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Acute Pentylenetetrazol Injection Reduces Rat $GABA_A$ Receptor mRNA Levels and GABA Stimulation of Benzodiazepine Binding with No Effect on Benzodiazepine Binding Site Density[1]

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ABSTRACT

The effects of a single convulsive dose of pentylenetetrazol (PTZ, 45 mg/kg i.p.) on rat brain γ-aminobutyric acid type A ($GABA_A$) receptors were studied. Selected $GABA_A$ receptor subunit mRNAs were measured by Northern blot analysis (with β-actin mRNA as a standard). Four hours after PTZ, the $GABA_A$ receptor γ_2-mRNA was decreased in hippocampus, cerebral cortex, and cerebellum; α_1-mRNA was decreased in cerebellum; and β_2 subunit mRNA was decreased in cortex and cerebellum. The α_5 subunit mRNA level was not altered. Those mRNAs that had been reduced were increased in some brain regions at the 24-h time point, and these changes reverted to control levels by 48 h. PTZ effect on $GABA_A$ receptors was also studied by autoradiographic binding assay with the benzodiazepine agonist [^{3}H]flunitrazepam (FNP), the $GABA_A$ agonist [^{3}H]muscimol, and the benzodiazepine antagonist [^{3}H]flumazenil. There was an overall decrease in [^{3}H]FNP binding 12 but not 24 h after PTZ treatment. In contrast, [^{3}H]muscimol binding was minimally affected, and [^{3}H]flumazenil binding was unchanged after PTZ treatment. Additional binding studies were performed with well-washed cerebral cortical homogenates to minimize the amount of endogenous GABA. There was no PTZ effect on specific [^{3}H]FNP binding. However, there was a significant reduction in the stimulation of [^{3}H]FNP binding by GABA. The results showed that an acute injection of PTZ caused transient changes in $GABA_A$ receptor mRNA levels without altering receptor number but affected the coupling mechanism between the GABA and benzodiazepine sites of the $GABA_A$ receptor.

γ-Aminobutyric acid (GABA) is the major neurotransmitter mediating fast inhibitory neurotransmission in the mammalian central nervous system. Activation of the $GABA_A$ receptor leads to opening of its intrinsic anion channel, with increased chloride conductance, typically resulting in an inhibitory postsynaptic potential. Several experimental models of epilepsy or increased seizure susceptibility have been shown to be associated with variation in $GABA_A$ receptor number or function (Löscher and Schwark, 1985; Olsen et al., 1985; Yu et al., 1986; Spreafico et al., 1993). Conversely, the occurrence of seizure activity may affect $GABA_A$ receptors (Shin et al., 1985; Corda et al., 1990; Titulaer et al., 1994), and such changes could be involved in altered neural excitability after a seizure episode, such as postictal depression or kindling.

Pentylenetetrazol (PTZ) is a chemical convulsant frequently used in the study of seizures. Some studies have indicated that the pharmacological effect of PTZ is at least partly mediated by interactions with the anion channel of the $GABA_A$ receptor (Squires et al., 1984). Either single or repeated PTZ administration may modify $GABA_A$ receptor number or function. Corda et al. (1990) found that repeated injection of a moderate dose of PTZ (e.g., 30 mg/kg), which will produce kindling, has several neurochemical effects, including a decrease in [^{3}H]GABA and [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) binding and in GABA-stimulated $^{36}Cl^-$ uptake, although a single PTZ injection had no effect on [^{35}S]TBPS binding. Two injections of a larger dose, sufficient to cause full tonic-clonic seizures 48 and 24 h before assay, were found to produce a decrease in GABA-mediated inhibition and decreased binding to $GABA_A$ receptors in hippocampus (Psarropoulou et al., 1994). In contrast to these reports of decreased $GABA_A$ receptor number or function after repeated PTZ injection, one study reported that a sin-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; PTZ, pentylenetetrazol; FNP, flunitrazepam; TBPS, *t*-butylbicyclophosphorothionate.

gle, acute tonic-clonic PTZ or electroshock seizure produced a dramatic increase in [^{3}H]diazepam binding within 30 min (Paul and Skolnick, 1978). A subsequent study in rats found that an acute convulsive dose of PTZ, administered 30 min before assay, had little effect on the binding of several ligands to various sites on the $GABA_A$ receptor in homogenates from seven regions of rat brain (Ito et al., 1986). Although there was an increase in the binding of [^{35}S]TBPS to striatal homogenates, there were no other changes in the binding of this ligand or of [^{3}H]flunitrazepam, [^{3}H]GABA, or [^{3}H]muscimol. Recent autoradiographic studies by Rocha et al. (1996) provided conflicting data in that a single, subconvulsive i.p. injection of PTZ produced a decrease in [^{3}H]FNP binding throughout the rat brain. Chronic PTZ administration also reduced benzodiazepine binding, and saturation analysis revealed a decrease in receptor number but not affinity. A saturation study for the effects of the single PTZ administration was not reported.

Another approach to evaluating the effect of seizure activity on $GABA_A$ receptor expression was used by Pratt et al. (1993), who reported that a single electroconvulsive shock seizure caused an increase in the mRNA levels for some of the $GABA_A$ receptor subunits in cerebellum between 4 and 8 h after the convulsion, but no change was evident as early as 2 h, and no changes were found in hippocampus or cerebral cortex. This showed that the level of mRNA may be a sensitive measure of the effect of seizure activity on expression of $GABA_A$ receptors and demonstrated the importance of the time interval between seizure activity and tissue collection for detecting changes in expression of $GABA_A$ receptors after seizure activity. In contrast to the results of electroshock seizure reported by Pratt et al. (1993), our preliminary studies indicated decreases in $GABA_A$ receptor mRNA after PTZ seizures.

This study was undertaken to investigate the effect of PTZ seizure activity on $GABA_A$ receptor expression by measuring mRNA levels and the binding of radioligands to the $GABA_A$ receptor at different time points after PTZ injection. Several subunit mRNAs were studied, including those in highest abundance and that contribute to receptors with benzodiazepine sites in the brain regions studied (α1, β2, and γ2) and α5, which may be of interest in relation to altered hippocampal function after PTZ seizures (Psarropoulou et al., 1994). Studying the cerebral cortex, cerebellum, and hippocampus might show whether the $GABA_A$ receptor subunit mRNAs are regulated differently by brain region. Binding assays were performed with quantitative autoradiographic methods for binding of [^{3}H]FNP, a benzodiazepine agonist, and [^{3}H]muscimol, a $GABA_A$ agonist. The results suggested additional studies with [^{3}H]flumazenil, a benzodiazepine antagonist, and an evaluation of GABA-stimulated [^{3}H]FNP binding in a well-washed tissue homogenate preparation.

Materials and Methods

PTZ Treatment. Male Sprague-Dawley rats (240–300 g, Harlan, Indianapolis, IN) were injected with 45 mg/kg PTZ i.p., in a volume of 1 ml/kg, to produce clonic convulsions. Injections were given between 8 and 9 AM. Rats that did not exhibit clonus (11%) were given a second injection 1 h after the first injection. Control rats were injected with 1 ml/kg physiological saline. For mRNA measurements, brains were collected 4 h after i.p. injection of PTZ or saline (or 3 h after a second PTZ injection, if required). In a second experiment, the interval was 24 or 48 h after PTZ or saline injection. In this second experiment, half of the saline-injected controls were assigned to each time point, and their data were analyzed as a group. For autoradiographic binding experiments, brains were collected 12 or 24 h after treatment. The time interval was chosen based on the preliminary results showing a decrease in $GABA_A$ receptor mRNA 4 h after PTZ (described below). It was expected that, with 12- and 24-h intervals, there would be time for translation and turnover of previously synthesized receptors, based on the reported turnover rate of benzodiazepine receptors in primary cultured neurons (Borden et al., 1984).

mRNA Isolation and Measurement. Oligodeoxynucleotide probes for $GABA_A$ receptor α_1 , α_5, β_2, and γ_2 subunits were synthesized by Oligos Etc., Inc. (Wilsonville, OR). The sequences of the probes and the preparation of the cDNA probe for β-actin were as reported previously (Wu et al., 1994; Zhao et al., 1994a,b). Oligodeoxynucleotide probes were labeled by 3′-tailing, and the cDNA probe was randomly labeled with [^{32}P]ATP.

Rats were decapitated and brains were quickly removed. Cerebral cortex, hippocampus, and cerebellum were isolated, frozen with liquid nitrogen, and stored at −70°C. With the same methods as in previous work (Wu et al., 1994; Zhao et al., 1994a, b), RNA was isolated with the proteinase K technique, enriched in poly(A)$^+$ RNA, separated by electrophoresis, transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), and immobilized.

Hybridization was performed under conditions previously reported (Zhao et al., 1994a,b). The Northern blots were prehybridized at 42°C for 4 h in 5× Denhardt's solution, 50% formamide, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA, 1 mM EDTA, at pH 8.0, and 2 × standard saline citrate. The appropriate ^{32}P-labeled probes (2×10^7 cpm) were added, and the hybridization was carried out for 18 to 20 h at 42°C. After posthybridization washes, the membranes were air dried and exposed to X-ray film (Kodak XAR; Kodak, Rochester, NY) at −70°C with intensifying screens. The intensities of the bands were determined with a Bio-Rad imaging densitometer (model GS-670; Bio-Rad, Richmond, CA). The membrane was stripped, as described previously (Zhao et al., 1994a), and reprobed several times for different subunits.

To evaluate the possibility that PTZ treatment might have affected β-actin, the relative densities of the bands from control samples on each membrane were averaged, and all samples were normalized to this value. The density of each $GABA_A$ receptor subunit mRNA band was expressed relative to its corresponding β-actin band (measured on the same blots). For each mRNA studied, the mean of these ratios in control samples was used to normalize the data.

Quantitative Autoradiography Binding Assay. After decapitation, the brains were quickly removed and immersed in methylbutane, cooled in an acetone-dry ice bath. The tissue was stored at −70°C in air-tight vials until the time of slide preparation. Parasagittal slices, 10-μm thick, were cut in a cryostat microtome (−14°C) 2.5 to 3.0 mm lateral from the midline (essentially at the level of the substantia nigra). Each slice was thaw-mounted onto a gelatin-coated slide (0.5% gelatin/0.05% chrome alum) and then transferred to ice-cold slide boxes and stored at −70°C until the time of binding assay.

For [^{3}H]benzodiazepine binding, slide-mounted brain slices were preincubated in 0.17 M Tris-HCl buffer (pH 7.4) at 4°C for 30 min. Slices were then incubated for 60 min at 4°C in 0.17 M Tris-HCl containing either 5 nM [^{3}H]FNP (85 Ci/mmol; New England Nuclear, Boston, MA) or 2 nM [^{3}H]flumazenil (87.0 Ci/mmol, New England Nuclear). The incubation was terminated by rapidly dipping the slides twice for 30 s in 0.17 M Tris-HCl buffer. Finally, the slides were quickly rinsed in cold distilled water and dried with a stream of cold air. Nonspecific binding was determined by incubating adjacent slices in the presence of the radiologand plus 1 μM clonazepam. The slides were exposed to tritium-sensitive film at room temperature for 2 weeks.

For [^{3}H]muscimol binding, slide-mounted sections were preincu-

bated twice for 15 min in 50 mM Tris-acetate buffer (pH 7.1) at 4°C. Slides were then incubated in a solution containing 50 mM Tris-acetate buffer and 5 nM [^{3}H]muscimol (19.1 Ci/mmol; New England Nuclear) for 60 min at 4°C. Nonspecific binding was determined by incubating adjacent sections in the presence of [^{3}H]muscimol and 100 μM GABA. Incubation was terminated by washing the slices twice (30 s) in Tris-acetate buffer (4°C), followed by a brief rinse in a 2.5% gluteraldehyde/acetone (v/v) solution at 4°C. The slides were placed in film cassettes and exposed to tritium-sensitive film at 4°C for 6 week.

Ligand binding was quantified with computer-assisted densitometry via NIH Image software. Tritium standards (courtesy of Dr. E.I. Tietz) were 10 disks of rat brain paste containing known amounts of [^{3}H]thymidine that had been cut on a microtome, mounted on a single slide, and fixed with paraformaldehyde vapor. For each region, ligand binding was determined by converting optical density measurement to picomoles per milligram protein (Tietz et al., 1986). For each rat brain, 33 different regions were measured on each of four sagittal sections, and the mean of the four values was used. Specific binding was the difference between total and nonspecific binding.

Homogenate Binding Assay. Immediately after decapitation, cerebral cortices were collected and stored at −70°C until the time of binding assay. Cerebral cortical tissues were prepared according to the method of Tietz et al. (1989). Tissue was homogenized in 15 vol of 0.32 M sucrose. The homogenates were centrifuged at 1000*g* for 10 min at 4°C. The supernatant was collected and then re-centrifuged at 20,000*g* for 20 min at 4°C. The resulting pellet was frozen overnight at −70°C. The following day, the pellet was thawed and then refrozen again for 30 min. After the freeze/thaw cycle, the pellet was lysed for 30 min in ice-cold 5 mM Tris-HCl (pH 7.4) and then recentrifuged 20 min at 20,000*g* (4°C). The resulting pellet was washed three times by resuspension in 20 vol 50 mM Tris-HCl at 4°C and centrifuged at 20,000*g* for 20 min. The final pellets were resuspended in 5 ml 50 mM Tris-HCl buffer (pH 7.4). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard.

Binding was performed by incubating 0.4 mg/ml protein with either no GABA or increasing concentrations of GABA (10^{-8}–10^{-4} M) plus 0.5 nM [^{3}H]FNP in 50 mM Tris-HCl buffer (pH 7.4) for 60 min at 4°C. Basal [^{3}H]FNP binding (no GABA added) and nonspecific binding (in the presence of 1 μM clonazepam) were done in triplicate; [^{3}H]FNP binding in the presence of each GABA concentration was done in duplicate. Incubation was terminated by rapidly adding 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) to the glass tube and rapid filtration. The filters were rinsed two more times with 5 ml of ice-cold 50 mM Tris-HCl buffer and were allowed to equilibrate overnight in CytoScint scintillation fluid (ICN, Costa Mesa, CA) before the radioactivity was counted.

Statistical Analysis. Results of autoradiographic binding assays were evaluated by two-way ANOVA, with treatment and brain region as independent variables. Homogenate binding assays were also evaluated by two-way ANOVA, with treatment and GABA concentrations as the independent variables. The basal [^{3}H]FNP binding (i.e., with no GABA added to the reaction vial) was evaluated by two-tailed Student's *t* test. For mRNA determinations, the effect of PTZ treatment on each subunit mRNA was analyzed with Student's *t* test. In all cases, $P < .05$ was considered to be statistically significant.

Results

Administration of PTZ (45 mg/kg i.p.) induced clonic convulsions in almost all of the animals after a single injection, typically within 3 min. However, 11% of animals needed a second injection to cause clonus. Each rat showed jerking or myoclonus followed by clonus or tonic-clonic convulsions. Two rats had lethal convulsions and were not used for this study. Preliminary evaluation of the results showed that data from the rats that needed a second PTZ injection did not differ from those of the other PTZ-treated rats, so the data were subsequently grouped together.

The effect of PTZ convulsions on $GABA_A$ receptors was examined by measuring mRNA levels for selected subunits 4, 24, and 48 h after injection of PTZ or saline. The recovery of mRNA, as judged by the A_{260} of the eluates, did not differ between treated and control samples or among the samples from the three time points in any of the three brain regions (data not shown). The $GABA_A$ receptor subunit oligodeoxynucleotide and β-actin cDNA probes labeled RNA species of the expected sizes and produced autoradiographs virtually identical to those reported previously from this laboratory (Wu et al., 1994; Zhao et al., 1994a,b). As shown in Fig. 1, PTZ treatment had no effect on the level of β-actin mRNA in cerebral cortex, cerebellum, or hippocampus.

PTZ injection resulted in significant changes in $GABA_A$ receptor subunit mRNAs (Table 1). At 4 h after PTZ injection, there was a 39% decrease in the level of α_1 subunit mRNA in cerebellum. However, there was no change in α_1 subunit mRNA in cerebral cortex or hippocampus. The time course of changes in cerebellar α_1 subunit mRNA was studied, and it was found that, in contrast to the decrease at 4 h, there was a significant 16% increase 24 h after the PTZ injection but no difference between control and treated cerebellum 48 h after PTZ (control, 1.00 ± 0.02; treated, 0.93 ± 0.04). Although there appeared to be a trend toward a decrease in α_5 subunit mRNA, statistical analysis revealed no significant effect on the level of α_5 subunit mRNA 4 h after PTZ injection in cerebral cortex or in hippocampus. The mRNA for the α_5 subunit was not detected in cerebellar tissue from either control or PTZ-treated rats. The level of β_2 subunit mRNA was significantly decreased in cortex and cerebellum 4 h after PTZ injection. There was a trend toward a decrease in hippocampus, but this was not statistically significant. By 24 h after PTZ treatment, the level of β_2 subunit mRNA had increased in cortex and in cerebellum, but the difference reached statistical significance only in cerebellum. There was no longer any effect of PTZ treatment at 48 h. A significant decrease in γ_2 subunit mRNA was detectable 4 h after PTZ injection in all three brain regions. This was followed by a

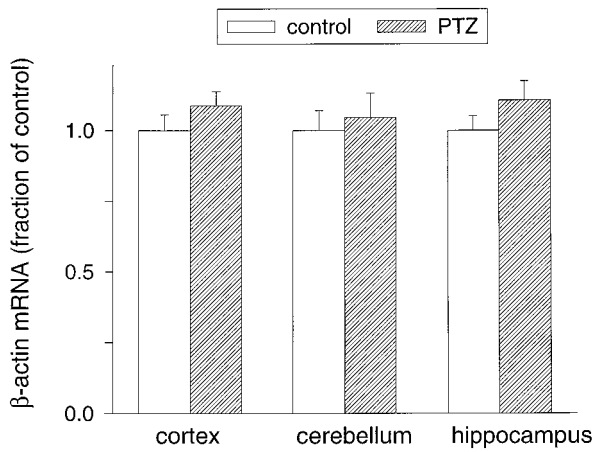

Fig. 1. PTZ pretreatment had no effect on the level of β-actin mRNA in the three brain areas studied. Bars, means and S.E.M.; n = 9–12.

TABLE 1
Effects of PTZ convulsions on $GABA_A$ receptor mRNA
Values are amount of mRNA relative to controls. n = 9–11.

	$GABA_A$ Receptor Subunits			
	α_1	α_5	β_2	γ_2
Cerebral cortex				
4 h after PTZ	0.99 ± 0.06	0.95 ± 0.03	0.76 ± 0.04[a]	0.72 ± 0.04[a]
24 h after PTZ	0.99 ± 0.06		1.28 ± 0.14	1.20 ± 0.10[a]
Cerebellum				
4 h after PTZ	0.61 ± 0.13[a]		0.70 ± 0.03[a]	0.78 ± 0.03[a]
24 h after PTZ	1.16 ± 0.03[a]		1.25 ± 0.06[a]	1.22 ± 0.05[a]
Hippocampus				
4 h after PTZ	0.99 ± 0.06	0.95 ± 0.03	0.91 ± 0.05	0.87 ± 0.03[a]
24 h after PTZ				0.95 ± 0.03

[a] $P < .05$.

significant increase (22%) in cerebellum at 24 h, which had reversed by 48 h after PTZ. A similar rebound appeared to occur in cerebral cortex, but this was not statistically significant. In hippocampus, the effect of PTZ on γ_2 mRNA was no longer present by 24 h after PTZ injection.

Figure 2A is a representative autoradiograph of a sagittal brain section from a control rat labeled with 5 nM [^{3}H]FNP. [^{3}H]FNP-binding density and distribution were similar to those reported previously (Tietz et al., 1986). In rats that had received PTZ 12 h before tissue collection, there was a widespread decrease in [^{3}H]FNP binding throughout the brain sections (Fig. 3). Statistical analysis revealed a significant PTZ effect on [^{3}H]FNP binding (F = 63; df = 1; $P < .001$) and a significant difference among brain areas (F = 11; df = 32; $P < .001$) but no significant interaction between brain region and treatment (F = 0.12; df = 32). Note that [^{3}H]FNP-binding density in the cerebral cortical areas was measured in three layers (corresponding to laminae I–III, VI, and V and VI), and the individual values were included in the statistical analysis. However, because the reduction in binding was similar (i.e., 15–20%) in each layer and to simplify the data presentation, the values presented in the figures are the average of [^{3}H]FNP binding across the layers in each of the various regions of the cerebral cortex indicated. Analysis of autoradiographs 24 h after PTZ treatment suggested a trend toward increased [^{3}H]FNP binding in the hippocampal and cerebellar regions (Fig. 4). However, there was no significant treatment effect (F = 0.64; df = 32) and no significant interaction between treatment and brain region (F = 0.36; df = 32).

The density and distribution of 5 nM [^{3}H]muscimol binding (Fig. 2B) were similar to those reported in previous studies (Olsen et al., 1990), with the highest level of binding found in the granule cell layer of the cerebellum. In contrast to the effect of PTZ on [^{3}H]FNP binding, PTZ had a lesser effect on [^{3}H]muscimol binding, with most areas showing little or no effect (Fig. 5). However, statistical analysis showed this small PTZ effect to be significant (F = 9.3; df = 1; $P < .01$). As expected, there was a significant effect of brain region (F = 340; df = 32; $P < .001$) but no significant interaction (F = 0.55; df = 32).

An example of the binding of 2 nM [^{3}H]flumazenil, the benzodiazepine antagonist, is shown in Fig. 2C. The distribution of [^{3}H]flumazenil binding was similar to that of the benzodiazepine agonist [^{3}H]FNP. However, in sharp contrast to the results with [^{3}H]FNP, there was no effect of PTZ treatment on [^{3}H]flumazenil binding (Fig. 6). Statistical analysis showed no PTZ treatment effect (F = 0.03; df = 1), a significant effect of brain region (F = 125; df = 32; $P < .001$), and no significant interaction between treatment and region (F = 0.56; df = 32).

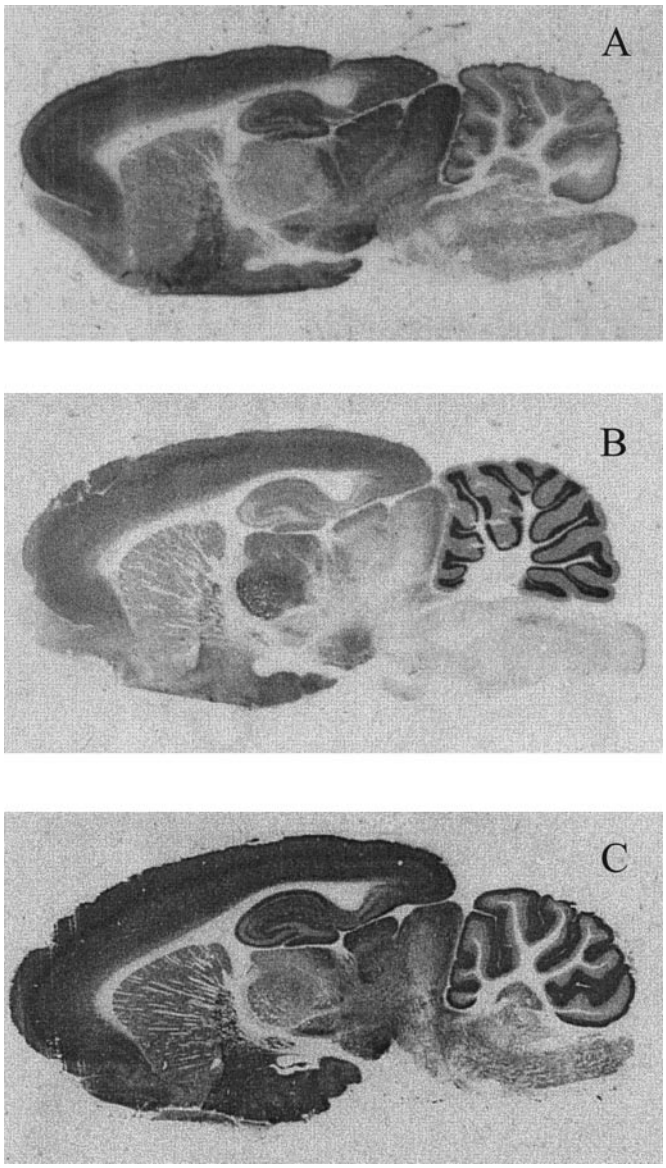

Fig. 2. Sample autoradiographs of radioligand binding to sagittal sections of rat brain showing binding of 5 nM [^{3}H]flunitrazepam (A), 5 nM [^{3}H]muscimol (B), and 2 nM [^{3}H]flumazenil (C).

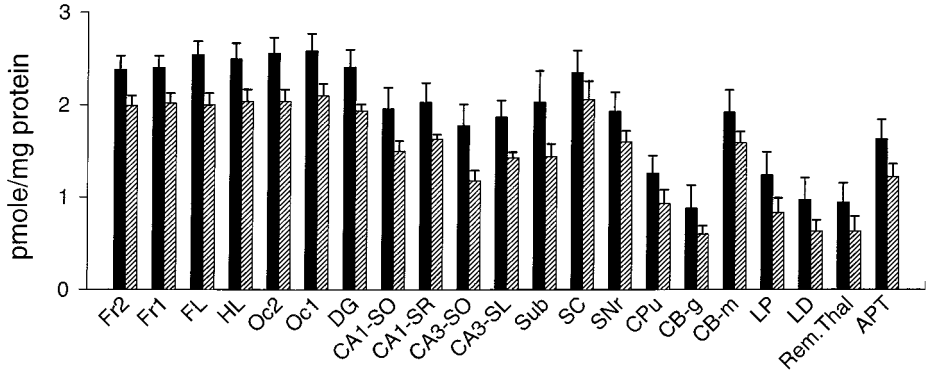

Fig. 3. Specific binding of 5 nM [^{3}H]flunitrazepam to brain sections 12 h after saline (solid bars) or PTZ injection (hatched bars). Each bar shows mean and S.E.M. of values from 5 rats. Fr2, frontal cortex, area 2; Fr1, frontal cortex, area 1; FL, forelimb area of motor cortex; HL, hindlimb area of cortex; Oc2, occipital cortex, area 2; Oc1, occipital cortex, area 1; DG, dentate gyrus; CA1-SO, hippocampal CA1 region, stratum oriens; CA1-SR, CA1, stratum radiatum; CA3-SO, CA3, stratum oriens; CA3-SL, CA3, stratum lucidum; sub, subiculum; SC, superior colliculus, SNr, substantia nigra, pars reticulata; CPu, caudate-putamen; CB-g, granule cell layer of cerebellum; CB-m, molecular layer of cerebellum; LP, lateroposterior thalamic nucleus; LD, laterodorsal thalamic nucleus; Rem.Thal., remaining thalamic areas; APT, anterior pretectal nucleus. There was a significant effect of PTZ and brain region but no significant interaction.

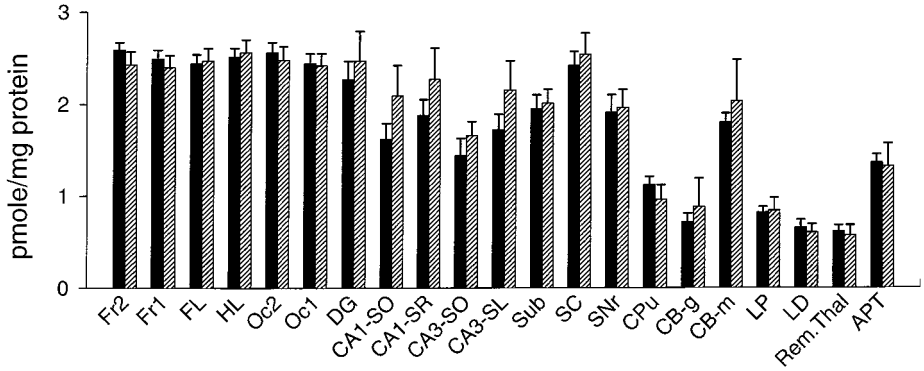

Fig. 4. Specific binding of 5 nM [^{3}H]flunitrazepam to brain sections 24 h after saline (solid bars) or PTZ (hatched bars) injection. Abbreviations as in Fig. 3. There was no significant PTZ effect.

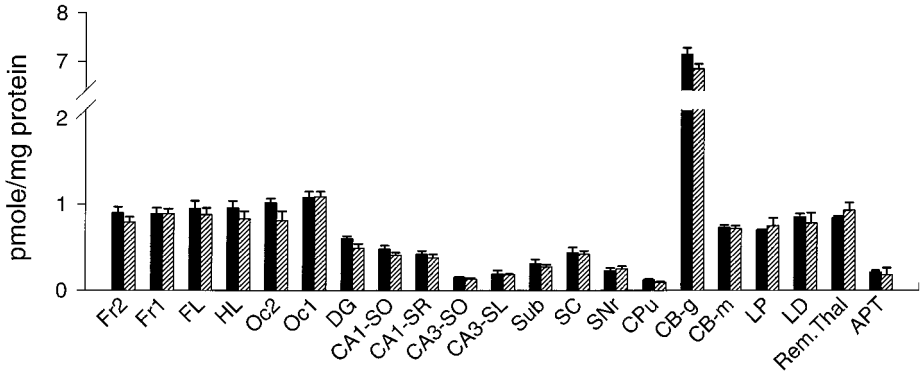

Fig. 5. Specific binding of 5 nM [^{3}H]muscimol to brain sections 12 h after saline (solid bars) or PTZ (hatched bars) injection. Abbreviations as in Fig. 3. There was a significant effect of PTZ and brain region but no significant interaction.

To reconcile the differing results with [^{3}H]FNP and [^{3}H]flumazenil, we examined [^{3}H]FNP binding with the well-washed membrane homogenate preparation in the absence and presence of added GABA. Without GABA, there was no difference (t test, $P = .5$, $n = 5$) in specific 0.5 nM [^{3}H]FNP binding to well washed cerebral cortical homogenates from control (386.8 ± 12.5 fmol/mg protein) and PTZ-treated (381.2 ± 11.4 fmol/mg protein) rats. As expected, there was concentration-dependent stimulation of specific [^{3}H]FNP binding by GABA, but this was less pronounced in brain homogenates taken from rats 12 h after the PTZ seizure (Fig. 7). Two-way ANOVA revealed a significant effect of GABA concentration ($F = 10$; df = 4; $P < .001$) and PTZ treatment ($F = 15$; df = 1; $P < .001$) but no significant interaction ($F = 0.70$; df = 4).

Discussion

The results of our study indicate that a single PTZ-induced convulsion is associated with rapid changes in $GABA_A$ receptors. These changes involve transient decreases and subsequent increases in some subunit mRNAs and a decrease in the coupling between the GABA and benzodiazepine recognition sites in the receptor.

mRNA determinations showed that a single PTZ convulsion has the potential to alter $GABA_A$ receptor expression for at least 24 h. The mRNAs for several $GABA_A$ receptor subunits were decreased at 4 h after PTZ. There was a rebound increase in some of these 24 h after PTZ injection and apparent normalization of mRNA levels by 48 h. Because the amount of poly(A)$^+$ RNA in the extracts and the density of

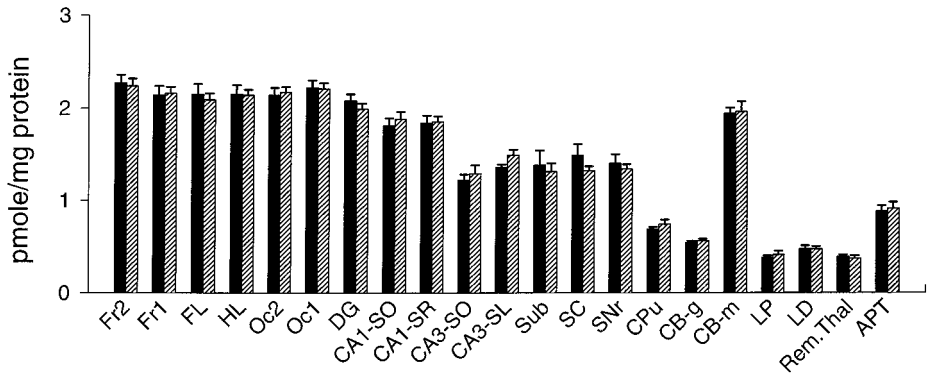

Fig. 6. Specific binding of 2 nM [^{3}H]flumazenil to brain sections 12 h after saline (solid bars) or PTZ (hatched bars) injection. Abbreviations as in Fig. 3. There was no significant PTZ effect.

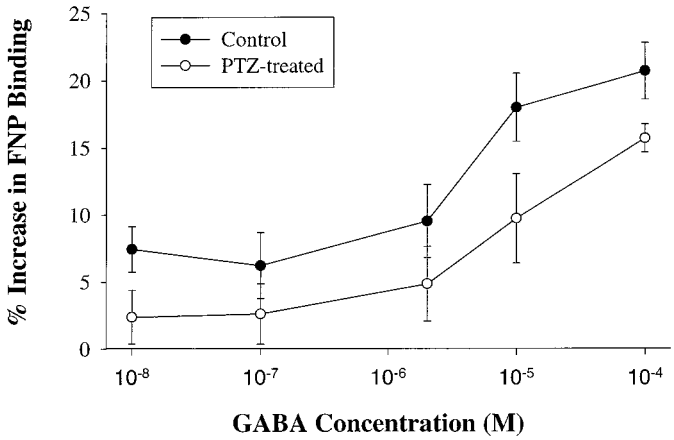

Fig. 7. Effect of GABA on specific binding of 0.5 nM [^{3}H]flunitrazepam in well-washed cerebral cortical homogenates. Each point is mean ± S.E.M. of values from 5 rats. Baseline [^{3}H]flunitrazepam binding (e.g., in absence of added GABA) was not affected by PTZ treatment. There was a significant effect of PTZ and GABA concentration but no significant interaction.

the β-actin bands did not differ between PTZ-treated and saline control rats, the results were probably not due to a generalized or nonspecific effect of PTZ or of the seizure. The observations that not all $GABA_A$ receptor subunit mRNAs were decreased and that the changes differed according to brain region also argue against a nonspecific effect. In contrast to the other subunit mRNAs studied, α_5-mRNA was not affected. In general, subunit mRNAs that decreased 4 h after PTZ injection did so to roughly to the same degree, but there were obvious differences among brain regions. The cerebellum appeared to be most susceptible, because α_1, β_2, and γ_2 were all decreased at 4 h and then significantly increased at 24 h. In cerebral cortical tissue, β_2 and γ_2 mRNA decreased about as much as in cerebellum, but the trend toward rebound at 24 h did not reach significance, and α_1 subunit mRNA did not change. The hippocampus was the most resistant region studied, with a decrease at 4 h only for γ_2 subunit mRNA. The methods used would not detect changes localized to small areas.

Previous studies have noted changes in mRNAs for $GABA_A$ receptor subunits and other receptor proteins after experimental seizures. For example, kainate-induced status epilepticus was associated with decreased mRNA for glutamate receptor GluR2 and GluR3 subunits and for the $GABA_A$ receptor α_1 subunit in hippocampal CA3/CA4 region but an increase in glutamate receptor mRNAs and no change in the $GABA_A$ α_1 mRNA in the dentate gyrus (Friedman et al., 1994). In rats that had been kindled by electrical stimulation of the Schaffer collateral/commissural fiber pathway, [^{3}H]muscimol binding and $GABA_A$ receptor β subunit mRNAs were altered differentially in various hippocampal fields (Kamphuis et al., 1994; Titulaer et al., 1994). Considering these types of observations and our findings of regional differences even when comparing large brain areas, it is reasonable to suspect that localized changes in the expression of $GABA_A$ receptor mRNA are present after PTZ seizures. Regional differences indicate that the changes in $GABA_A$ receptor mRNA levels after PTZ may be determined at least in part by regional differences in $GABA_A$ receptor subunit composition and/or neuronal function. $GABA_A$ receptor mRNAs may also change according to the experimental seizure used. Unlike our results for PTZ seizures, electroshock seizures in mice produced no change in the levels of α_1, α_2, β_2, β_3, γ_1, or γ_2 subunit mRNAs in cerebral cortex or hippocampus but did produce an increase in cerebellar levels of α_1 and β_2 subunit mRNAs at 4 and 8 (but not 2 or 24) h after the seizure (Pratt et al., 1993).

Transient variations in $GABA_A$ receptor mRNAs after single seizures may be associated with alterations in $GABA_A$ receptor number or function. The decreases in $GABA_A$ receptor mRNAs seen after PTZ, if maintained long enough, could lead to decreased numbers of $GABA_A$ receptors and a reduction in GABAergic inhibition. In one study, two PTZ-induced tonic-clonic seizures over 2 days did produce a decrease in hippocampal $GABA_A$ binding sites accompanied by a decrease in GABAergic inhibition (Psarropoulou et al., 1994). More prolonged PTZ treatment to produce kindling was also associated with a decrease in [^{3}H]GABA and [^{35}S]TBPS binding and in GABA-stimulated $^{36}Cl^-$ uptake (Corda et al., 1990, 1992). However, the results of our study suggest that, although a single PTZ convulsion did affect $GABA_A$ receptor mRNA levels and may have affected receptor function, the number of $GABA_A$ receptors was not altered.

In autoradiographic binding studies, the decrease in [^{3}H]FNP binding suggested that the reduced levels of $GABA_A$ receptor mRNA would result in reduced availability of receptors. However, there was no change in [^{3}H]flumazenil binding and minimal change in [^{3}H]muscimol binding. This suggested that the reduction in [^{3}H]FNP binding was not due to a loss in $GABA_A$ receptors or even to a general change in the affinity of the benzodiazepine recognition site. Rather, the results pointed to some change in receptors that would affect the benzodiazepine agonist [^{3}H]FNP but not the antagonist [^{3}H]flumazenil. One possibility involves the coupling between the GABA and benzodiazepine sites of the $GABA_A$

receptor. A manifestation of this is the ability of $GABA_A$ agonists to increase the binding affinity of benzodiazepine agonists (Tallman et al., 1978; Wastek et al., 1978; Chiu and Rosenberg, 1979) but not of the antagonist flumazenil (Möhler and Richards, 1981; Chiu and Rosenberg, 1983). GABA-benzodiazepine coupling was evaluated with a well washed homogenate preparation, in which GABA concentration is greatly reduced. In the absence of exogenous GABA, there was no difference in [^{3}H]FNP-specific binding. However, there was a decrease in GABA stimulation of [^{3}H]FNP binding, indicating that the acute PTZ injection had affected coupling between the GABA and benzodiazepine recognition sites of the $GABA_A$ receptor. Because the method used in the autoradiography binding assay would probably not have reduced GABA concentration below that affecting benzodiazepine binding (McCabe et al., 1988), reduced GABA-benzodiazepine coupling might be expected to reduce [^{3}H]FNP binding but have no effect on [^{3}H]flumazenil binding in the autoradiographic binding assay, although the rather high (5 nM) concentration of [^{3}H]FNP would have tended to reduce the magnitude of GABA stimulation. Another possibility, not evaluated in this study, involves compensatory changes in other subunits. For example, compared with receptors with the γ_2 subunit, receptors with γ_3 have substantially lower affinity for FNP but not flumazenil (Benke et al., 1996), so that a combined decrease in γ_2 and increase in γ_3 might produce the uncoupling reported above.

Mechanisms for GABA-benzodiazepine uncoupling after a PTZ convulsion might involve transcription of regulatory elements that interact with the $GABA_A$ receptor to alter its expression or posttranslational modifications or may involve more direct effects, for example, by regulating receptor phosphorylation. Acute PTZ seizure has been shown to cause a transient increase in c-*fos* and c-*jun* mRNA expression, with an increase in activator protein 1-binding sites, suggesting up-regulation of transcription factors (Saffen et al., 1988; Sonnenberg et al., 1989). This could provide a conceptual basis for postulating alterations in any number of neuronal components after seizure activity. Currently, not enough is known about the target genes of Fos and Jun proteins to determine the specific role they might play with respect to alterations in $GABA_A$ or other receptors. However, available information at least suggests that the excessive neural activity associated with a PTZ seizure could affect expression of regulatory elements somehow related to GABA-benzodiazepine coupling at the $GABA_A$ receptor.

One possibility is that GABA-benzodiazepine coupling is affected by the phosphorylation state of the receptor. $GABA_A$ receptor subunits contain consensus sequences for phosphorylation by various protein kinases and can be phosphorylated by them (McDonald and Moss, 1997). Altering receptor phosphorylation can modify $GABA_A$ receptor function (Leidenheimer et al., 1991; Moss et al., 1992) and may modify benzodiazepine potentiation of GABA-mediated currents (Leidenheimer et al., 1993). Several studies have indicated a relationship between seizure activity and phosphorylation. For example, the single-locus mutant mouse tottering, a model system for absence epilepsy, exhibits ataxia and myoclonic seizures. In microsacs prepared from the brain of these mice, there is an overall decrease in $GABA_A$ receptor function, as determined by $^{36}Cl^-$ flux, and large concentrations of intravesicular PKA, which may have suppressed $GABA_A$ receptor function by intracellular phosphorylation of the receptor subunits (Tehrani and Barnes, 1995). In another example, Chen (1994a,b) demonstrated that both electroshock seizure and convulsive doses of PTZ can increase activity of the PKC γ isoform. Although it is not as yet known whether such an increase in PKC activity would indeed phosphorylate $GABA_A$ receptors after a PTZ convulsion, there is sufficient information to hypothesize that increased protein kinase activity after seizure activity may phosphorylate substrates, including the $GABA_A$ receptors, and subsequently alter GABAergic function. Furthermore, the rapid rise in PKC activity could also be associated with expression of Fos and Jun (Karin et al., 1997), providing a possible link between c-*fos* and c-*jun* gene expression and phosphorylation. Further studies examining the phosphorylation of $GABA_A$ receptor subunits after seizure activity and the functional consequences may reveal a role for phosphorylation in the changes in $GABA_A$ receptor function associated with seizure activity.

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Acute Pentylenetetrazol Injection Reduces Rat $GABA_A$ Receptor mRNA Levels and GABA Stimulation of Benzodiazepine Binding with No Effect on Benzodiazepine Binding Site Density[1]

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ABSTRACT

The effects of a single convulsive dose of pentylenetetrazol (PTZ, 45 mg/kg i.p.) on rat brain γ-aminobutyric acid type A ($GABA_A$) receptors were studied. Selected $GABA_A$ receptor subunit mRNAs were measured by Northern blot analysis (with β-actin mRNA as a standard). Four hours after PTZ, the $GABA_A$ receptor γ_2-mRNA was decreased in hippocampus, cerebral cortex, and cerebellum; α_1-mRNA was decreased in cerebellum; and β_2 subunit mRNA was decreased in cortex and cerebellum. The α_5 subunit mRNA level was not altered. Those mRNAs that had been reduced were increased in some brain regions at the 24-h time point, and these changes reverted to control levels by 48 h. PTZ effect on $GABA_A$ receptors was also studied by autoradiographic binding assay with the benzodiazepine agonist [^{3}H]flunitrazepam (FNP), the $GABA_A$ agonist [^{3}H]muscimol, and the benzodiazepine antagonist [^{3}H]flumazenil. There was an overall decrease in [^{3}H]FNP binding 12 but not 24 h after PTZ treatment. In contrast, [^{3}H]muscimol binding was minimally affected, and [^{3}H]flumazenil binding was unchanged after PTZ treatment. Additional binding studies were performed with well-washed cerebral cortical homogenates to minimize the amount of endogenous GABA. There was no PTZ effect on specific [^{3}H]FNP binding. However, there was a significant reduction in the stimulation of [^{3}H]FNP binding by GABA. The results showed that an acute injection of PTZ caused transient changes in $GABA_A$ receptor mRNA levels without altering receptor number but affected the coupling mechanism between the GABA and benzodiazepine sites of the $GABA_A$ receptor.

γ-Aminobutyric acid (GABA) is the major neurotransmitter mediating fast inhibitory neurotransmission in the mammalian central nervous system. Activation of the $GABA_A$ receptor leads to opening of its intrinsic anion channel, with increased chloride conductance, typically resulting in an i.p.s.p. Several experimental models of epilepsy or increased seizure susceptibility have been shown to be associated with variation in $GABA_A$ receptor number or function (Löscher

and Schwark, 1985; Olsen et al., 1985; Yu et al., 1986; Spreafico et al., 1993). Conversely, the occurrence of seizure activity may affect $GABA_A$ receptors (Shin et al., 1985; Corda et al., 1990; Titulaer et al., 1994), and such changes could be involved in altered neural excitability after a seizure episode, such as postictal depression or kindling.

Pentylenetetrazol (PTZ) is a chemical convulsant frequently used in the study of seizures. Some studies have indicated that the pharmacological effect of PTZ is at least partly mediated by interactions with the anion channel of the $GABA_A$ receptor (Squires et al., 1984). Either single or repeated PTZ administration may modify $GABA_A$ receptor number or function. Corda et al. (1990) found that repeated injection of a moderate dose of PTZ (e.g., 30 mg/kg), which will produce kindling, has several neurochemical effects, including a decrease in [^{3}H]GABA and [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) binding and in GABA-stimulated $^{36}Cl^-$ uptake, although a single PTZ injection had no effect on [^{35}S]TBPS binding. Two injections of a larger dose, sufficient to cause full tonic-clonic seizures 48 and 24 h before assay, were found to produce a decrease in GABA-mediated inhibition and decreased binding to $GABA_A$ receptors in hippocampus (Psarropoulou et al., 1994). In contrast to these reports of decreased $GABA_A$ receptor number or function after repeated PTZ injection, one study reported that a single, acute tonic-clonic PTZ or electroshock seizure produced a dramatic increase in [^{3}H]diazepam binding within 30 min (Paul and Skolnick, 1978). A subsequent study in rats found that an acute convulsive dose of PTZ, administered 30 min before assay, had little effect on the binding of several ligands to various sites on the $GABA_A$ receptor in homogenates from seven regions of rat brain (Ito et al., 1986). Although there was an increase in the binding of [^{35}S]TBPS to striatal homogenates, there were no other changes in the binding of this ligand or of [^{3}H]flunitrazepam, [^{3}H]GABA, or [^{3}H]muscimol. Recent autoradiographic studies by Rocha et al. (1996) provided conflicting data in that a single, subconvulsive i.p. injection of PTZ produced a decrease in [^{3}H]FNP binding throughout the rat brain. Chronic PTZ administration also reduced benzodiazepine binding, and saturation analysis revealed a decrease in receptor number but not affinity. A saturation study for the effects of the single PTZ administration was not reported.

Another approach to evaluating the effect of seizure activity on $GABA_A$ receptor expression was used by Pratt et al. (1993), who reported that a single electroconvulsive shock seizure caused an increase in the mRNA levels for some of the $GABA_A$ receptor subunits in cerebellum between 4 and 8 h after the convulsion, but no change was evident as early as 2 h, and no changes were found in hippocampus or cerebral cortex. This showed that the level of mRNA may be a sensitive measure of the effect of seizure activity on expression of $GABA_A$ receptors and demonstrated the importance of the time interval between seizure activity and tissue collection for detecting changes in expression of $GABA_A$ receptors after seizure activity. In contrast to the results of electroshock seizure reported by Pratt et al. (1993), our preliminary studies indicated decreases in $GABA_A$ receptor mRNA after PTZ seizures.

This study was undertaken to investigate the effect of PTZ seizure activity on $GABA_A$ receptor expression by measuring mRNA levels and the binding of radioligands to the $GABA_A$ receptor at different time points after PTZ injection. Several subunit mRNAs were studied, including those in highest abundance and that contribute to receptors with benzodiazepine sites in the brain regions studied (α1, β2, and γ2) and in α5, which may be of interest in relation to altered hippocampal function after PTZ seizures (Psarropoulou et al., 1994). Studying the cerebral cortex, cerebellum, and hippocampus might show whether the $GABA_A$ receptor subunit mRNAs are regulated differently by brain region. Binding assays were performed with quantitative autoradiographic methods for binding of [^{3}H]FNP, a benzodiazepine agonist, and [^{3}H]muscimol, a $GABA_A$ agonist. The results suggested additional studies with [^{3}H]flumazenil, a benzodiazepine antagonist, and an evaluation of GABA-stimulated [^{3}H]FNP binding in a well-washed tissue homogenate preparation.

Materials and Methods

PTZ Treatment. Male Sprague-Dawley rats (240–300 g, Harlan, Indianapolis, IN) were injected with 45 mg/kg PTZ i.p., in a volume of 1 ml/kg, to produce clonic convulsions. Injections were given between 8 and 9 AM. Rats that did not exhibit clonus (11%) were given a second injection 1 h after the first injection. Control rats were injected with 1 ml/kg physiological saline. For mRNA measurements, brains were collected 4 h after i.p. injection of PTZ or saline (or 3 h after a second PTZ injection, if required). In a second experiment, the interval was 24 or 48 h after PTZ or saline injection. In this second experiment, half of the saline-injected controls were assigned to each time point, and their data were analyzed as a group. For autoradiographic binding experiments, brains were collected 12 or 24 h after treatment. The time interval was chosen based on the preliminary results showing a decrease in $GABA_A$ receptor mRNA 4 h after PTZ (described below). It was expected that, with 12- and 24-h intervals, there would be time for translation and turnover of previously synthesized receptors, based on the reported turnover rate of benzodiazepine receptors in primary cultured neurons (Borden et al., 1984).

mRNA Isolation and Measurement. Oligodeoxynucleotide probes for $GABA_A$ receptor α_1 , α_5, β_2, and γ_2 subunits were synthesized by Oligos Etc., Inc. (Wilsonville, OR). The sequences of the probes and the preparation of the cDNA probe for β-actin were as reported previously (Wu et al., 1994; Zhao et al., 1994a,b). Oligodeoxynucleotide probes were labeled by 3′-tailing, and the cDNA probe was randomly labeled with [^{32}P]ATP.

Rats were decapitated and brains were quickly removed. Cerebral cortex, hippocampus, and cerebellum were isolated, frozen with liquid nitrogen, and stored at −70°C. With the same methods as in previous work (Wu et al., 1994; Zhao et al., 1994a, b), RNA was isolated with the proteinase K technique, enriched in poly(A)$^+$ RNA, separated by electrophoresis, transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), and immobilized.

Hybridization was performed under conditions previously reported (Zhao et al., 1994a,b). The Northern blots were prehybridized at 42°C for 4 h in 5× Denhardt's solution, 50% formamide, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA, 1 mM EDTA, at pH 8.0, and 2 × standard saline citrate. The appropriate ^{32}P-labeled probes ($2 \times 10^{[minus]7}$ cpm) were added, and the hybridization was carried out for 18 to 20 h at 42°C. After posthybridization washes, the membranes were air dried and exposed to X-ray film (Kodak XAR; Kodak, Rochester, NY) at −70°C with intensifying screens. The intensities of the bands were determined with a Bio-Rad imaging densitometer (model GS-670; Bio-Rad, Richmond, CA). The membrane was stripped, as described previously (Zhao et al., 1994a), and reprobed several times for different subunits.

To evaluate the possibility that PTZ treatment might have affected β-actin, the relative densities of the bands from control sam-

ples on each membrane were averaged, and all samples were normalized to this value. The density of each $GABA_A$ receptor subunit mRNA band was expressed relative to its corresponding β-actin band (measured on the same blots). For each mRNA studied, the mean of these ratios in control samples was used to normalize the data.

Quantitative Autoradiography-Binding Assay. After decapitation, the brains were quickly removed and immersed in methylbutane, cooled in an acetone-dry ice bath. The tissue was stored at −70°C in air-tight vials until the time of slide preparation. Parasagittal slices, 10-μm thick, were cut in a cryostat microtome (−14°C) 2.5 to 3.0 mm lateral from the midline (essentially at the level of the substantia nigra). Each slice was thaw-mounted onto a gelatin-coated slide (0.5% gelatin/0.05% chrome alum) and then transferred to ice-cold slide boxes and stored at −70°C until the time of binding assay.

For [^{3}H]benzodiazepine binding, slide-mounted brain slices were preincubated in 0.17 M Tris-HCl buffer (pH 7.4) at 4°C for 30 min. Slices were then incubated for 60 min at 4°C in 0.17 M Tris-HCl containing either 5 nM [^{3}H]FNP (85 Ci/mmol; New England Nuclear, Boston, MA) or 2 nM [^{3}H]flumazenil (87.0 Ci/mmol, New England Nuclear). The incubation was terminated by rapidly dipping the slides twice for 30 s in 0.17 M Tris-HCl buffer. Finally, the slides were quickly rinsed in cold distilled water and dried with a stream of cold air. Nonspecific binding was determined by incubating adjacent slices in the presence of the radiologand plus 1 μM clonazepam. The slides were exposed to tritium-sensitive film at room temperature for 2 weeks.

For [^{3}H]muscimol binding, slide-mounted sections were preincubated twice for 15 min in 50 mM Tris-acetate buffer (pH 7.1) at 4°C. Slides were then incubated in a solution containing 50 mM Tris-acetate buffer and 5 nM [^{3}H]muscimol (19.1 Ci/mmol; New England Nuclear) for 60 min at 4°C. Nonspecific binding was determined by incubating adjacent sections in the presence of [^{3}H]muscimol and 100 μM GABA. Incubation was terminated by washing the slices twice (30 s) in Tris-acetate buffer (4°C), followed by a brief rinse in a 2.5% gluteraldehyde/acetone (v/v) solution at 4°C. The slides were placed in film cassettes and exposed to tritium-sensitive film at 4°C for 6 week.

Ligand binding was quantified with computer-assisted densitometry via NIH Image software. Tritium standards (courtesy of Dr. E.I. Tietz) were 10 disks of rat brain paste containing known amounts of [^{3}H]thymidine that had been cut on a microtome, mounted on a single slide, and fixed with paraformaldehyde vapor. For each region, ligand binding was determined by converting optical density measurement to picomoles per milligram protein (Tietz et al., 1986). For each rat brain, 33 different regions were measured on each of four sagittal sections, and the mean of the four values was used. Specific binding was the difference between total and nonspecific binding.

Homogenate-Binding Assay. Immediately after decapitation, cerebral cortices were collected and stored at −70°C until the time of binding assay. Cerebral cortical tissues were prepared according to the method of Tietz et al. (1989). Tissue was homogenized in 15 vol of 0.32 M sucrose. The homogenates were centrifuged at 1000*g* for 10 min at 4°C. The supernatant was collected and then re-centrifuged at 20,000*g* for 20 min at 4°C. The resulting pellet was frozen overnight at −70°C. The following day, the pellet was thawed and then refrozen again for 30 min. After the freeze/thaw cycle, the pellet was lysed for 30 min in ice-cold 5 mM Tris-HCl (pH 7.4) and then recentrifuged 20 min at 20,000*g* (4°C). The resulting pellet was washed three times by resuspension in 20 vol 50 mM Tris-HCl at 4°C and centrifuged at 20,000*g* for 20 min. The final pellets were resuspended in 5 ml 50 mM Tris-HCl buffer (pH 7.4). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard.

Binding was performed by incubating 0.4 mg/ml protein with either no GABA or increasing concentrations of GABA (10^{-8}–10^{-4} M) plus 0.5 nM [^{3}H]FNP in 50 mM Tris-HCl buffer (pH 7.4) for 60 min at 4°C. Basal [^{3}H]FNP binding (no GABA added) and nonspecific binding (in the presence of 1 μM clonazepam) were done in triplicate; [^{3}H]FNP binding in the presence of each GABA concentration was done in duplicate. Incubation was terminated by rapidly adding 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) to the glass tube and rapid filtration. The filters were rinsed two more times with 5 ml of ice-cold 50 mM Tris-HCl buffer and were allowed to equilibrate overnight in CytoScint scintillation fluid (ICN, Costa Mesa, CA) before the radioactivity was counted.

Statistical Analysis. Results of autoradiographic binding assays were evaluated by two-way ANOVA, with treatment and brain region as independent variables. Homogenate binding assays were also evaluated by two-way ANOVA, with treatment and GABA concentrations as the independent variables. The basal [^{3}H]FNP binding (i.e., with no GABA added to the reaction vial) was evaluated by two-tailed Student's *t* test. For mRNA determinations, the effect of PTZ treatment on each subunit mRNA was analyzed with Student's *t* test. In all cases, $P < .05$ was considered to be statistically significant.

Results

Administration of PTZ (45 mg/kg i.p.) induced clonic convulsions in almost all of the animals after a single injection, typically within 3 min. However, 11% of animals needed a second injection to cause clonus. Each rat showed jerking or myoclonus followed by clonus or tonic-clonic convulsions. Two rats had lethal convulsions and were not used for this study. Preliminary evaluation of the results showed that data from the rats that needed a second PTZ injection did not differ from those of the other PTZ-treated rats, so the data were subsequently grouped together.

The effect of PTZ convulsions on $GABA_A$ receptors was examined by measuring mRNA levels for selected subunits 4, 24, and 48 h after injection of PTZ or saline. The recovery of mRNA, as judged by the A_{260} of the eluates, did not differ between treated and control samples or among the samples from the three time points in any of the three brain regions (data not shown). The $GABA_A$ receptor subunit oligodeoxynucleotide and β-actin cDNA probes labeled RNA species of the expected sizes and produced autoradiographs virtually identical to those reported previously from this laboratory (Wu et al., 1994; Zhao et al., 1994a,b). As shown in Fig. 1, PTZ treatment had no effect on the level of β-actin mRNA in cerebral cortex, cerebellum, or hippocampus.

PTZ injection resulted in significant changes in $GABA_A$ receptor subunit mRNAs (Table 1). At 4 h after PTZ injection, there was a 39% decrease in the level of α_1 subunit mRNA in cerebellum. However, there was no change in α_1 subunit mRNA in cerebral cortex or hippocampus. The time course of changes in cerebellar α_1 subunit mRNA was studied, and it was found that, in contrast to the decrease at 4 h, there was a significant 16% increase 24 h after the PTZ injection but no difference between control and treated cerebellum 48 h after PTZ (control, 1.00 ± 0.02; treated, 0.93 ± 0.04). Although there appeared to be a trend toward a decrease in α_5 subunit mRNA, statistical analysis revealed no significant effect on the level of α_5 subunit mRNA 4 h after PTZ injection in cerebral cortex or in hippocampus. The mRNA for the α_5 subunit was not detected in cerebellar tissue from either control or PTZ-treated rats. The level of β_2 subunit mRNA was significantly decreased in cortex and cerebellum 4 h after PTZ injection. There was a trend toward a decrease in hippocampus, but this was not statistically significant. By

24 h after PTZ treatment, the level of β_2 subunit mRNA had increased in cortex and in cerebellum, but the difference reached statistical significance only in cerebellum. There was no longer any effect of PTZ treatment at 48 h. A significant decrease in γ_2 subunit mRNA was detectable 4 h after PTZ injection in all three brain regions. This was followed by a significant increase (22%) in cerebellum at 24 h, which had reversed by 48 h after PTZ. A similar rebound appeared to occur in cerebral cortex, but this was not statistically significant. In hippocampus, the effect of PTZ on γ_2 mRNA was no longer present by 24 h after PTZ injection.

Figure 2A is a representative autoradiograph of a sagittal brain section from a control rat labeled with 5 nM [^{3}H]FNP. [^{3}H]FNP-binding density and distribution were similar to those reported previously (Tietz et al., 1986). In rats that had received PTZ 12 h before tissue collection, there was a widespread decrease in [^{3}H]FNP binding throughout the brain sections (Fig. 3). Statistical analysis revealed a significant PTZ effect on [^{3}H]FNP binding ($F = 63$; df = 1; $P < .001$) and a significant difference among brain areas ($F = 11$; df = 32; $P < .001$) but no significant interaction between brain region and treatment ($F = 0.12$; df = 32). Note that [^{3}H]FNP-binding density in the cerebral cortical areas was measured in three layers (corresponding to laminae I–III, VI, and V and VI), and the individual values were included in the statistical analysis. However, because the reduction in binding was similar (i.e., 15–20%) in each layer and to simplify the data presentation, the values presented in the figures are the average of [^{3}H]FNP binding across the layers in each of the various regions of the cerebral cortex indicated. Analysis of autoradiographs 24 h after PTZ treatment suggested a trend toward increased [^{3}H]FNP binding in the hippocampal and cerebellar regions (Fig. 4). However, there was no significant treatment effect ($F = 0.64$; df = 32) and no significant interaction between treatment and brain region ($F = 0.36$; df = 32).

The density and distribution of 5 nM [^{3}H]muscimol binding (Fig. 2B) were similar to those reported in previous studies (Olsen et al., 1990), with the highest level of binding found in the granule cell layer of the cerebellum. In contrast to the effect of PTZ on [^{3}H]FNP binding, PTZ had a lesser effect on [^{3}H]muscimol binding, with most areas showing little or no effect (Fig. 5). However, statistical analysis showed this small PTZ effect to be significant ($F = 9.3$; df = 1; $P < .01$). As expected, there was a significant effect of brain region ($F = 340$; df = 32; $P < .001$) but no significant interaction ($F = 0.55$; df = 32).

An example of the binding of 2 nM [^{3}H]flumazenil, the benzodiazepine antagonist, is shown in Fig. 2C. The distribution of [^{3}H]flumazenil binding was similar to that of the benzodiazepine agonist [^{3}H]FNP. However, in sharp contrast to the results with [^{3}H]FNP, there was no effect of PTZ treatment on [^{3}H]flumazenil binding (Fig. 6). Statistical analysis showed no PTZ treatment effect ($F = 0.03$; df = 1), a significant effect of brain region ($F = 125$; df = 32; $P < .001$), and no significant interaction between treatment and region ($F = 0.56$; df = 32).

To reconcile the differing results with [^{3}H]FNP and [^{3}H]flumazenil, we examined [^{3}H]FNP binding with the well-washed membrane homogenate preparation in the absence and presence of added GABA. Without GABA, there was no difference (t test, $P = .5$, $n = 5$) in specific 0.5 nM [^{3}H]FNP binding to well washed cerebral cortical homogenates from control (386.8 ± 12.5 fmol/mg protein) and PTZ-treated (381.2 ± 11.4 fmol/mg protein) rats. As expected, there was concentration-dependent stimulation of specific [^{3}H]FNP binding by GABA, but this was less pronounced in brain homogenates taken from rats 12 h after the PTZ seizure (Fig. 7). Two-way ANOVA revealed a significant effect of GABA concentration ($F = 10$; df = 4; $P < .001$) and PTZ treatment ($F = 15$; df = 1; $P < .001$) but no significant interaction ($F = 0.70$; df = 4).

Discussion

The results of our study indicate that a single PTZ-induced convulsion is associated with rapid changes in $GABA_A$ receptors. These changes involve transient decreases and subsequent increases in some subunit mRNAs and a decrease in the coupling between the GABA and benzodiazepine recognition sites in the receptor.

mRNA determinations showed that a single PTZ convulsion has the potential to alter $GABA_A$ receptor expression for at least 24 h. The mRNAs for several $GABA_A$ receptor subunits were decreased at 4 h after PTZ. There was a rebound increase in some of these 24 h after PTZ injection and apparent normalization of mRNA levels by 48 h. Because the amount of poly(A)$^+$ RNA in the extracts and the density of the β-actin bands did not differ between PTZ-treated and saline control rats, the results were probably not due to a generalized or nonspecific effect of PTZ or of the seizure. The observations that not all $GABA_A$ receptor subunit mRNAs were decreased and that the changes differed according to brain region also argue against a nonspecific effect. In contrast to the other subunit mRNAs studied, α_5-mRNA was not affected. In general, subunit mRNAs that decreased 4 h after PTZ injection did so to roughly to the same degree, but there were obvious differences among brain regions. The cerebellum appeared to be most susceptible, because α_1, β_2, and γ_2 were all decreased at 4 h and then significantly increased at 24 h. In cerebral cortical tissue, β_2 and γ_2 mRNA decreased about as much as in cerebellum, but the trend toward rebound at 24 h did not reach significance, and $\alpha_{1\ \mathrm{subunit}}$ mRNA did not change. The hippocampus was the most resistant region studied, with a decrease at 4 h only for γ_2 subunit mRNA. The methods used would not detect changes localized to small areas.

Previous studies have noted changes in mRNAs for $GABA_A$ receptor subunits and other receptor proteins after experimental seizures. For example, kainate-induced status epilepticus was associated with decreased mRNA for glutamate receptor GluR2 and GluR3 subunits and for the $GABA_A$ receptor α_1 subunit in hippocampal CA3/CA4 region but an increase in glutamate receptor mRNAs and no change in the $GABA_A$ α_1 mRNA in the dentate gyrus (Friedman et al., 1994). In rats that had been kindled by electrical stimulation of the Schaffer collateral/commissural fiber pathway, [^{3}H]muscimol binding and $GABA_A$ receptor β subunit mRNAs were altered differentially in various hippocampal fields (Kamphuis et al., 1994; Titulaer et al., 1994). Considering these types of observations and our findings of regional differences even when comparing large brain areas, it is reasonable to suspect that localized changes in the expression of $GABA_A$ receptor mRNA are present after PTZ seizures. Re-

gional differences indicate that the changes in $GABA_A$ receptor mRNA levels after PTZ may be determined at least in part by regional differences in $GABA_A$ receptor subunit composition and/or neuronal function. $GABA_A$ receptor mRNAs may also change according to the experimental seizure used. Unlike our results for PTZ seizures, electroshock seizures in mice produced no change in the levels of α_1, α_2, β_2, β_3, γ_1, or γ_2 subunit mRNAs in cerebral cortex or hippocampus but did produce an increase in cerebellar levels of α_1 and β_2 subunit mRNAs at 4 and 8 (but not 2 or 24) h after the seizure (Pratt et al., 1993).

Transient variations in $GABA_A$ receptor mRNAs after single seizures may be associated with alterations in $GABA_A$ receptor number or function. The decreases in $GABA_A$ receptor mRNAs seen after PTZ, if maintained long enough, could lead to decreased numbers of $GABA_A$ receptors and a reduction in GABAergic inhibition. In one study, two PTZ-induced tonic-clonic seizures over 2 days did produce a decrease in hippocampal $GABA_A$ binding sites accompanied by a decrease in GABAergic inhibition (Psarropoulou et al., 1994). More prolonged PTZ treatment to produce kindling was also associated with a decrease in [^{3}H]GABA and [^{35}S]TBPS binding and in GABA-stimulated $^{36}Cl^-$ uptake (Corda et al., 1990, 1992). However, the results of our study suggest that, although a single PTZ convulsion did affect $GABA_A$ receptor mRNA levels and may have affected receptor function, the number of $GABA_A$ receptors was not altered.

In autoradiographic binding studies, the decrease in [^{3}H]FNP binding suggested that the reduced levels of $GABA_A$ receptor mRNA would result in reduced availability of receptors. However, there was no change in [^{3}H]flumazenil binding and minimal change in [^{3}H]muscimol binding. This suggested that the reduction in [^{3}H]FNP binding was not due to a loss in $GABA_A$ receptors or even to a general change in the affinity of the benzodiazepine recognition site. Rather, the results pointed to some change in receptors that would affect the benzodiazepine agonist [^{3}H]FNP but not the antagonist [^{3}H]flumazenil. One possibility involves the coupling between the GABA and benzodiazepine sites of the $GABA_A$ receptor. A manifestation of this is the ability of $GABA_A$ agonists to increase the binding affinity of benzodiazepine agonists (Tallman et al., 1978; Wastek et al., 1978; Chiu and Rosenberg, 1979) but not of the antagonist flumazenil (Möhler and Richards, 1981; Chiu and Rosenberg, 1983). GABA-benzodiazepine coupling was evaluated with a well washed homogenate preparation, in which GABA concentration is greatly reduced. In the absence of exogenous GABA, there was no difference in [^{3}H]FNP-specific binding. However, there was a decrease in GABA stimulation of [^{3}H]FNP binding, indicating that the acute PTZ injection had affected coupling between the GABA and benzodiazepine recognition sites of the $GABA_A$ receptor. Because the method used in the autoradiography binding assay would probably not have reduced GABA concentration below that affecting benzodiazepine binding (McCabe et al., 1988), reduced GABA-benzodiazepine coupling might be expected to reduce [^{3}H]FNP binding but have no effect on [^{3}H]flumazenil binding in the autoradiographic binding assay, although the rather high (5 nM) concentration of [^{3}H]FNP would have tended to reduce the magnitude of GABA stimulation. Another possibility, not evaluated in this study, involves compensatory changes in other subunits. For example, compared with receptors with the γ_2 subunit, receptors with γ_3 have substantially lower affinity for FNP but not flumazenil (Benke et al., 1996), so that a combined decrease in γ_2 and increase in γ_3 might produce the uncoupling reported above.

Mechanisms for GABA-benzodiazepine uncoupling after a PTZ convulsion might involve transcription of regulatory elements that interact with the $GABA_A$ receptor to alter its expression or posttranslational modifications or may involve more direct effects, for example, by regulating receptor phosphorylation. Acute PTZ seizure has been shown to cause a transient increase in c-fos and c-jun mRNA expression, with an increase in activator protein 1-binding sites, suggesting up-regulation of transcription factors (Saffen et al., 1988; Sonnenberg et al., 1989). This could provide a conceptual basis for postulating alterations in any number of neuronal components after seizure activity. Currently, not enough is known about the target genes of Fos and Jun proteins to determine the specific role they might play with respect to alterations in $GABA_A$ or other receptors. However, available information at least suggests that the excessive neural activity associated with a PTZ seizure could affect expression of regulatory elements somehow related to GABA-benzodiazepine coupling at the $GABA_A$ receptor.

One possibility is that GABA-benzodiazepine coupling is affected by the phosphorylation state of the receptor. $GABA_A$ receptor subunits contain consensus sequences for phosphorylation by various protein kinases and can be phosphorylated by them (McDonald and Moss, 1997). Altering receptor phosphorylation can modify $GABA_A$ receptor function (Leidenheimer et al., 1991; Moss et al., 1992) and may modify benzodiazepine potentiation of GABA-mediated currents (Leidenheimer et al., 1993). Several studies have indicated a relationship between seizure activity and phosphorylation. For example, the single-locus mutant mouse tottering, a model system for absence epilepsy, exhibits ataxia and myoclonic seizures. In microsacs prepared from the brain of these mice, there is an overall decrease in $GABA_A$ receptor function, as determined by $^{36}Cl^-$ flux, and large concentrations of intravesicular PKA, which may have suppressed $GABA_A$ receptor function by intracellular phosphorylation of the receptor subunits (Tehrani and Barnes, 1995). In another example, Chen (1994a,b) demonstrated that both electroshock seizure and convulsive doses of PTZ can increase activity of the PKC γ isoform. Although it is not as yet known whether such an increase in PKC activity would indeed phosphorylate $GABA_A$ receptors after a PTZ convulsion, there is sufficient information to hypothesize that increased protein kinase activity after seizure activity may phosphorylate substrates, including the $GABA_A$ receptors, and subsequently alter GABAergic function. Furthermore, the rapid rise in PKC activity could also be associated with expression of Fos and Jun (Karin et al., 1997), providing a possible link between c-*fos* and c-*jun* gene expression and phosphorylation. Further studies examining the phosphorylation of $GABA_A$ receptor subunits after seizure activity and the functional consequences may reveal a role for phosphorylation in the changes in $GABA_A$ receptor function associated with seizure activity.

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