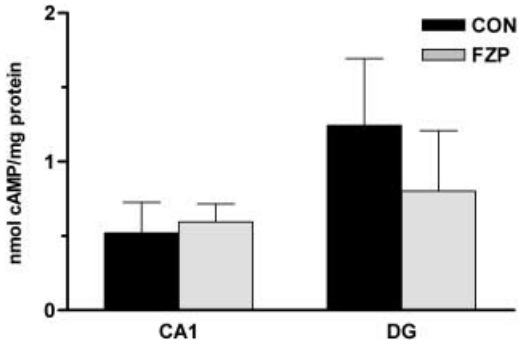

Fig. 2 Chronic FZP treatment does not affect hippocampal cAMP levels. cAMP levels were assayed in CA1 and DG 2 days after the end of 1-week FZP treatment. Alterations in PKA activity (Fig. 1) are not accounted for by an alteration in endogenous cAMP levels. CA1: 86% of control; DG: 65% of control $n = 6$ rats/group. ■, CON; □, FZP.

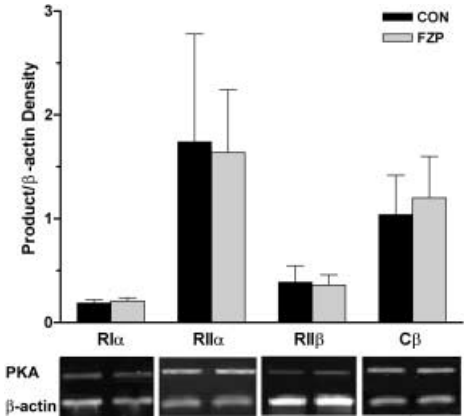

Fig. 3 Chronic FZP treatment does not affect PKA isoform mRNA expression in the hippocampus. Total mRNA was isolated from rat hippocampus as described in Methods. Top: RIα, RIIα, RIIβ, and Cβ mRNA levels were compared between FZP-treated and control rats using semiquantitative RT–PCR: RIα (110% of control), RIIα (90% of control), RIIβ (98% of control), Cβ (120% of control). One-week FZP-treatment did not significantly affect the transcript levels of these PKA isoforms. $n = 5$ rats/group. Bottom: Representative bands depicting control and FZP-treated samples. ■, CON; □, FZP.

(Fig. 1a). Chronic FZP treatment resulted in a statistically significant reduction in both basal (CON: 0.65 ± 0.13, FZP 0.47 ± 0.04 pmol ATP/min/μg protein) and stimulated (CON: 3.72 ± 0.32, FZP 2.88 ± 0.32 pmol ATP/min/μg protein) PKA activity in the insoluble CA1 fraction (Fig. 1b). There was no effect of FZP treatment on PKA activity in the soluble or insoluble DG fractions. Moreover, 2 days after acute desalkyl-FZP treatment, PKA activity was not significantly changed in the soluble (CA1 basal: CON 3.31 ± 0.27, FZP 3.43 ± 0.41; total: 6.90 + 0.62, 7.72 + 0.95; DG basal: 3.73 ± 0.80, FZP 3.40 ± 0.35; total: 7.08 + 0.68, 6.34 + 1.07) and insoluble (CA1 basal: CON 0.28 ± 0.09, FZP 0.25 ± 0.05; total: 0.90 + 0.15, 0.90 + 0.08; DG basal: 0.26 ± 0.09, FZP 0.29 ± 0.09; total: 1.10 + 0.40, 1.00 + 0.17) fractions ($n = 3$ rats/group).

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

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

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

Chronic FZP treatment changed PKA isoform protein levels

Because the mRNA samples for RT–PCR were collected from whole hippocampus (see Methods), and as such may not pick up specific regional differences in PKA isoform expression, we chose to examine the protein expression of PKA subunit RIIβ and Cβ in CA1 and DG of hippocampus. RIIβ is of particular interest because it is the major regulatory PKA isoform in the brain, and is localized to the membrane cytoskeleton, i.e. the same fraction in which consistent decreases in PKA activity were shown (Fig. 1b). An alteration in RIIβ levels may have implications for kinase targeting or sensitivity to cAMP (Skalhegg and Tasken 1997; Colledge and Scott 1999). Cβ was also investigated because of its relative abundance in the rat brain.

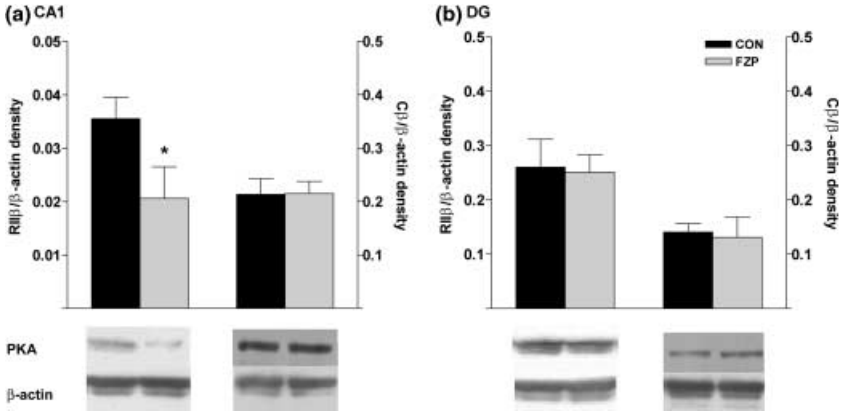

Fig. 4 Chronic FZP treatment altered PKA isoform protein levels in hippocampal CA1 area. Insoluble fraction of hippocampal (a) CA1, but not (b) DG, exhibited a significant 40% reduction in PKA RIIβ protein 2 days after 1-week FZP treatment. PKA Cβ isoform levels in (a) CA1 or (b) DG were unaffected by chronic FZP treatment. $n = 5$ rats/group. Bottom: Representative bands from western blots depicting RIIβ and Cβ levels from control and FZP-treated samples. ■, CON; □, FZP.

Although a change in PKA RIIβ mRNA was not detected in whole hippocampus (Fig. 3), chronic FZP treatment resulted in a significant 40% decrease ($n = 5$ rats/group) in RIIβ protein in CA1, but did not affect RIIβ protein levels in the DG (Fig. 4). Whether PKA RIIβ mRNA and protein are regulated in parallel in this region of the hippocampus could best be asssessed by *in situ* hybridization studies. When hippocampal Cβ protein levels were compared between control and FZP-treated rats, there was no change in the expression of this subunit in CA1 or DG regions of the hippocampus.

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

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

VIP is contained in subsets of GABAergic interneurons that synapse on CA1 pyramidal cell somata and proximal dendrites and is released in an activity-dependent manner, eliciting post-synaptic increases in cAMP and subsequent activation of PKA (Giachetti *et al*. 1977; Ascady *et al*. 1996; Brenneman *et al*. 2000). To determine whether the effect of 8-bromo cAMP on GABAR mIPSC amplitude in slices from FZP-treated rats could be replicated with an endogenous modulator of PKA, we assessed the effect of VIP (0.03–1 μM) on GABAR function. Again the baseline mIPSC was significantly reduced in cells from FZP-treated rats (CON − 24.7 ± 3.4, FZP − 14.3 ± 2.9 pA). In agreement with the effects of 8-bromo-cAMP, VIP significantly increased mIPSC amplitude (30 nM, $p \leq 0.05$) in CA1 neurons from FZP-treated rats (Fig. 6b). The maximal effect of VIP resulted in full restoration of mIPSC amplitude in FZP-treated cells. After this point VIP showed a trend to inhibit mIPSCs in both control and FZP neurons, although this effect did not reach statistical significance. Although a previous report described pre-synaptic effects of VIP (Wang *et al*. 1997), VIP did not significantly alter mIPSC frequency at the concentrations used, which were well below that used in the former study. This suggests that the alteration of GABA mIPSCs by VIP was a post-synaptic phenomenon.

There are three VIP receptors in the rat CNS (VPAC1, VPAC2, and PAC1) with different affinities for the peptide ($IC_{50} = 1$, 3, and 1000 nM, respectively; Harmar *et al*. 1998). We explored the possibility that the differential effect of VIP on mIPSC amplitude was associated with an alteration in VPAC2 expression. Reasons for the focus on VPAC2 include VPAC2′s abundance in CA1 hippocampus (Sheward *et al*. 1995), the relative lack of VPAC1 mRNA in the hippocampus (data not shown) and the low affinity of PAC1 for VIP (Harmar *et al*. 1998). Because a specific antibody to VPAC2 is unavailable, we compared the transcript levels of VPAC2 by semiquantitative RT–PCR. There was no effect of chronic FZP treatment on VPAC2 receptor mRNA expression (96% of control, Fig. 6c, $n = 5$/

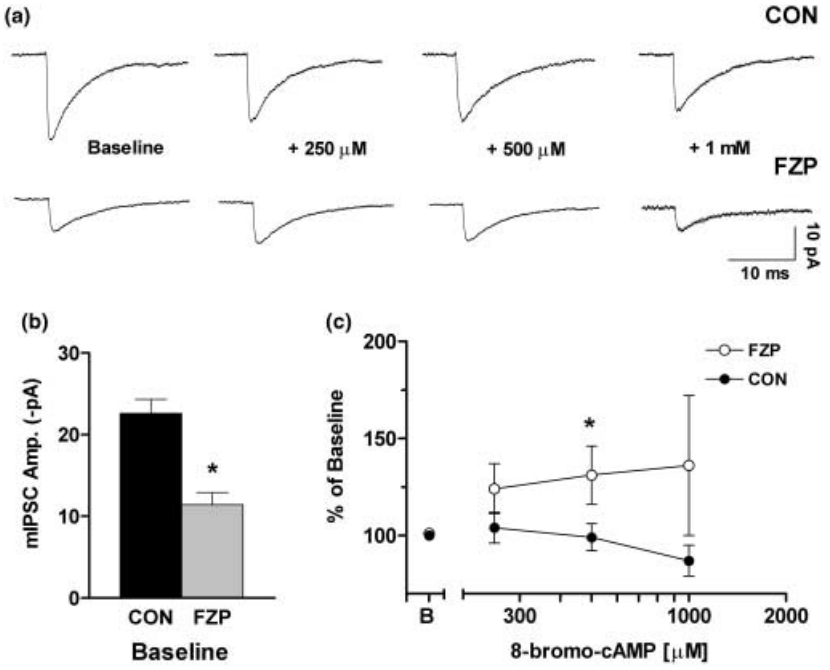

Fig. 5 8-bromo-cAMP positively modulated mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats. (a) Representative traces of mIPSC events before and during superfusion of increasing concentrations (250–1000 μM) 8-bromo-cAMP. (b) Baseline mIPSC amplitude, averaged across 5 min recording, was significantly reduced (50% of control) 2 days following chronic FZP treatment. These same cells were subsequently exposed to 8-bromo-cAMP (CON: 10 cells/10 rats, FZP: nine cells/nine rats). (c) The membrane permeable cAMP analog, 8-bromo-cAMP, significantly increased mIPSC amplitude in FZP-treated (+30% at 500 μM, $p \leq 0.05$), but not control, pyramidal cells. At the highest concentration (1000 μM), mIPSC amplitude was partially suppressed by 8-bromo-cAMP in control cells. ●, CON; ○, FZP. Asterisks denote significant differences between control and FZP-treated groups, $^*p \leq 0.05$.

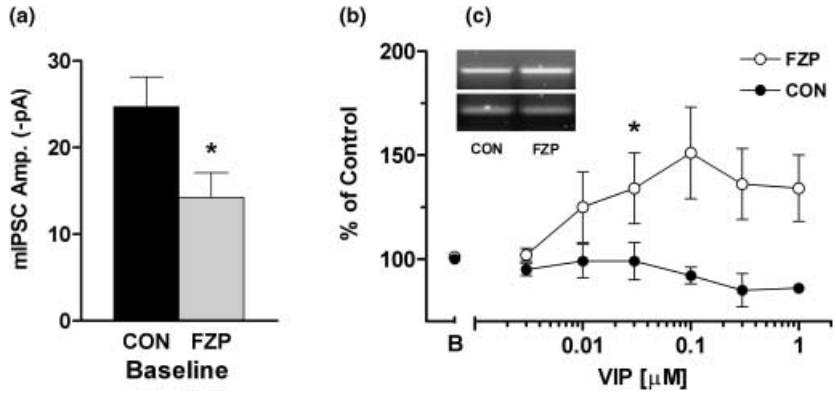

Fig. 6 VIP positively modulated mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats. (a) mIPSC amplitude, averaged across 5 min recording, was significantly reduced (58% of control) 2 days following chronic FZP treatment. These same cells were subsequently exposed to VIP (CON: eight cells/eight rats, FZP: seven cells/seven rats). (b) Like 8-bromo-cAMP, VIP increased mIPSC amplitude in cells from FZP-treated (36% at 30 nM, $p \leq 0.05$), but not control rats. (c) VPAC2 mRNA expression (top) assessed by RT–PCR in control and FZP-treated rats relative to β-actin mRNA levels (bottom). The differential susceptibility of FZP-treated cells to VIP is not accompanied by a change in VPAC2 mRNA (96% of control level). A representative comparison is shown. $n = 5$ rats/group. ●, CON; ○, FZP. Asterisks denote significant differences between control and FZP-treated groups, $^*p \leq 0.05$.

group). Concurrent studies indicated that PAC1 expression was also unaltered (data not shown). This finding suggests that changes in VPAC2 receptor mRNA do not underlie the enhanced sensitivity of FZP-treated cells to VIP.

Discussion

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

Interestingly, others have shown that RIIβ subunit disruption occurs with a reduction in PKA activity and catalytic

subunit protein (Brandon *et al*. 1998). In the present study the reduction in CA1 PKA activity occurred with reduced PKA RIIβ but not Cβ subunit protein. The type of regulatory subunit contained in the PKA holoenzyme affects cAMP sensitivity and subcellular localization (Skalhegg and Tasken 1997). Thus, a change in the level of one regulatory subunit may have considerable effects on both the likelihood of substrate phosphorylation and the proximity of substrates. Although not assessed in the present study, the Cα isoform has been detected in CA1 of mouse hippocampus (Cadd and Mcknight 1989), where it may comprise part of the RIIβ holoenzyme in the normal animal.

Alterations in hippocampal PKA activity due to chronic psychoactive drug treatments have previously been reported, concomitant with changes in PKA isoform expression (e.g. Mori *et al*. 1998; Dwivedi *et al*. 2002). Chronic clozapine treatment, for example, significantly decreased PKA activity in both the soluble and insoluble fractions of hippocampus and was accompanied by a reduction in hippocampal GABAR expression (Farnbach-Pralong *et al*. 1998; Dwivedi *et al*. 2002). In the present study, there was a significant reduction in membrane-associated PKA activity paralleled by a reduction in PKA RIIβ protein in the CA1 region of hippocampus, but not the DG. Notably, these changes occurred after chronic, but not acute, FZP treatment, and were temporally (evident 2 days after chronic FZP treatment) and spatially (prominent in CA1, but not DG) consistent with the reductions in GABAR subunit protein and mIPSC amplitude (Poisbeau *et al*. 1997; Chen *et al*. 1999; Tietz *et al*. 1999a, 1999b). Thus, PKA may play a role in the GABAR alterations induced by chronic FZP.

Phenomena that are common to chronic BZ treatments and models of BZ tolerance include an uncoupling of GABA/BZ binding sites, internalization of the GABAR, and subsequent alterations in subunit expression (Barnes 1996). Others have indicated that the preliminary steps, i.e. BZ-induced uncoupling and GABAR internalization, are mimicked by inhibition of PKA and reversed by PKA activation (Brown and Bristow 1996; Ali and Olsen 2001). Hence, the reduction in mIPSC amplitude, which likely reflects a loss of functional receptors at the post-synaptic membrane (Poisbeau *et al*. 1997; Nusser *et al*. 1998), could be a result of decreased local PKA activity (Fig. 1b). Changes more persistent than those resulting from receptor internalization might be expected to result from alterations in subunit gene expression. Like other models of BZ tolerance (Heninger *et al*. 1990; Holt *et al*. 1996; Impagnatiello *et al*. 1996; Costa *et al*. 2002), the FZP treatment used here causes selective reductions in GABAR subunit expression, notably of α1 and β3 subunit mRNA and protein (Chen *et al*. 1999; Tietz *et al*. 1999a). Transcription of the α1 subunit gene is partially controlled by cAMP-responsive element binding protein (CREB; Bateson *et al*. 2002), which likely subserves the PKA-dependent increases in α1 expression induced by increased cAMP levels (Thompson *et al*. 2000). Interestingly, the ability of cortical neurons to transduce cAMP increases to CREB activation relies on the intact expression of the PKA RIIβ subunit (Paolillo *et al*. 1999), which was significantly reduced following chronic FZP treatment (Fig. 4). If reduced PKA activity and RIIβ protein results in a suppression of CREB-mediated transcription, this scheme may provide a mechanism for the selective downregulation of α1 subunit in hippocampal CA1 neurons following chronic FZP treatment, and the associated functional sequalae. Thus, PKA may play a critical role in both the transient and sustained reductions of GABAR function characteristic of use-dependent regulation (Barnes 1996), by regulating receptor internalization and GABAR gene expression, respectively. The reduced GABAR mIPSC amplitude is likely a functional manifestation of these GABAR reductions (Nusser *et al*. 1998).

The sensitivity of GABARs from FZP-treated animals to PKA modulators suggests that, apart from the role PKA may play in the acquisition of GABAR dysfunction following chronic FZP treatment, PKA activation may be important for the recovery of GABAR function in the FZP-treated rat. To screen the sensitivity of GABARs to PKA modulators we utilized both 8-bromo-cAMP and VIP, an endogenous modulator of PKA activity. In CA1 of hippocampus, VIP is present in a subset of GABAergic interneurons that synapse on CA1 pyramidal cell somata and proximal dendrites (Ascady *et al*. 1996). Similar to the present findings, post-synaptic effects of VIP on GABAR function have been described in dissociated bipolar and retinal ganglion cells, where VIP increased GABAR currents in a PKA-dependent fashion (Veruki and Yeh 1992, 1994).

The acute effects of PKA on GABAR function have been investigated in a wide variety of cell types and brain areas. However, there is not an absolute consensus regarding the acute effects of PKA and its modulators on GABAR function. Nonetheless, the effects reported so far are largely attributed to phosphorylation of the β1 and β3 subunits (McDonald *et al*. 1998; Brandon *et al*. 2002), which are both expressed in CA1 pyramidal cells (Wisden *et al*. 1992; Chen *et al*. 1999; Tietz *et al*. 1999a; Pirker *et al*. 2000). In recombinant systems, forskolin caused a direct phosphorylation of β1 subunits and subsequent reduction of GABAR currents, while β3 phosphorylation resulted in an enhancement of GABAR function (McDonald *et al*. 1998). The latter findings were subsequently extended to olfactory granule neurons (Nusser *et al*. 1999). However, others demonstrated reduced GABAR currents in olfactory granule neurons when PKA activity was indirectly enhanced by agonism of the D_1-dopamine receptor (Bruning *et al*. 1999), a phenomenon also evident in the striatum and accompanied by increased phosphorylation of the β1/3 subunit (Flores-Hernandez *et al*. 2000).

Both the subunit composition of the GABAR and the mechanism of PKA stimulation account for some of the

reported discrepancies regarding the effects of PKA on GABAR function. Although there was a trend toward reduced mIPSC amplitude in control cells with greater PKA activation, these effects only approximated those reported previously with intracellularly applied PKA (Poisbeau *et al*. 1999). This discrepancy may reflect different methods of PKA activation. Alternatively, GABARs in control cells may exhibit greater basal phosphorylation and be less responsive to PKA activation.

Because PKA's capacity to modify GABAR function is partially based on the subunit complement of the GABAR, the sensitivity of CA1 pyramidal cells from FZP-treated rats to PKA modulators may be a reflection of altered subunit composition. For example, the increased β2 and the decreased β3 subunit expression 2 days after cessation of chronic FZP treatment (Chen *et al*. 1999; Tietz *et al*. 1999a) could conceivably unmask the functional effects of β1 subunit-mediated phosphorylation on CA1 pyramidal cells. However, this would arguably reduce GABAR function in neurons (McDonald *et al*. 1998; Poisbeau *et al*. 1999), and the present study indicates that PKA activation in FZP-treated cells causes a preliminary increase in GABAR function. Another possibility is that chronic FZP treatment results in a change in the endogenous phosphorylation of the GABAR. Poisbeau *et al*. (1999) reported that GABARs in CA1 pyramidal cells, but not DG neurons, are under the tonic control of PKA-mediated phosphorylation, which serves to suppress GABAR function. An increase in β2 mRNA following FZP treatment could result in the expression of GABAR isoforms less amenable to endogenous phosphorylation, but perhaps more sensitive to changes in PKA activity. In this manner, both the altered GABAR subunit expression and local reductions in PKA activity may act in tandem to pre-dispose CA1 neuron GABARs to modulation by PKA. Nevertheless, PKA activation in these neurons reportedly serves to depress GABAR function, and the present study indicates that PKA activation in fact increases mIPSC amplitude in CA1 neurons from FZP-treated rats.

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

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

In summary, the presence of alterations in PKA activity in the hippocampal CA1 area, but not the DG, supports previous work indicating the different responses of these neurons to protein kinases and to regulation by chronic FZP treatment (Poisbeau *et al*. 1997, 1999; Chen *et al*. 1999; Tietz *et al*. 1999a; Van Sickle and Tietz 2002). These studies support an association between the acquired GABAR dysfunction following chronic FZP treatment and the reduced local PKA activity and RIIβ expression in CA1 pyramidal cells. Collectively, suppressed PKA activity is likely related to the FZP-induced GABAR dysfunction, and subsequent elevation of PKA activity may be important for the return to normal function in CA1 pyramidal cells.

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