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Treatment with an antisense oligodeoxynucleotide to the $GABA_A$ receptor γ_2 subunit increases convulsive threshold for β-CCM, a benzodiazepine 'inverse agonist', in rats

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Abstract

The γ_2 subunit of the γ-aminobutyric acid type-A ($GABA_A$) receptor is associated with the actions of benzodiazepines and related drugs. A phosphorothioate-modified antisense oligodeoxynucleotide directed against the γ_2 subunit was given by i.c.v. injection (18 μg in 2 μl saline) to male Sprague-Dawley rats every 12 h for 3 days. Controls received the corresponding sense oligodeoxynucleotide. 4–6 h after the last i.c.v. treatment, rats were given methyl-β-carboline-3-carboxylate (β-CCM), a benzodiazepine 'inverse agonist', by slow i.v. infusion. Compared to naive rats, the β-CCM threshold dose was not affected by the sense oligodeoxynucleotide, but was increased 87% in antisense oligodeoxynucleotide-treated rats. The treatment had no effect on the seizure threshold for picrotoxin. Both antisense and sense oligodeoxynucleotide treatments slightly increased the threshold for strychnine seizures. The results suggest that antisense oligodeoxynucleotide treatment altered $GABA_A$ receptor composition and interfered with the actions of a benzodiazepine receptor ligand in vivo, and may provide a tool for studying regulation of receptor structure and function.

Keywords: $GABA_A$ receptor; γ_2 Subunit ; Antisense oligodeoxynucleotide; Methyl-β-carboline-3-carboxylate; Strychnine; Picrotoxin; Convulsion

1. Introduction

The amino acid γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the central nervous system (CNS). The fast inhibitory postsynaptic potential generated by GABA release is mediated by $GABA_A$ receptors, which are hetero-oligomeric protein complexes consisting of several homologous membrane-spanning glycoprotein subunits (α, β, γ, δ and ρ). Most of these glycoprotein subunits exist in several isoforms, and each isoform is encoded by a different gene (e.g., α_{1-6}, β_{1-4}, and γ_{1-4}). Each subunit protein may confer specific pharmacological functions to the $GABA_A$ receptor (Macdonald and Olsen, 1994). The functional and pharmacological properties of the $GABA_A$ receptors are related to the underlying subunit composition, and this subunit composition may be affected by various treatments, such as chronic benzodiazepine administration. Benzodiazepines act via the benzodiazepine receptor, which is a modulatory site located on the $GABA_A$ receptor. Benzodiazepines do not bind to the same site as GABA, though the benzodiazepine binding site may partially involve the same domain as the GABA site (Smith and Olsen, 1995). The presence of a γ subunit in the $GABA_A$ receptor is necessary for benzodiazepine potentiation of the GABA response (Pritchett et al., 1989). Previous studies from our laboratory demonstrated that chronic benzodiazepine treatment was associated with reduced expression of γ_2 subunit mRNA (Zhao et al., 1994a). Down-regulation of benzodiazepine receptors in brains of animals chronically treated with a benzodiazepine (Rosenberg and Chiu, 1981a,b; Tietz et al., 1986) also suggests that the γ_2 subunit may be altered. Chronic benzodiazepine treatment is also associated with changes in other $GABA_A$ receptor subunit mRNAs (Kang and Miller, 1991; O'Donovan et al., 1992; Primus and Gallager, 1992; Tietz et al., 1994; Wu et al., 1994; Zhao et al., 1994b). $GABA_A$ receptor subunit mRNA expression can also be regulated by such treatments as chronic ethanol exposure (Mhatre and Ticku, 1994) and exposure to GABA (Montpied et al., 1991). However, such treatments may not

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selectively target the expression of a particular receptor subunit. The use of antisense oligodeoxynucleotide technology may allow a more selective approach to studying $GABA_A$ receptors.

Although the precise mechanism of action of antisense oligodeoxynucleotides is not completely understood, they have been used to selectively inhibit gene expression in vitro and in vivo, including use for investigating specific functions of proteins in the CNS. For example, antisense oligodeoxynucleotides have been injected i.c.v. to alter brain neuropeptide Y receptors, resulting in behavioral and biochemical changes in the animals (Whalestedt et al., 1993). Introduction of an antisense oligodeoxynucleotide selective for the κ-opioid receptor by i.c.v. injection (Adams et al., 1994), and of an oligo selective for the δ-opioid receptor by intrathecal administration (Tseng et al., 1994) each produced an appropriate, receptor subtype-selective inhibition of analgesia. More recently, antisense oligodeoxynucleotides were used to study $GABA_A$ receptors in cell culture by using an oligo against the α_1 and α_2 subunits of the receptor (Brussaard and Baker, 1995). In the present study, an antisense oligodeoxynucleotide for the γ_2 subunit of the rat $GABA_A$ receptor was administered by i.c.v. injection to examine the possibility of using this technique to affect $GABA_A$ receptor expression in vivo, and the contribution of the γ_2 subunit to seizure behavior elicited by several convulsant drugs. It was hypothesized that such treatment should reduce the number of benzodiazepine recognitions sites, and thereby decrease the effectiveness of drugs acting at this site.

2. Materials and methods

2.1. Oligodeoxynucleotides

Based on the sequence for the rat $GABA_A$ receptor γ_2 subunit mRNA (Shivers et al., 1989), we designed a 17-base phosphorothioate oligodeoxynucleotide for the region starting at position 2 following the initiation codon. Antisense (5′-CATGTATTTGGCGAACT-3′) and sense (5′-AGTTCGCCAAATACATG-3′) phosphorothioate oligodeoxynucleotides were synthesized by Oligos Etc. (Wilsonville, OR). These were dissolved in sterile, filtered saline for i.c.v. injection.

2.2. Animals

Male Sprague-Dawley rats weighing between 250–300 g were kept under standard conditions of a 12 h light/12 h dark cycle (light on at 5 a.m.) 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. Naive rats were housed 3–7 days and, except for routine care, were not handled or otherwise treated.

2.3. 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 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 following surgery before treatment. Oligodeoxynucleotide solution was administered into the intracerebroventricular 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 antisense oligodeoxynucleotide injection (18 μg in 2 μl saline) every 12 h 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. Though the treatment duration was limited to 3 days by the obvious weight loss of the antisense oligodeoxynucleotide treated rats (described below), it was expected that this should be sufficient time to allow for significant turnover of benzodiazepine receptors, based on the kinetics of receptor turnover in neuronal cell culture (Borden et al., 1984).

2.4. Measurement of convulsant threshold

Picrotoxin and strychnine were purchased from Sigma Chemical Co. (St. Louis, MO) and were dissolved with normal saline on the same day they were to be used. Methyl-β-carboline-3-carboxylate (β-CCM, Research Biochemicals International, Natick, MA) was dissolved in a small volume of 1 N HCl, then diluted with normal saline and the pH was adjusted to 4.0 with NaOH just prior to use (a precipitate often formed at higher pH).

4–6 h after the last i.c.v. injection with oligodeoxynucleotide solution, rats were given a slow i.v. infusion of a convulsant. To measure the threshold for β-CCM-induced convulsive activity, a 0.3 mg/ml solution of β-CCM was infused at a constant rate of 0.54 ml/min via a catheter in a lateral tail vein. The time to onset of convulsive activity was recorded and used to calculate the threshold convulsant dose. A similar technique was used for picrotoxin and strychnine. Picrotoxin, 0.8 mg/ml, was made fresh daily with normal saline and infused into a lateral tail vein at a rate of 0.21 ml/min. Strychnine, 0.4 mg/ml, was also

made freshly and infused into a tail vein at a rate of 0.54 ml/min. For strychnine, the onset of tonic extension of the front legs was used as the endpoint. In all of the experiments, each rat was tested only once.

2.5. Statistical analysis

The effect of γ_2 antisense treatment was determined by the convulsive threshold for each convulsant drug in antisense oligodeoxynucleotide treated rats, sense oligodeoxynucleotide treated rats and in naive rats. On each test day, the treated rats, the corresponding sense oligodeoxynucleotide treated rats, and the naive rats were tested in parallel. The seizure activity was evaluated by analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant. In the case of a significant treatment effect, groups were further compared by Tukey's test.

3. Results

The body weight of the rats had returned to their presurgical values by 10 days after cannula implantation. Rats were randomly chosen to receive antisense or sense oligodeoxynucleotide treatment. There were no obvious behavioral effects of the treatment during the first treatment day. However, by the last of the six i.c.v. injections, rats that received antisense oligodeoxynucleotide treatment had a 13% weight loss, while the sense oligodeoxynucleotide treated rats lost 5% of their weight. The weights at the time of the first and sixth injections were compared by repeated measures ANOVA, with the treatment as a grouping variable. There was a significant interaction between the weight change and treatment group ($F = 20.5$, $P = 0.0002$). Further analysis by planned comparisons showed that the weights of the two groups did not differ on the first treatment day ($P = 0.22$), but the treated rats weighed significantly less than the sense oligodeoxynucleotide treated rats by the third treatment day ($P = 0.0001$). Though the food intake was not monitored, there was apparently a decrease in food consumption since there was a dramatic decrease in the number of fecal pellets in the cages of treated rats. In addition, the antisense oligodeoxynucleotide treated, but not the sense oligodeoxynucleotide treated rats showed a generalized tremor, and had an obvious decrease in spontaneous motor behavior (though they responded as expected when handled).

The γ_2 antisense oligodeoxynucleotide treatment decreased the sensitivity of the rats to the convulsant effects of β-CCM (Fig. 1). Compared to naive control rats, there was a significant 87% increase in the threshold dose for β-CCM-induced convulsive activity in the rats that received γ_2 antisense oligodeoxynucleotide. In contrast, there was no significant change in β-CCM threshold in the rats that received sense oligodeoxynucleotide. The threshold dose (mg/kg) for β-CCM-induced convulsions was 0.36 ± 0.06 for naive rats, 0.36 ± 0.05 for sense rats, and 0.66 ± 0.10 for antisense rats. There was a significant effect of treatment ($F = 5.5$, $P = 0.02$). There was a significant difference (Tukey's test, $P < 0.05$) between naive and antisense oligodeoxynucleotide rats, but no significant difference between naive and sense oligodeoxynucleotide rats.

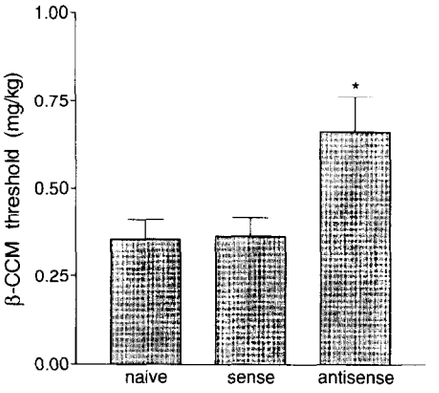

Fig. 1. Threshold β-CCM dose in rats tested 4–6 h after the last oligodeoxynucleotide injection for rats given 3-day antisense oligodeoxynucleotide treatment ($n = 5$) or sense oligodeoxynucleotide treatment ($n = 4$), and naive rats ($n = 5$). β-CCM (0.3 mg/ml) was infused at a rate of 0.54 ml/min via the lateral tail vein. * Significantly different from naive and sense rats.

No change in sensitivity to the convulsant effect of picrotoxin was found (Fig. 2). The dose (mg/kg) for picrotoxin-induced convulsions was 4.21 ± 0.13 for naive rats, 3.93 ± 0.10 for sense oligodeoxynucleotide-treated rats, and 3.98 ± 0.17 for antisense oligodeoxynucleotide rats. These values were not significantly different ($F = 1.4$, $P = 0.28$).

Unexpectedly, pretreatment with either antisense or sense oligodeoxynucleotide increased the seizure threshold for strychnine-induced tonus about 17% (Fig. 3). The dose (mg/kg) for strychnine-induced tonus was 0.84 ± 0.03 for

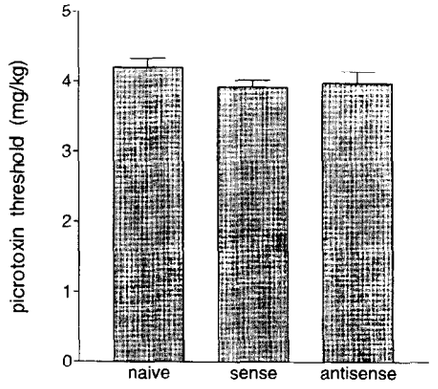

Fig. 2. Threshold picrotoxin dose for clonus in rats tested 4–6 h after the last oligodeoxynucleotide injection for rats given a 3-day antisense oligodeoxynucleotide treatment ($n = 5$) or sense oligodeoxynucleotide treatment ($n = 5$), and naive rats ($n = 7$). Picrotoxin (0.8 mg/ml) was infused at a rate of 0.21 ml/min via the lateral tail vein.

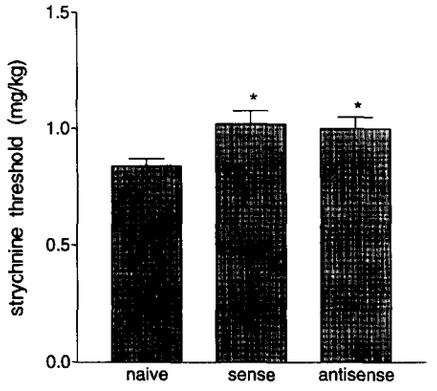

Fig. 3. Threshold strychnine dose for tonus in rats tested 4–6 h after the last oligodeoxynucleotide injection for 3-day antisense oligodeoxynucleotide treatment ($n = 5$) or sense treatment ($n = 5$), and naive rats ($n = 11$). Strychnine (0.4 mg/ml) was infused at a rate of 0.54 ml/min via the lateral tail vein. * Significantly different from naive rats.

naive rats, 1.02 ± 0.06 for sense oligodeoxynucleotide rats, and 1.00 ± 0.05 for antisense oligodeoxynucleotide rats. Though there was a significant treatment effect ($F = 6.7$, $P = 0.007$), there was no significant difference between antisense and sense oligodeoxynucleotide rats (Tukey's test, $P > 0.05$). On the other hand, the strychnine threshold in both of these groups was significantly greater than in naive rats.

4. Discussion

Benzodiazepine agonists act by binding to a modulatory site of the $GABA_A$ receptor, and increasing the frequency of opening of the intrinsic Cl^- channel in response to GABA, essentially increasing the efficiency of the neurotransmitter. A γ subunit appears to be required for benzodiazepine binding and modulation of $GABA_A$ receptor function, and the γ_2 subunit has been most closely associated with typical benzodiazepine actions at the $GABA_A$ receptor (Macdonald and Olsen, 1994; Mihic et al., 1995; Pritchett et al., 1989). Studies of the physiologic and pharmacologic roles of $GABA_A$ receptor subunits in specific regions of intact brain might profit from the availability of methods to selectively block the expression of, or actions associated with particular subunits. The use of the antisense oligodeoxynucleotide technique to reduce γ_2 subunit expression, and thus receptor function associated with the presence of the γ_2 subunit, offers the possibility of selectively modifying the availability of one subunit without altering other $GABA_A$ receptor subunits. If successful, such an approach could be used to address questions related to the requirement for expression of this subunit to form functional $GABA_A$ receptors in adult neurons, and possible alternate forms of the receptor based on availability of subunit protein.

To detect the effect of treatment, the convulsant action of a β-carboline benzodiazepine inverse agonist was examined. Several β-carbolines have been shown to be competitive ligands of central type benzodiazepine receptors and have been reported to display either agonist, inverse agonist, or antagonist activities in vivo (Potier et al., 1988), though the agonist and inverse agonist binding sites may overlap rather than correspond exactly (Smith and Olsen, 1995). β-CCM was reported to be an inverse agonist at the benzodiazepine receptor, with pharmacological properties opposite to those of benzodiazepines such as diazepam (Macdonald and Olsen, 1994; Polc, 1988; Rosenberg et al., 1989). In the present study, the rats treated with an antisense oligodeoxynucleotide directed against the γ_2 subunit of the $GABA_A$ receptor required a larger dose of β-CCM to induce convulsions, which was the expected result if treatment had successfully inhibited benzodiazepine receptor synthesis. Since treatment with the sense oligodeoxynucleotide had no effect on β-CCM convulsive threshold, the effect of treatment can not be attributed to any general toxic effect of i.c.v. oligodeoxynucleotides, or to an effect of the surgery and handling.

A recent report has described the effects on [^{3}H]flunitrazepam binding to brain homogenates from rats given only two daily i.c.v. infusions of a γ_2 antisense oligodeoxynucleotide that was very similar to the one used in the present study (Karle and Nielsen, 1995). In that study, it was found that there was a 9–15% decrease in [^{3}H]flunitrazepam or [^{3}H]flumazenil binding to homogenates from cerebral cortex or striatum. A similar or greater decrease in benzodiazepine binding might be expected to have been present in the rats in the present study, which were given twice daily infusions for three days. In fact, our preliminary results of [^{3}H]flunitrazepam binding, studied autoradiographically, show a 15–25% decrease in benzodiazepine binding in several brain regions (data not shown).

In contrast to the results with β-CCM, treatment with γ_2 antisense oligodeoxynucleotide had no effect on picrotoxin convulsive threshold, with very similar doses required in all three groups. This finding further suggests that the effect on β-CCM convulsive threshold was a specific effect. If the effect on β-CCM threshold were a non-specific effect of treatment, or secondary to another antisense oligodeoxynucleotide effect, such as weight loss, there should have been a similar increase in picrotoxin convulsive threshold. Picrotoxin, which is not a structural analogue of GABA, exerts a noncompetitive inhibition of GABA currents via a site distinct from that for GABA itself (Macdonald and Olsen, 1994; Olsen, 1981). The precise nature of the picrotoxin binding site has not been identified biochemically, but picrotoxin binding to this site may allosterically prevent the conformational change of the GABA receptor protein or physically block the Cl^- channel (Macdonald and Olsen, 1994). The lack of effect on picrotoxin convulsive threshold suggests that the presence of a γ_2 subunit may not be necessary for picrotoxin

to block the function of the $GABA_A$ receptor Cl^- channel. Indeed, in studies of receptors expressed from only α plus β subunits, which lack γ_2 subunits and do not support benzodiazepine binding or actions, picrotoxin does block GABA current (Levitan et al., 1988; Pritchett et al., 1988). In fact, the subunit composition of $GABA_A$ receptors appeared to play little role in the effect of picrotoxin (Sigel et al., 1990). In may be of interest to note that the γ_2 antisense oligodeoxynucleotide treatment employed by Karle and Nielsen (1995) caused no measurable change in [^{3}H]muscimol binding to cerebral cortex, in which [^{3}H]flunitrazepam binding had been reduced, suggesting that the treatment may not have decreased the number of GABA recognition sites associated with $GABA_A$ receptors.

Since strychnine causes convulsions by acting as a glycine receptor antagonist, the treatment should have had no effect on strychnine convulsive threshold. As expected, there was no difference in the strychnine threshold dose between antisense and sense oligodeoxynucleotide treated rats. However, pretreatment with both oligodeoxynucleotides slightly increased the strychnine seizure threshold compared to naive rats. This unanticipated result may have been related to the (presumably) stressful manipulation during 3-day oligodeoxynucleotide treatment, or may have been indicative of a nonspecific toxic effect of i.c.v. phosphorothioate oligodeoxynucleotide treatment.

Though picrotoxin threshold was not changed by oligodeoxynucleotide treatment, it is unlikely that $GABA_A$ receptor function was unaffected, other than by a reduction in benzodiazepine binding sites, since inclusion of a γ_2 subunit in expressed receptors does affect GABA gating of the Cl^- channel (Sigel et al., 1990; Verdoorn et al., 1990). Thus, some effect of γ_2 antisense oligodeoxynucleotide treatment, resulting from changing GABA neurotransmission, was not entirely unexpected. The weight loss and apparent decreased food consumption were quite possibly a result of altered GABA neurotransmission, based on its apparent importance in brain areas functionally related to the regulation of feeding behavior. For example, i.c.v. injection of the $GABA_A$ agonist, muscimol, increased food consumption, and this was completely abolished by administration of the $GABA_A$ receptor antagonist, bicuculline (Baldwin et al., 1990; Morley et al., 1981; Olgiati et al., 1980). Benzodiazepines also increase food consumption in many mammalian species by an action at benzodiazepine recognition sites, and the drug-induced hyperphagia is reversed by a benzodiazepine receptor antagonist (Cooper and Gilbert, 1985). The other behavioral changes noted, especially tremor, may have also been related to reduced GABA function, either because of reduced numbers of functioning receptors, or because of changes in receptor subunit composition that affected GABA actions.

In a recent study, Günther et al. (1995) also addressed the role of the γ_2 subunit in $GABA_A$ receptor function by producing mutant mice that lacked this subunit. The results in neonatal mice, which were consistent with the presence of $GABA_A$ receptors lacking γ_2 subunits, included a lack of behavioral response to benzodiazepines, 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), and changes in $GABA_A$ receptor physiological and pharmacological properties consistent with a deletion of the γ_2 subunit. In addition, the mutant mice failed to thrive, displayed abnormalities in sensorimotor behavior, and survived only a short time (Günther et al., 1995). Though the experimental approach was very different from that used in the present study, the behavioral changes were reminiscent of the effects of γ_2 antisense oligodeoxynucleotide treatment, which underlines the likelihood of physiological changes in $GABA_A$ receptors resulting from loss of the γ_2 subunit.

Selective deletion of one subunit of a ligand-gated ion channel receptor has also been used in vitro to evaluate the role of particular subunits of the nicotinic acetylcholine receptor in sympathetic ganglia neurons (Listerud et al., 1991), and of the $GABA_A$ receptor in cultured visual cortical slices (Brussard and Baker, 1995). In the present study, the results are consistent with the selective deletion of the γ_2 subunit from $GABA_A$ receptors. Further studies will determine if this in vivo treatment results in a reduction in numbers of $GABA_A$ receptors, or modification of the subunit composition and function of the receptors.

Acknowledgements

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