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Mice Lacking the Long Splice Variant of the γ2 Subunit of the $GABA_A$ Receptor Are More Sensitive to Benzodiazepines

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QUINLAN, J. J., L. L. FIRESTONE AND G. E. HOMANICS. *Mice lacking the long splice variant of the γ2 subunit of the $GABA_A$ receptor are more sensitive to benzodiazepines*. PHARMACOL BIOCHEM BEHAV **66**(2) 371–374, 2000.—The γ2 subunit is required for benzodiazepine modulation of the $GABA_A$ receptor. Alternate splicing of precursor $GABA_A$ γ2 mRNA results in two splice variants, a short (γ2S) and a long (γ2L) variant. We investigated the roles of these splice variants in benzodiazepine pharmacology using mice lacking genes for the γ2L splice variant. Sleep time responses to midazolam and zolpidem were 20 and 18% greater, respectively, in null allele mice compared with wild-type mice, while responses to nonbenzodiazepine agents such as etomidate and pentobarbital were unchanged. Although the $GABA_A$ receptor number was not altered in null allele mice, there was a corresponding increase in affinity of brain membranes for benzodiazepine agonists (midazolam, diazepam, and zolpidem), while affinity for benzodiazepine inverse agonists (βCCM and Ro15-4513) was decreased. These changes were not observed in inbred mice of the parental strains (C57BL/6J and 129/SvJ) used to create the genetically altered mice, indicating that differences between γ2L null allele and wild-type mice were unlikely to be simply due to cosegregation of linked alleles. Absence of the γ2L splice variant increases the affinity of receptors for benzodiazepine agonists, and is associated with a modest increase in behavioral sensitivity to benzodiazepine agonists. Lack of the γ2L subunits may shift the $GABA_A$ receptor from an inverse agonist-preferring toward an agonist-preferring configuration. © 2000 Elsevier Science Inc.

$GABA_A$ receptor γ2 Subunit: γ2 splice variant Gene deletion Benzodiazepines Diazepam Midazolam Zolpidem βCCM Ro15-4513 Mice

GENE targeting techniques have enabled the creation of animals that lack specific genes such as those encoding $GABA_A$ receptor subunits (e.g., α1–6, β1–3, γ1–3, and δ). $GABA_A$ receptor subunit composition confers unique pharmacology. For example, mice lacking the γ2 subunit are insensitive to benzodiazepines, indicating that this subunit is critical to benzodiazepine sensitivity (2). Alternate splicing of the precursor γ2 mRNA results in two splice variants, a short and a long variant. The latter possesses eight additional amino acids (residues 338–345) in the intracellular loop between the third and fourth transmembrane domains that contain a consensus protein kinase C phosphorylation site (18). These splice variants have markedly different distributions in the brain. In the rat, the long splice variant is more abundant that the short variant in the inferior colliculus, medulla, and cerebellar Purkinje cells, while the opposite is true in the hippocampus, cerebral cortex, and olfactory bulb (3). The roles of these splice variants in benzodiazepine pharmacology are unknown. We previously created mice lacking the long splice variant of the γ2 $GABA_A$ receptor subunit (6). In the present study, we investigate the function of this particular splice variant in benzodiazepine pharmacology.

METHOD

Wild-type (γ2L +/+) and null allele (γ2L −/−) mice were created using homologous recombination and genotyped as previously described (6). Male and female mice (7–12 weeks of age) of the F4 generation with a mixed C57BL/6J and strain 129/Sv/SvJ genetic background were used. C57BL/6J and 129/SvJ mice of the same age range were obtained from Jackson Labs, Bar Harbor, ME.

Sleep time was measured in wild-type and null-allele mice of the same litters after intraperitoneal injection of midazolam (45 mg/kg), zolpidem (60 mg/kg), etomidate (20 mg/

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TABLE 1

SLEEP TIME OF γ2L WILD-TYPE AND NULL-ALLELE MICE

	Wild Type		Null Allelle	
Agent	*n*	Sleep Time (min)	*n*	Sleep Time (min)
Midazolam*	25	52.8 ± 3.4	22	66.0 ± 4.0
Zolpidem*	21	33.1 ± 1.6	22	40.5 ± 2.4
Etomidate	20	42.0 ± 4.8	16	43.1 ± 5.6
Pentobarbital	24	26.3 ± 2.7	23	28.4 ± 1.9

*$p < 0.05$.

kg), and pentobarbital (45 mg/kg) by a blinded observer using the endpoint of loss-of-righting reflex (n = 16–25 per group) (10). Data were compared using student's t-test.

Binding to mouse brain homogenates of ^{3}H-Ro15-4513, ^{3}H-muscimol, and ^{3}H-flumazenil, as well as inhibition of 5 nM ^{3}H-flumazenil binding by unlabeled midazolam, diazepam, zolpidem, and β-CCM were determined using an ultracentrifugation assay (12). Briefly, whole brains from four to five mice were harvested to prepare pooled brain membrane homogenates using ultracentrifugation. The membrane was washed with 0.05% Triton X-100 to remove endogenous GABA prior to experiments to determine ^{3}H-muscimol binding. Radiolabeled ligand was incubated with membrane protein suspension at room temperature for 20 min. Five individual determinations were performed at each concentration of radioligand per experiment. Nonspecific binding was determined in the presence of excess unlabeled ligand (either muscimol or flumazenil), also using five individual determinations. Inhibition of 5 nM ^{3}H-flumazenil binding by the benzodiazepine ligands was measured by coincubating with a competing ligand in a range of concentrations. Measurements were again performed in quintiplicate for each experiment. Membrane bound radioligand was separated from free radioligand using ultracentrifugation. The supernatant was sampled to determine free radioligand concentration, while the pellet was counted to determine bound radioligand using liquid scintillation counting. Specific binding data from at least four separate experiments were pooled and fit to a logistic equation, yielding half-effect concentrations (K_d, or K_i for inhibition experiments), slopes, maximal receptor number (B_{max}) and an estimate of their respective standard errors (17). Null-allele and wild-type parameters were statistically compared by referring the variance ratio to a standard normal distribution (1).

TABLE 2

BINDING CHARACTERISTICS OF γ2L WILD-TYPE AND NULL-ALLELE WHOLE BRAIN

	Wild Type (+/+)	Null Allele (−/−)
^{3}H-Flumazenil		
B_{max} (pmol/mg)	1.3 ± 0.1	1.4 ± 0.1
Slope	0.9 ± 0.2	0.9 ± 0.2
K_d (nM)	3.2 ± 0.6	4.7 ± 1.1
^{3}H-Ro15-4513		
B_{max} (pmol/mg)	2.0 ± 0.2	1.7 ± 0.2
Slope	0.7 ± 0.1	1.0 ± 0.1
K_d (nM)	21.8 ± 7.2	68.3 ± 12.3*
^{3}H-Muscimol		
B_{max} (pmol/mg)	3.9 ± 0.4	4.1 ± 0.2
Slope	0.7 ± 0.2	1.0 ± 0.2
K_d (nM)	9.8 ± 3.7	3.2 ± 0.3

*$p = 0.001$.

TABLE 3

INHIBITION OF ^{3}H-FLUMAZENIL BINDING IN γ2L WILD-TYPE AND NULL-ALLELE MOUSE BRAIN

	Wild Type (+/+)	Null Allele (−/−)
Midazolam		
Slope	1.0 ± 0.1	0.9 ± 0.1
K_I	7.3 ± 0.3	6.6 ± 0.2
Diazepam		
Slope	0.9 ± 0.1	0.9 ± 0.1
K_I	33.2 ± 1.3	28.7 ± 0.8*
Zolpidem		
Slope	0.8 ± 0.1	0.9 ± 0.1
K_I	49.0 ± 1.9	42.3 ± 1.5†
β-CCM		
Slope	0.8 ± 0.1	0.9 ± 0.1
K_I	17.2 ± 0.8	24.4 ± 1.3‡

*$p = 0.003$.
†$p = 0.006$.
‡$p < 0.001$.

RESULTS

Hypnotic responses of wild-type mice to drugs were similar to values previously reported by our lab (7,13). Sleep time after midazolam and zolpidem was 20 and 18% greater, respectively, in null-allele mice compared with wild-type mice (Table 1). Null-allele and wild-type mice did not differ in their responses to etomidate and pentobarbital (Table 1).

Benzodiazepine receptor density as determined by ^{3}H-flumazenil binding was essentially identical in the wild-type and null-allele mouse brain (Table 2). Parallel slopes allowed valid comparison of flumazenil affinity, which was also unchanged (Table 2). Affinity for inhibition of flumazenil binding by the benzodiazepine agonists midazolam, diazepam, and zolpidem was 10–14% greater in null allele mice (Table 3). Conversely, affinity for the inverse agonist βCCM and Ro15-4513 was substantially decreased (50% and threefold decrease, respectively) in null-allele mice; although affinity for ^{3}H-muscimol tended to be greater in null-allele mice, this did not achieve statistical significance ($p = 0.08$) (Table 2).

TABLE 4

BINDING CHARACTERISTICS OF C57BL/6J AND STRAIN 129/SvJ WHOLE BRAIN

	C57BL/6J	Strain 129/SvJ
^{3}H-Ro15-4513		
B_{max} (pmol/mg)	2.9 ± 0.4	3.4 ± 0.6
Slope	0.8 ± 0.2	1.1 ± 0.7
K_d (nM)	19.4 ± 6.9	15.2 ± 6.0
^{3}H-Flumazenil		
B_{max} (pmol/mg)	1.3 ± 0.1	1.0 ± 0.1
Slope	1.1 ± 0.3	1.2 ± 0.5
K_d (nM)	10.2 ± 3.7	3.6 ± 0.8*

*$p = 0.05$.

Binding of ^{3}H-Ro15-4513 to brain membranes of inbred mice of the parental strains used to create the genetically altered mice, namely C57BL/6J and 129/SvJ, were also indistinguishable (Table 4). Although the total number of benzodiazepine binding sites were similar in C57BL/6J and Strain 129/SvJ mice, affinity for ^{3}H-flumazenil was significantly greater in Strain 129/SvJ mice (Table 4), which precluded analysis of other benzodiazepine agents via inhibition of ^{3}H-flumazenil binding.

DISCUSSION

Although the identity of the α subunit present in a given $GABA_A$ receptor significantly modifies its benzodiazepine pharmacology (e.g., low affinity for benzodiazepine agonist when the α6 subunit is incorporated) (9), the presence of the γ subunit is even more crucial to benzodiazepine action. In the absence of the γ2 subunit, diazepam fails to produce sedation or loss-of-righting reflex (2). Different γ subunits also confer differences in benzodiazepine pharmacology. For example, Ro15-4513 has mild agonist effects at receptors containing γ1 subunits, but displays its usual inverse agonist effects at receptors containing γ2 subunits (15). The two variants of the γ2 subunit may also influence pharmacological profiles. Early work suggested that the phosphorylation site of the γ2L subunit was necessary for ethanol to potentiate GABA action (16), but this hypothesis remains controversial due to conflicting data (5,11,14). The finding that mice lacking the γ2L subunit do not differ in their behavioral and electrophysiologic responses to ethanol suggests the γ2L subunit is not absolutely required for ethanol to exert its effects (6). The importance of the γ2 splice variants in the behavioral effects of benzodiazepines is unknown. The γ2L wild-type and null-allele mice offer a unique tool to investigate the behavioral consequences of these splice variants.

Our data indicate that the γ2S subunits replace missing γ2L subunits to maintain a normal number of benzodiazepine sites in null-allele mice. Absence of the γ2L splice variant increases the affinity of receptors for benzodiazepine agonists, and is associated with a modest increase in behavioral sensitivity to benzodiazepine agonists. A three-state model (agonist-, antagonist-, and inverse agonist-preferring state) has been proposed to explain the interaction of $GABA_A$ receptors with different types of benzodiazepine ligands (4,12). The increase in affinity for the agonists midazolam, diazepam, and zolpidem, and decrease in affinity for the inverse agonist βCCM suggests that γ2S subunits might shift the $GABA_A$ receptor from an inverse agonist-preferring toward an agonist-preferring configuration. A similar switch from an inverse agonist-preferring to an agonist-preferring receptor has been observed in receptors containing a mutant α6 subunit that differs by four key amino acids from the wild-type receptor (19). Such a shift towards an benzodiazepine agonist-preferring conformation might be expected to result in a null allele animal with lower anxiety levels than the wild type. However, this is not the case. Our initial behavioral characterization of γ2L null-allele mice using an elevated plus-maze to measure baseline anxiety found that they had modest but significant reductions of both entries onto and percent of time spent on the open arms of the maze, suggesting that γ2L null-allele mice have higher baseline levels of anxiety (6). This difference in baseline anxiety levels complicates attempts to assay the relative sensitivity of null-allele and wild-type mice to inverse agonists such as βCCM or Ro15-4513 in vivo.

The vast majority of gene targeted mice are developed on a mixed genetic background of inbred mouse strains C57BL/6J and Strain 129. These strains differ in their responses to many drugs, due to the possession of polymorphic alleles, which determine their pharmacological responses (8). These differences are a potential confounding variable in the analysis of any gene-targeting experiment. If some of these alleles affect the phenotype that is being tested (e.g., sleep time), and if these alleles are genetically linked to the targeted mutation, then these linked alleles will cosegregate with the mutation and influence the response being tested. In this case, a difference in response between wild-type and null-allele mice may simply reflect the different underlying genetic backgrounds of the mouse strains in use, rather than a difference due to the targeted mutation. For this reason, the responses of wild-type and null-allele mice must be compared to the pattern of responses found in the parental mouse strains used. In the present study, genes linked to the γ2L mutation would be derived from Strain 129/SvJ, while genes linked to the wild-type γ2L gene would stem from C57BL/6J. We recently found that these two strains of mice have similar hypnotic responses to ethanol and pentobarbital; however, Strain 129/SvJ mice are markedly less sensitive to the hypnotic effects of midazolam, zolpidem, and propofol (sleep times 30, 65, and 52% of the C57BL/6J values, respectively), while Strain 129/SvJ are significantly more resistant to etomidate (30% greater sleep time than C57BL/6J) (8). Because the pattern of individual drug sensitivities of null-allele and wild-type mice does not parallel the responses of Strain 129/SvJ and C57BL/6J mice, it is unlikely that the differences between null-allele and wild-type mice are simply due to cosegregation of linked genes. The additional data that the greater sensitivity to benzodiazepine agonists observed in null allele mice is accompanied by a corresponding increase in agonist affinity and decreased in inverse agonist affinity strengthens this inference. Furthermore, affinity for inverse agonists in inbred 129/SvJ mice is similar to that of C57BL/6J mice, so it is extremely unlikely that the substantial decrease in inverse agonist affinity observed in null-allele mice reflects gene linkage.

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