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Decreased Benzodiazepine Binding with Little Effect on γ-Aminobutyric Acid Binding in Rat Brain After Treatment with Antisense Oligodeoxynucleotide to the γ-Aminobutyric Acid$_A$ Receptor *Gamma*-2 Subunit[1,2]

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ABSTRACT

Benzodiazepine potentiation of γ-aminobutyric acid (GABA) neurotransmission is associated with the presence of a *gamma*-2 subunit in the GABA$_A$ receptor. A method was developed to modify the *gamma*-2 subunit expression in adult rat brain. Unilateral intracerebroventricular (i.c.v.) infusion of a 17-base phosphorothioate-modified antisense oligodeoxynucleotide (ASO) was performed every 12 hr for 3 days. Controls were treated with a sense oligodeoxynucleotide. Parasagittal brain sections were used for quantitative autoradiographic analysis of radioligand binding. ASO treatment caused a 15% to 25% decrease of specific [^{3}H]flunitrazepam binding in most brain areas, with statistically significant decreases in frontal cortex, cerebellar molecular layer, zona reticulata of substantia nigra and CA3 of hippocampus. In contrast, [^{3}H]muscimol binding was not changed. [^{3}H]GABA binding was also unchanged, except for a 10% decrease in cerebellar granule cell layer. The effect on the chloride channel of the GABA$_A$ receptor complex was examined by 4′-ethynyl-4-*n*-[2,3-^{3}H$_2$]propylbicycloorthobenzoate binding; most brain areas showed small decreases in 4′-ethynyl-4-*n*-[2,3-^{3}H$_2$]propylbicycloorthobenzoate binding. However, hippocampal regions showed much larger decreases. Binding of the adenosine A$_1$ receptor antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine was used to examine possible secondary effects of the ASO. There was a decrease in [^{3}H]8-cyclopentyl-1,3-dipropylxanthine binding, but this was much smaller than the change in [^{3}H]flunitrazepam binding, and no area showed a significant effect. Quantitative immunoblotting with a monoclonal antibody that recognizes GABA$_A$ receptor *beta*-2 and *beta*-3 subunits showed no change in immunoreactivity in cerebellar tissue after ASO treatment. The results indicate a selective effect on benzodiazepine binding to GABA$_A$ receptors and a possible change in receptor subunit composition.

The GABA$_A$ receptor, a ligand-gated anion channel, carries sites of action for several clinically important compounds, such as benzodiazepines and barbiturates. The receptors consist of several glycoprotein subunits, most of which exist in several isoforms, encoded by different genes (*e.g.*, *alpha*-1–6, *beta*-1–3, *gamma*-1–3) (Macdonald and Olsen, 1994). The distribution of these subunits in the brain shows regional and cellular differences (Wisden *et al.*, 1992). It is thought that there are several GABA$_A$ receptor subtypes in brain due to the differential assembly of subunits. GABA$_A$ receptors can be investigated using radioligands recognizing different binding sites, including the benzodiazepine binding site, GABA binding site and TBPS site (associated with the ion channel domain of the receptor). Benzodiazepines act *via* the benzodiazepine binding site, which is a modulatory site located on the GABA$_A$ receptor. The presence of a *gamma* subunit in the GABA$_A$ receptor is necessary for formation of a benzodiazepine recognition site and for potentiation of the GABA response by benzodiazepines (Pritchett *et al.*, 1989b; Günther *et al.*, 1995). This binding site may be located at the interface between *alpha* and *gamma* subunits (Sigel and Buhr, 1997; Stephenson, 1995). In contrast, the GABA recognition site may be at the interface of *alpha* and *beta* subunits (Sigel and Buhr, 1997). In the rat brain, the *gamma*-2 isoform is the predominant *gamma* isoform, is widely expressed throughout most regions of the rat brain and most often is associated with *alpha*-1 and *beta*-2 subunits (Benke *et al.*, 1994).

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ABBREVIATIONS: ASO, antisense oligodeoxynucleotide; β-CCM, methyl-β-carboline-3-carboxylate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GABA, γ-aminobutyric acid; [^{3}H]EBOB, 4′-ethynyl-4-*n*-[2,3-^{3}H$_2$]propylbicycloorthobenzoate; TBOB, *t*-butylbicycloorthobenzoate; TBPS, *t*-buytlbicyclophosphorothionate; i.c.v., intracerebroventricular.

The physiological and pharmacological properties of $GABA_A$ receptors are largely determined by the receptor subunit composition (Macdonald and Olsen, 1994; Sieghart, 1995). Compounds that are selective for receptors incorporating particular subunit combinations might then be useful tools for studying the contribution of individual subunits to the properties of receptors in adult brain cells. So far, only a few such ligands are known; these include the benzodiazepine site ligands zolpidem, which has a relative preference for binding to $GABA_A$ receptor containing the *alpha*-1 subunit (Arbilla *et al*., 1986; Wu *et al*., 1994a), and RY-80, which is selective for receptors containing the *alpha*-5 subunit (Skolnick *et al*., 1997). Several studies using various radioligands have shown that the $GABA_A$ receptor population in adult brain is plastic. For example, chronic flurazepam treatment was associated with reduced brain benzodiazepine binding (Rosenberg and Chiu, 1981a, 1981b; Tietz *et al*., 1986). Using the selective ligand zolpidem, an even greater loss of zolpidem-sensitive sites was demonstrated, suggesting a change in receptor composition (Wu *et al*., 1994a). This demonstrated the usefulness of such selective ligands as tools. Chronic benzodiazepine treatment was also associated with decreased levels of mRNAs for several $GABA_A$ receptor subunits, including *gamma*-2 (Zhao *et al*., 1994; Wu *et al*., 1994b).

Another possibility for studying the role of $GABA_A$ receptor subunits in adult brain is the use of ASO technology to reduce the expression of selected proteins. Several studies have shown this to be a useful approach for investigating the function of individual proteins in the central nervous system. ASOs directed against the *alpha*-1, *alpha*-2, *alpha*-6 or *gamma*-2 subunits of the $GABA_A$ receptor have been used to study receptors in cell culture (Brussaard and Baker, 1995; Zhu *et al*., 1996). More recently, Smith *et al*. (1998) showed that pretreating rats with an ASO against the *alpha*-4 subunit of the $GABA_A$ receptor prevented the increased seizure susceptibility observed after progesterone withdrawal, which was apparently related to an increase in *alpha*-4 expression. In other studies, an ASO for the $GABA_A$ receptor *gamma*-2 subunit was infused into rat brain and shown to reduce benzodiazepine binding but with some indication of toxic effects (Karle *et al*., 1997a, 1997b). In a previous study from our laboratory, a similar ASO directed against rat *gamma*-2 subunit was also used to study $GABA_A$ receptor regulation in adult rat brain. It was found that the convulsive threshold dose for β-CCM, a benzodiazepine "inverse agonist," was increased 87% in rats infused by the i.c.v. route with *gamma*-2 ASO (every 12 hr for 3 days) but was not affected by the sense (control) oligodeoxynucleotide (Zhao *et al*., 1996). In contrast, there was no change in picrotoxin seizure threshold and no difference in strychnine threshold between sense and ASO-treated rats. These results suggested that ASO treatment had interfered with the actions of the benzodiazepine binding site *in vivo* and that this may have been selective for the benzodiazepine recognition site of $GABA_A$ receptors. In the present study, the same ASO treatment method against *gamma*-2 subunit was used to study the effect of such treatment on the binding of several ligands to the $GABA_A$ receptor. It was hypothesized that *gamma*-2 ASO treatment would decrease benzodiazepine binding without changing the number of $GABA_A$ receptor complexes.

Methods

Oligodeoxynucleotides. A 17-base ASO for the region starting at position 2 after the initiation codon was based on the sequence for the rat $GABA_A$ receptor *gamma*-2 subunit mRNA (Shivers *et al*., 1989). Antisense (5′-CATGTATTTGGCGAACT-3′) and sense (5′-AGTTCGCCAAATACATG-3′) phosphorothioate-modified oligodeoxynucleotides were synthesized by Oligos Etc., Inc. (Wilsonville, OR). These were dissolved in sterile, filtered saline for i.c.v. injection.

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were kept under standard conditions of a 12-hr light/dark cycle with free access to standard rat food and water. After an acclimation period of 4 days, surgery was performed to implant the guide cannula for i.c.v. injections.

Cannula implantation and oligodeoxynucleotide administration. Rats were implanted with unilateral stainless steel guide cannulae aimed at a point 2.0 mm above the lateral ventricle. Under sodium pentobarbital anesthesia (45 mg/kg i.p.), animals were placed in a Kopf rat stereotaxic device. Using sterile technique, the dorsal surface of the skull was exposed, and a hole was drilled to yield an implantation site corresponding to −0.5 mm (caudal) and 1.5 mm lateral to bregma according to the atlas of Paxinos and Watson (1986). A sterilized guide cannula was lowered into the brain tissue 2 mm below skull surface and fixed with dental cement and a screw. A close-fitting stainless steel obturator was used to occlude the cannula. The animals were allowed to recover for 10 days before beginning treatment. Oligodeoxynucleotide solution was administered into the right i.c.v. space of the conscious animals *via* an injection cannula that extended 2.0 mm beyond the tip of the guide cannula. The rats received the ASO injection (18 μg in 2 μl saline) every 12 hr for 3 days, beginning in the evening. The control group received the corresponding sense oligodeoxynucleotide. Solutions were slowly infused over 1 min using a Harvard infusion pump, and the injection cannula was left in place for an additional 1 min before it was slowly withdrawn and replaced with the obturator. The treatment duration was limited to 3 days by the obvious weight loss of ASO-treated rats (Zhao *et al*., 1996).

Brain slice preparation. Rats were sacrificed by decapitation 6 hr after the final i.c.v. injection. Brains were removed quickly and immersed in isopentane cooled in an acetone-dry ice bath. Sagittal sections (10 μm) were cut from the right side of each brain in a cryostat microtome (−14°C) and were thaw-mounted onto slides previously coated with 0.5% gelatin and 0.05% chrome alum. Slide-mounted tissue sections were transferred to ice-cold slide boxes and stored at −70°C.

[^{3}H]Flunitrazepam binding. Slices were preincubated in 170 mM Tris-HCl buffer (pH 7.4) at 4°C for 30 min and rapidly dried with a cold stream of air. Incubation was performed for 60 min at 4°C in the same buffer in the presence of 5 nM [^{3}H]flunitrazepam (86.0 Ci/mmol, Amersham, Arlington Heights, IL). Nonspecific binding was determined by incubating an adjacent section in the presence of radioligand plus 1 μM clonazepam. Incubation was terminated by rinsing sections twice (30 sec each time) in the Tris-HCl buffer. The slides were then dipped in deionized water and finally dried with a stream of cool air. The dried sections were placed next to tritium-sensitive film (Hyperfilm-[^{3}H], Amersham), which was exposed at 4°C for 12 days.

[^{3}H]Muscimol binding. [^{3}H]Muscimol binding was performed according to the method of Johnson *et al*. (1994). Briefly, slide-mounted sections were preincubated twice in 50 mM Tris-acetate buffer, pH 7.1, at room temperature for 15 min. Sections were then transferred to buffer containing 5 nM [^{3}H]muscimol (19.1 Ci/mmol, New England Nuclear, Boston, MA) for 60 min. To determine nonspecific binding, unlabeled 100 μM GABA was added. After the incubation, the slides were rinsed twice (30 sec each time), followed by a final rinse in glutaraldehyde/acetone (2.5% v/v) at 4°C. The dried sections were placed in x-ray cassettes and exposed to tritium-sensitive film at 4°C for 6 weeks.

[^{3}H]GABA binding. The method for [^{3}H]GABA binding was adopted from Bristow and Martin (1988). Slide-mounted tissue sections were incubated in the presence of 50 nM [^{3}H]GABA (92.0 Ci/mmol, Amersham) for 20 min at room temperature in buffer (pH 7.4) including 50 mM Tris-HCl, 190 mM sucrose and 100 μM baclofen to displace binding to $GABA_B$ receptors. Nonspecific binding was assayed in the presence of 100 μM isoguvacine along with 100 μM baclofen. Incubation was terminated by rinsing sections twice (3 sec each time) in 50 mM Tris-HCl buffer at 4°C. Then the slides were dipped in deionized water and finally dried with a stream of cool air. The dried sections were placed in film cassettes and exposed to tritium-sensitive film at 4°C for 14 days.

[^{3}H]EBOB binding. The picrotoxin site on the $GABA_A$ receptor complex, thought to be associated with the anion channel, was examined by [^{3}H]EBOB binding. The assay was performed according to the method of Kume and Albin (1994). Tissue sections were prewashed three times (10 min each time) in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA at 4°C and dried under a stream of cool air. Binding of 3 nM [^{3}H]EBOB (44.6 Ci/mmol, New England Nuclear) was carried out for 120 min in 50 mM Tris-HCl and 120 mM NaCl buffer (pH 7.4) at room temperature. Nonspecific binding was assessed in the presence of 20 μM picrotoxin. Incubation was terminated by washing twice (60 min each time) in 50 mM Tris-HCl, pH 7.4, at 4°C. Then the slides were dipped in deionized water and finally dried with a stream of cool air. The dried sections were placed in x-ray cassettes with tritium-sensitive film at 4°C for 3 weeks.

[^{3}H]DPCPX binding. The method for [^{3}H]DPCPX binding was as previously described (Fastbom and Fredholm, 1990). The slide-mounted brain slices were preincubated in 170 mM Tris-HCl buffer (pH 7.4), containing 2 IU/ml adenosine deaminase (Boehringer-Mannheim, Indianapolis, IN), for 120 min at room temperature, and then those slides were incubated in the presence of 0.8 nM [^{3}H]DPCPX (120 Ci/mmol, New England Nuclear) in the above-mentioned buffer for 120 min. Nonspecific binding was studied by adding 30 μM (−)-N^6-phenylisopropyladenosine. After the incubation, the slides were washed twice (2 min each time) at 4°C in 170 mM Tris-HCl buffer and then dried under a stream of cold air. The dried sections were placed in x-ray cassettes along with tritium-sensitive film at 4°C for 8 days.

Quantitative autoradiography. Autoradiograms were generated by exposing the ligand-labeled tissue slides to tritium-sensitive film in cassettes along with standards containing known amounts of radioactivity. Ligand binding was quantified with computer-assisted densitometry using the NIH Image software. To quantify ligand binding density, the optical density of coexposed standards were determined, and a standard curve was generated. The tritium standards (courtesy of Dr. E. I. Tietz) were 10 disks of rat brain paste containing known amounts of [^{3}H]thymidine, mounted on a single slide and fixed with paraformaldehyde vapor. The amount of each ligand bound in the rat brain regions was determined by converting optical density measurement to pmol/mg protein (Tietz *et al*., 1986). For each brain, 14 regions were measured on 4 sagittal sections, and the mean of the 4 values for each region was used. Specific binding was the difference between total and nonspecific binding.

Immunoblotting and quantitative densitometry. The left side of the cerebellum was rapidly dissected and stored at −70°C. The cerebellar tissues were homogenized in 3.0 ml of ice-cold medium containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.0), 1 mM sodium EDTA and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin). The homogenates were centrifuged at 1000 × g for 10 min. The supernatants were collected and centrifuged at 10,000 × g for 20 min. The resulting pellets were washed once with 5 ml of 10 mM Tris-HCl, pH 7.0, to remove the protease inhibitors. Aliquots of protein, 10 μg from each sample, were loaded onto the gel, and subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide) then transferred to nitrocellulose membrane overnight at 4°C. The nitrocellulose membrane was treated for 1 hr at room temperature with blocking reagent, consisting of TBS buffer (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl), 0.1% Tween 20 and 5% nonfat dry milk. The membrane was then exposed to anti-$GABA_A$ receptor *beta*-2/*beta*-3 subunit monoclonal antibody (bd-17; Boehringer-Mannheim) 1.0 μg/ml, overnight at 4°C. The membrane was then exposed to anti-mouse IgG (dilution 1:1000; Boehringer-Mannheim) for 2 hr at room temperature. The membrane was washed and placed in ECL reagent solution (Boehringer-Mannheim) for 1 min. The blot was then placed in an autoradiography cassette along with light-sensitive autoradiography film (Hyperfilm ECL, Amersham) for varying periods as needed to achieve adequate band density but staying well below the saturation level of the film. Autoradiographs were analyzed with a BioRad imaging densitometer (model GS-670). For each set of data on an autoradiograph, the mean density of the control samples was set as 1.0, and then the relative density of each band was taken as a fraction of this value.

Statistical analyses. Each set of the antisense and sense groups were treated in parallel throughout. For analysis of radioligand binding, the analyses for the antisense and sense groups were carried out using analysis of variance (ANOVA), with treatment and brain region as variables. In the case of a significant treatment effect, planned comparisons were further performed for each brain region. Results of the immunoblot analysis were evaluated by Student's *t* test. In all cases, $P < .05$ was considered statistically significant.

Results

[^{3}H]Flunitrazepam binding. Figure 1A shows a representative autoradiogram of 5 nM [^{3}H]flunitrazepam binding in a brain section obtained from a rat after treatment with the sense oligodeoxynucleotide. [^{3}H]Flunitrazepam binding was unevenly distributed throughout the rat brain, with a pattern like that in previous reports (Tietz *et al*., 1986; Suzuki *et al*., 1996). Nonspecific binding ranged from 2% to 20% of total binding in those areas examined. As shown in figure 2, there was a 15% to 25% decrease of specific [^{3}H]flunitrazepam binding in most brain areas in rats that had received *gamma*-2 ASO, compared with rats treated with a sense oligodeoxynucleotide. Analysis of these results by ANOVA revealed a significant difference among areas ($F = 69.1$, df = 14, $P < .0001$) and a significant ASO treatment effect ($F = 34.2$, df = 1, $P < .0001$) but no significant interaction between brain region and treatment ($F = 0.9$, df = 14). The *post-hoc* analysis showed a significant decrease of [^{3}H]flunitrazepam binding in several brain regions, including frontal cortex, cerebellar molecular layer, zona reticulata of substantia nigra and CA3 of hippocampus.

[^{3}H]Muscimol binding. As reported by Olsen *et al*.(1990), the abundance of [^{3}H]muscimol binding was the highest in cerebellar granule cell layer, whereas the molecular layer displayed much lower binding. Intermediate levels were seen in the thalamus and frontal cortex, with lower levels in hippocampus and substantia nigra (fig. 1B). Nonspecific binding in the presence of 100 μM GABA was at the level of film background. ASO treatment had no effect on [^{3}H]muscimol binding (fig. 3a). Analysis of the results showed a significant difference among areas ($F = 178.3$, df = 13, $P < .0001$) but no significant ASO treatment effect ($F = 0.1$, df = 1) and no significant interaction between brain region and treatment ($F = 0.01$, df = 13).

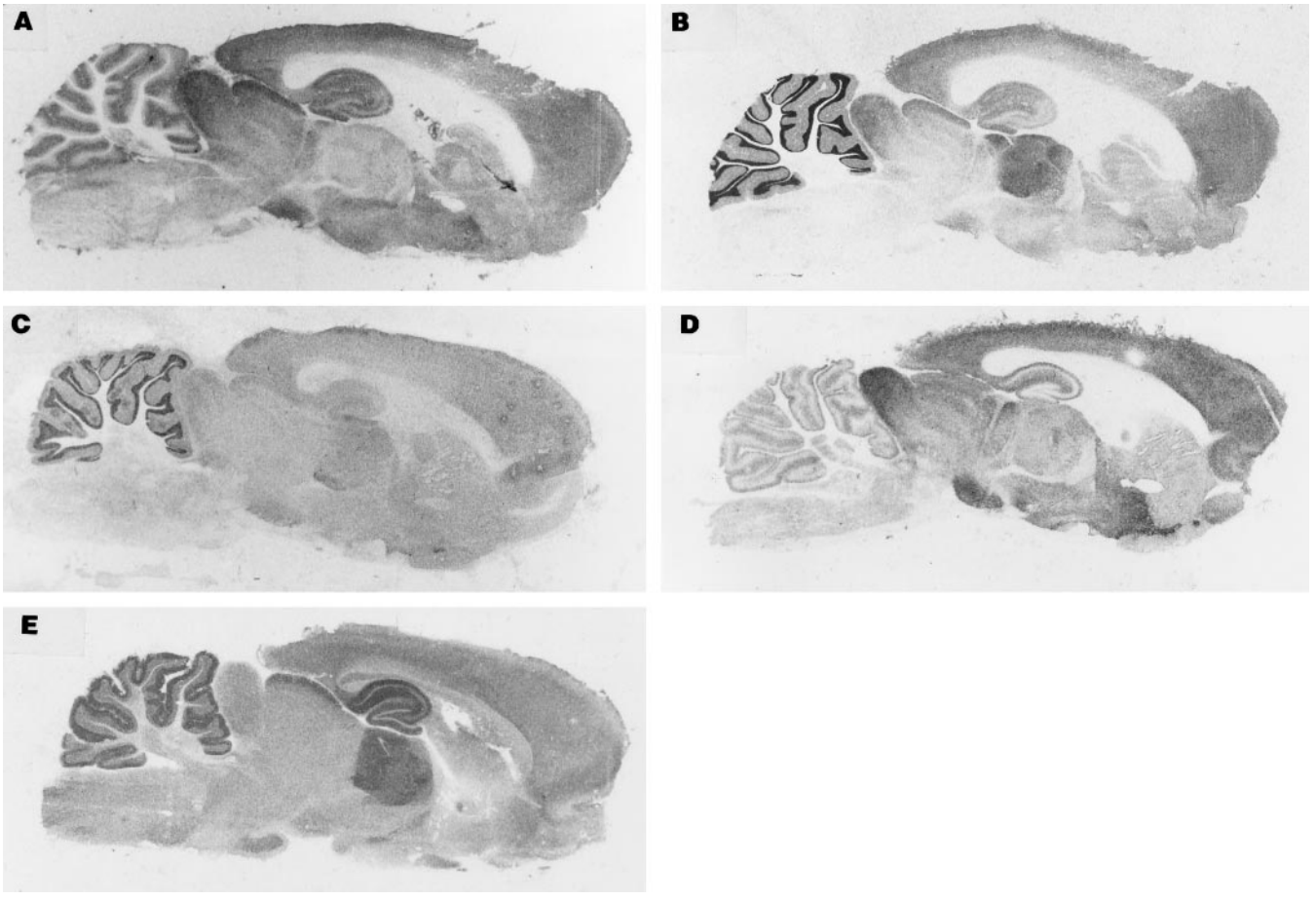

Fig. 1. Sample autoradiographs of radioligand binding to rat brain tissue after 3-day i.c.v. sense (control) oligodeoxynucleotide treatment. Results are shown for (A) 5 nM [^{3}H]flunitrazepam binding, (B) 5 nM [^{3}H]muscimol binding, (C) 50 nM [^{3}H]GABA binding in the presence of 100 μM baclofen, (D) 3 nM [^{3}H]EBOB binding and (E) 0.8 nM [^{3}H]DPCPX binding in the presence of adenosine deaminase. The regional distribution of binding density for each radioligand was similar to that expected from published studies (see text for references).

[^{3}H]GABA binding. As seen in a sample autoradiogram (fig. 1C) [^{3}H]GABA binding, in the presence of baclofen, was greatest in the granule cell layer of the cerebellum, with a lower level in the molecular layer, in agreement with a previous study (Bristow and Martin, 1988). The location of $GABA_A$ sites labeled with [^{3}H]GABA correlated well with the [^{3}H]muscimol binding. Nonspecific binding ranged from 10% to 45% of total [^{3}H]GABA binding in those areas examined. There appeared to be a modest effect of ASO treatment on GABA binding (fig. 3b). Data analysis by ANOVA showed a significant difference among areas ($F = 140.90$, $df = 13$, $P < .0001$) and a significant ASO treatment effect ($F = 6.26$, $df = 1$, $P < .02$) but no significant interaction. The *post-hoc* analysis showed a significant change in only the cerebellar granule cell layer, where [^{3}H]GABA binding was decreased 10%.

[^{3}H]EBOB binding. The results showed that the regional distribution of [^{3}H]EBOB binding was similar to that reported for TBOB (Olsen *et al*., 1990) and a previous [^{3}H]EBOB study (Kume and Albin, 1994). Nonspecific binding was 3% to 6% of total binding at 3 nM [^{3}H]EBOB. High levels of binding were found in cortex, inferior colliculus, superior colliculus and substantia nigra, whereas low levels of binding were found in cerebellar granule cell layer (fig. 1D). ASO treatment appeared to have the greatest effect on [^{3}H]EBOB

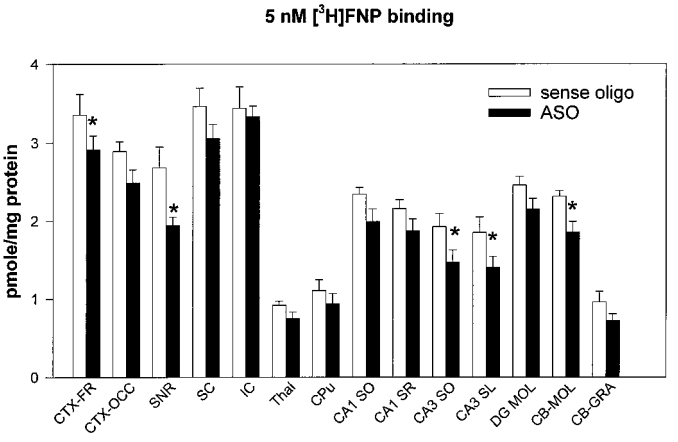

Fig. 2. Specific binding of 5 nM [^{3}H]flunitrazepam to rat brain tissue after i.c.v. infusion of *gamma*-2 ASO or sense oligodeoxynucleotide for 3 days. The bars represent the mean ± S.E. ($n = 4$–5). ANOVA revealed a significant difference among areas and a significant ASO treatment effect but no significant interaction. $*P < .05$ *vs.* sense oligodeoxynucleotide. CTX-FR, frontal cortex; CTX-OCC, occipital cortex; SNR, zona reticulata of substantia nigra; SC, superior colliculus; IC, inferior colliculus; thal, thalamus; CPu, caudate-putamen; CA1 SO, hippocampal CA1 region, stratum oriens; CA1 SR, CA1 stratum radiatum; CA3 SO, CA3 stratum oriens; CA3 SL, CA3 stratum lucidum; DG MOL, molecular layer of dentate gyrus; CB-GRA, granular layer of cerebellum; CB-MOL, molecular layer of cerebellum.

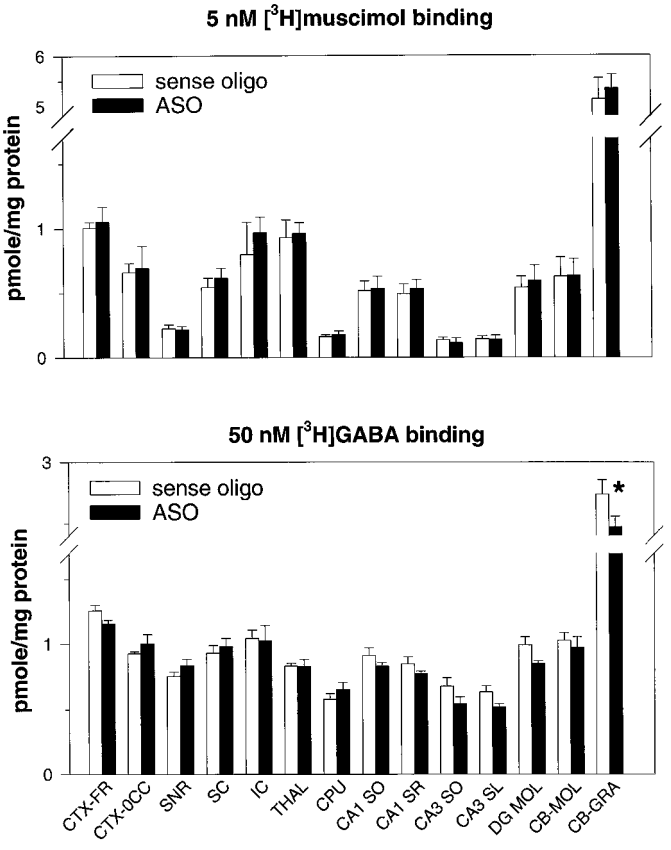

Fig. 3. Specific binding of GABA recognition site ligands. a, Binding of 5 nM [^{3}H]muscimol to rat brain tissue after i.c.v. infusion of *gamma*-2 ASO or sense oligodeoxynucleotide for 3 days. The bars represent the mean ± S.E. (n = 4–5). ANOVA revealed a significant difference among areas but no significant ASO treatment effect and no significant interaction. Abbreviations as in figure 2. b, Specific binding of 50 nM [^{3}H]GABA, in the presence of 100 μM baclofen, after i.c.v. infusion of *gamma*-2 ASO or sense oligodeoxynucleotide for 3 days. The bars represent the mean ± S.E. (n = 4–5). ANOVA revealed a significant difference among areas and a significant ASO treatment effect but no significant interaction. $*P < .05$ *vs.* sense oligodeoxynucleotide. Abbreviations as in figure 2.

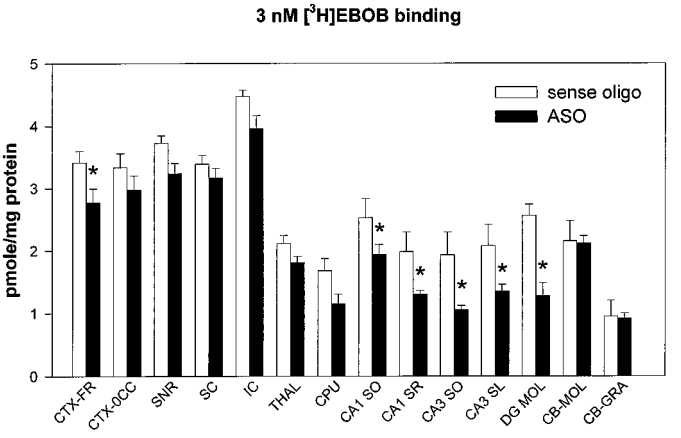

Fig. 4. Specific binding of 3 nM [^{3}H]EBOB after i.c.v. infusion of *gamma*-2 ASO or sense oligodeoxynucleotide for 3 days. The bars represent the mean ± S.E. (n = 4–5). ANOVA revealed a significant difference among areas and a significant ASO treatment effect but no significant interaction. $*P < .05$ *vs.* sense oligodeoxynucleotide. Abbreviations as in figure 2.

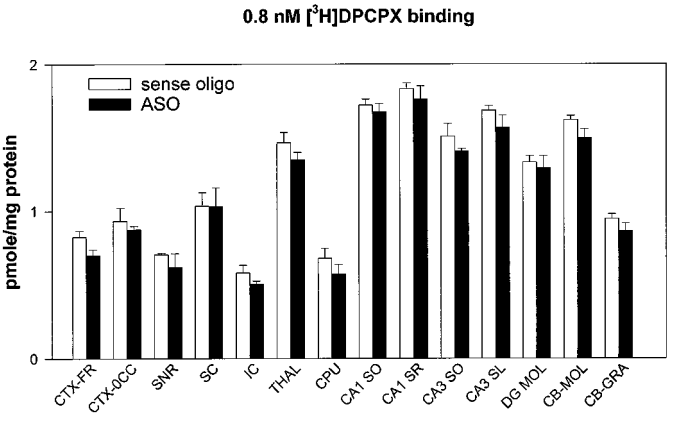

Fig. 5. Specific 0.8 nM [^{3}H]DPCPX binding after i.c.v. infusion of *gamma*-2 ASO or sense oligodeoxynucleotide for 3 days. The bars represent the mean ± S.E. (n = 4–5). ANOVA revealed a significant difference among areas and a significant ASO treatment effect but no significant interaction. *Post-hoc* analysis found no single region with a significant treatment effect. Abbreviations as in figure 2.

binding in hippocampal regions (fig. 4). There was a significant difference among brain areas ($F = 45.80$, $df = 13$, $P < .0001$) and a significant ASO treatment effect ($F = 47.14$, $df = 1$, $P < .0001$) but no significant interaction. The *post-hoc* analysis showed that a significant decrease of [^{3}H]EBOB binding was limited to frontal cortex and all measured areas of the hippocampal formation, where the binding was reduced by 24% to 45%.

[^{3}H]DPCPX binding. As shown in figure 1E, [^{3}H]DPCPX binding showed a wide distribution of A1 receptor in CNS. The distribution of [^{3}H]DPCPX binding was in agreement with a previous study (Fastbom and Fredholm, 1990). High densities of binding were seen in hippocampus, molecular layer of cerebellum and thalamus, whereas moderate levels of binding were seen in other regions. Nonspecific binding of [^{3}H]DPCPX could not be differentiated from background in any brain region. ASO treatment produced only a modest effect [^{3}H]DPCPX binding (fig. 5). Data analysis by ANOVA showed a significant difference among areas ($F = 92.6$, $df = 13,82$, $P < .0001$) and a significant ASO treatment effect ($F = 11.3$, $df = 1$, $P < .02$) but no significant interaction. Despite the significant treatment effect, *post-hoc* analysis showed that no single area had a significant difference between ASO and sense oligodeoxynucleotide treatments ($P \geq .15$ in each area).

Immunoblot for $GABA_A$ receptor *beta*-2/*beta*-3 subunit in cerebellum. Quantitative Western blot analysis was used to determine *beta*-2/*beta*-3 receptor subunit level after *gamma*-2 ASO treatment. The monoclonal antibody bd 17 recognized two different proteins, corresponding to the *beta*-2 and *beta*-3 subunits, with molecular weights of 56 and 58 kDa, respectively, in the cerebellar membrane preparation. These results were in agreement with a previous study in which this antibody was used to detect *beta*-2/*beta*-3 subunits in a brain membrane homogenate preparation (Mhatre and Ticku., 1994). The ASO treatment had no significant effect on the intensity of bands seen in the immunoblot (fig. 6).

Discussion

In this study, it was expected that the ASO treatment would reduce the availability of *gamma*-2 protein, which should interfere with normal assembly $GABA_A$ benzodiaz-

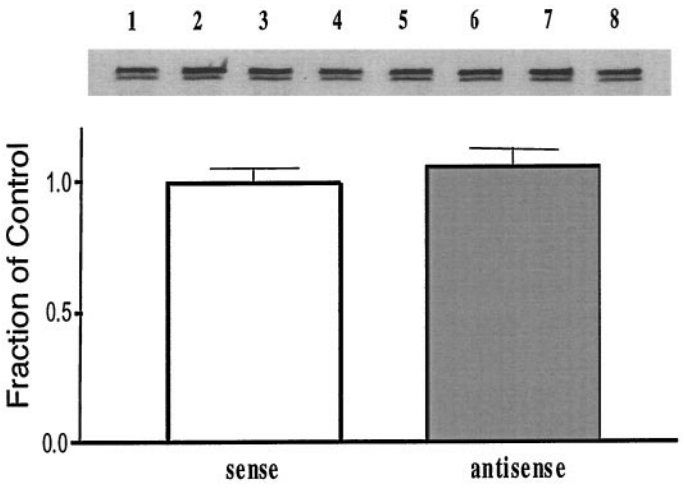

Fig. 6. Top, representative autoradiogram showing β subunit protein immunoreactivity in the cerebellum. The results shown in the autoradiogram depict immunoreactivity levels from sense (lanes 2, 4, 6, 8) and *gamma*-2 ASO (lanes 1, 3, 5, 7)-treated rats. Bottom, results of densitometry. The mean density of the four controls was set to 1.0 and used to compare each sense and ASO-treated sample (mean ± S.E., $n = 4$). There was no significant treatment effect (Student's t test).

epine receptors. The ability of benzodiazepines to bind to a $GABA_A$ receptor and to increase the GABA-dependent gating of the intrinsic Cl^- channel, requires a *gamma* subunit in the receptor, and the *gamma*-2 subunit has been most closely associated with typical benzodiazepine actions (Pritchett *et al*., 1989a; Macdonald and Olsen, 1994). Günther *et al*. (1995) also addressed the role of the *gamma*-2 subunit in $GABA_A$ receptor function by producing mutant mice that lacked this subunit. The neonatal mice expressed $GABA_A$ receptors lacking *gamma*-2 subunits, with almost complete absence of [^{3}H]flumazenil binding (benzodiazepine recognition sites), but a much smaller reduction in [^{3}H]SR-95531 binding (GABA recognition sites). These mice failed to thrive, displayed abnormalities in sensorimotor behavior and survived only a short time, indicating the likelihood of physiological changes in $GABA_A$ receptors resulting from loss of the *gamma*-2 subunit.

The ASO treatment produced a widespread decrease in binding of the benzodiazepine ligand [^{3}H]flunitrazepam. This was consistent with our previous observation of a significant increase in convulsive threshold for β-CCM, a benzodiazepine "inverse agonist," after the same 3-day i.c.v. treatment. The 10% to 25% decrease in [^{3}H]flunitrazepam binding was similar to the results reported by Karle and Nielsen (1995), who noted a 9% to 15% reduction in benzodiazepine binding in cerebral cortex subsequent to a 2-day treatment with i.c.v. infusions of a *gamma*-2 ASO that differed somewhat from the one used in the present study.

It had been hypothesized that the *gamma*-2 ASO treatment might result in the selective loss of benzodiazepine binding sites without changing the other binding sites present on $GABA_A$ receptors. Indeed, the GABA recognition site was largely unaffected by the ASO treatment. [^{3}H]Muscimol binding showed no change in any brain region, including cerebellar granule cell layer. This region has the highest level of [^{3}H]muscimol binding and demonstrated a 25% decrease of [^{3}H]flunitrazepam binding after 3-day ASO i.c.v. infusion. [^{3}H]GABA binding was only decreased 10% in this area, with no significant change in other regions, including those that had shown diminished benzodiazepine binding. The effects of *gamma*-2 ASO treatment suggested that even though $GABA_A$ receptors had been affected, as indicated by the reduced availability of benzodiazepine recognition sites ([^{3}H]flunitrazepam binding), there was little effect on the availability of GABA recognition sites ([^{3}H]muscimol and [^{3}H]GABA binding). These findings suggest that *gamma*-2-deficient receptors, of unknown subunit composition, were being expressed in ASO-treated rats. Under the experimental conditions used for the autoradiographic binding, [^{3}H]muscimol and [^{3}H]GABA show dramatic differences in relative binding among brain regions (fig. 3; Olsen *et al*., 1990). In recent studies, it was shown that high affinity [^{3}H]muscimol binding was dramatically reduced in the cerebellar granule cell layer of *alpha*-6-null/*delta*-deficient mice (Jones *et al*., 1997; Mäkelä *et al*., 1997), indicating that receptors containing these subunits are responsible for much of the high affinity [^{3}H]muscimol binding, particularly in cerebellar granule cells. Moreover, *delta* subunit protein and mRNA disribution closely approximate the distribution of [^{3}H]muscimol binding (Jones *et al*., 1997) and, in forebrain, alpha-4 and delta subunit distributions are similar (Wisden *et al*., 1992; Jones *et al*., 1997). Such findings indicate that [^{3}H]muscimol especially labels a population of $GABA_A$ receptors containing *alpha*-4 *delta* or *alpha*-6 *delta*. Thus, the lack of *gamma*-2 ASO effect on [^{3}H]muscimol binding could also suggest that these receptors do not include a *gamma*-2 subunit.

It is known that $GABA_A$ receptors can be expressed from only *alpha* and *beta* subunits and that such receptors will respond to GABA in a picrotoxin- and bicuculline-sensitive fashion but will be insensitive to benzodiazepine (Verdoorn *et al*., 1990; Angelotti and Macdonald, 1993). However, the presence or absence of a *gamma* subunit does affect the GABA response of such receptors (Verdoorn *et al*., 1990), so that receptors affected by ASO treatment may have had an altered GABA response, resulting in decreased GABA-mediated inhibitory transmission. This was thought to be the basis for some behavioral changes in ASO- but not sense oligonucleotide-treated rats (Zhao *et al*., 1996). Reduced GABA neurotransmission might also eventually result in cell damage or even loss of neurons. In their initial report, Karle and Nielsen (1995) noted only a modest decrease in [^{3}H]flunitrazepam binding and no loss of [^{3}H]muscimol binding in cerebral cortex after the 2-day ASO treatment. However, when this treatment was continued for up to 6 days, there was a greater decrease in [^{3}H]flunitrazepam binding (21%) and a 12% decrease in [^{3}H]muscimol binding in cortex (Karle *et al*., 1997a). The decreased GABA binding appeared to be at least partially dependent on the duration of *gamma*-2 ASO treatment and may be a secondary result of the *gamma*-2 ASO treatment. Subsequent work by the same group, using intrahippocampal ASO infusion, suggests this to be the case (Karle *et al*., 1997b). In our own study, behavioral effects of the ASO (but not sense) treatment, which includes serious weight loss (Zhao *et al*., 1996), limited the treatment to 3 days. The reasons for this are not clear but likely involve less effective GABA function in $GABA_A$ receptors that are deficient in the *gamma*-2 subunit (Verdoorn *et al*., 1990), as discussed above.

No change was found in *beta* subunit immunoreactivity in

the cerebellum after *gamma*-2 ASO treatment. As the GABA recognition site is thought to be associated with *beta* subunits (Sigel and Buhr, 1997), this was in keeping with the minimal effects of ASO treatment on [^{3}H]GABA and [^{3}H]muscimol binding. In the cerebellum, *beta*-2 is the most abundant isoform, followed by the *beta*-3 subunit, whereas *beta*-1 is present in a very small proportion of cerebellar $GABA_A$ receptors (Li and de Blas, 1997). The lack of change in the *beta* subunit immunoreactivity also indicated that the reduction in the *gamma*-2 subunit was not compensated by altering the levels of the *beta* subunits and adds further support for the idea that the *gamma*-2 ASO treatment affected neither the subunits involved in the GABA binding site nor the number of $GABA_A$ receptor complexes.

The apparent effect of treatment on the chloride channel of the $GABA_A$ receptor complex, as expressed by [^{3}H]EBOB binding, differed greatly among brain regions. Although several areas, such as substantia nigra pars reticulata, showed small, statistically insignificant decreases, there was no significant effect of ASO treatment here, or in the cerebellum, where there was a 25% decrease in [^{3}H]flunitrazepam binding. However, all areas of the hippocampal formation showed large decreases in [^{3}H]EBOB binding. In contrast to other regions of the rat brain, the cut surface of the hippocampal tissue of *gamma*-2 ASO-treated rats appeared edematous and discolored, with a less distinct laminar structure. A similar change in hippocampus after local *gamma*-2 ASO injection was observed by Karle *et al*. (1995). In that study, the intrahippocampal ASO treatment also resulted in a 51% decrease in [^{35}S]TBPS binding, and, in contrast to the present study, significant decreases in hippocampal [^{3}H]muscimol and [^{3}H]quinuclidinyl benzilate binding (for the muscarinic acetylcholine receptor), and a decrease in protein content, suggesting loss of hippocampal cells (Karle *et al*., 1995). In the present study, it is possible that the greater loss of [^{3}H]EBOB binding in the hippocampus may be related to the proximity of this region to the site of ASO infusion, resulting in a higher local ASO concentration. Other possibilities include the sensitivity of hippocampal structures to the loss of inhibitory function, resulting in increased excitatory activity and subsequent neuronal damage. Such damage may be associated with release of endogenous substances, such as fatty acids, which have been shown to inhibit the binding of a similar ligand (TBPS) to the chloride channel portion of the $GABA_A$ receptor (Koenig and Martin, 1992). To further evaluate possible nonspecific effects of the *gamma*-2 ASO treatment, [^{3}H]DPCPX binding was used as an indicator of possible changes in brain tissue secondary to decreased GABA-ergic neurotransmission. There was a small but significant ASO treatment effect on [^{3}H]DPCPX binding. However, no single area showed a significant change after 3-day *gamma*-2 ASO treatment. This modest change indicated that the 3-day ASO treatment did cause moderate secondary effects, especially in hippocampus. Because there was no loss of GABA, muscimol or DPCPX binding in hippocampus, it is unlikely that the ASO treatment had caused a loss of neurons, and any secondary changes that might have eventually resulted in neuronal loss with further treatment were still in a reversible stage. This is in accord with the recent findings by Karle *et al*. (1997b).

In summary, the *gamma*-2 ASO treatment reduced benzodiazepine binding with little effect on GABA binding. The findings support the hypothesis that, at least up to 3 days, the ASO treatment described can selectively alter the expression of *gamma*-2 subunits and thereby alter the composition and function of the $GABA_A$ receptor. Reducing the availability of one $GABA_A$ receptor subunit might be expected, through feedback mechanisms, to alter the transcription and/or translation of that subunit, as well as for other subunits. It is anticipated that the pattern of that response may provide insight into the regulation of $GABA_A$ receptor subunit expression and assembly in adult central neurons.

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