

Deletion of the *fyn*-Kinase Gene Alters Sensitivity to GABAergic Drugs: Dependence on $\beta 2/\beta 3$ GABA_A Receptor Subunits

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ABSTRACT

Tyrosine phosphorylation can modulate GABA_A receptor function, and deletion of the *fyn*-kinase gene alters GABAergic function in olfactory bulb neurons, as reported by Kitazawa, Yagi, Miyakawa, Niki, and Kawai (*J Neurophysiol* 1998;**79**:137–142). Our goal was to determine whether *fyn* gene deletion altered behavioral and functional actions of compounds that act on GABA_A receptors. Such evidence might suggest a role for *fyn*-kinase in modulating GABA_A receptor function, possibly via direct interactions between the kinase and receptor. Using the loss of righting reflex test, we found that null mutants were less sensitive to the hypnotic effects of THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), a GABA_A receptor agonist. Subunit specificity was suggested by the observation that null

mutants were also less sensitive to the hypnotic effects of etomidate, a GABAergic compound that is selective for receptors possessing $\beta 2$ and/or $\beta 3$ receptor subunits. The genotypes did not differ in sensitivity to zolpidem, an $\alpha 1$ -selective GABAergic drug. GABA_A receptor functional assays ($^{36}\text{Cl}^-$ influx) supported our behavioral results; the actions of the GABA_A agonists, THIP and muscimol, were reduced in the cerebellar membranes of *fyn*-null mutant mice. Importantly, similar results were seen with etomidate. Binding of [^3H]flunitrazepam supported the idea that this is due to a decrease in functional GABA_A receptor density. These data suggest that *fyn*-kinase may alter the function of GABA_A receptors, perhaps via actions on $\beta 2$ and/or $\beta 3$ receptor subunits.

Fyn-kinase, a member of the *src* family of nonreceptor tyrosine kinases, is localized at the growth cones and postsynaptic membranes of neurons and can be found in a number of brain regions including the olfactory bulb, cerebellum, hippocampus, and limbic system (Yagi et al., 1993). Studies have shown that *fyn*-kinase phosphorylates the NMDA receptor (Suzuki and Okumura-Noji, 1995; Yaka et al., 2003), modulating its function. Indeed, Miyakawa et al. (1997) demonstrated that *fyn* phosphorylation of the NR2B subunit is necessary for the development of acute tolerance to alcohol's inhibitory effects on NMDA receptor-mediated currents in hippocampal neurons. These authors argued that this process explained the enhanced sensitivity to the hypnotic effects of alcohol seen in *fyn*-deficient mice; because *fyn*-kinase is not available to phosphorylate the NR2B subunit, these mice cannot develop acute tolerance to alcohol. However, *fyn*-kinase may also phosphorylate the GABA_A receptor, and this receptor system is known to modulate ethanol's hypnotic effects (Blednov et al., 2003). One study showed that granule cells in the olfactory bulb of *fyn*-defi-

cient mice exhibit reduced sensitivity to bicuculline and picrotoxin, antagonists at the GABA_A receptor (Kitazawa et al., 1998). Moreover, several studies have shown that *src* tyrosine kinase, closely related to *fyn*-kinase, interacts with or phosphorylates the $\gamma 2$ (Valenzuela et al., 1995; Brandon et al., 2001) and β (Valenzuela et al., 1995; Wan et al., 1997; Brandon et al., 2001) subunits of the GABA_A receptor. Thus, the available evidence suggests that deletion of the *fyn*-kinase gene may, in addition to the NMDA receptor, interact with the GABA_A receptor.

The present studies examined the potential interaction between *fyn*-kinase and GABA_A receptors in *fyn*-null mutant and wild-type mice. We assessed hypnotic sensitivity to the GABA_A receptor agonist, tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), and the GABA_A receptor positive allosteric modulators, alfaxalone, pentobarbital, and flurazepam. Because we were interested in establishing whether the changes in GABAergic sensitivity were subunit-selective, we also assessed the hypnotic effects of the $\alpha 1$ -selective drug, zolpidem, and the $\beta 2/\beta 3$ -selective drug, etomidate. GABA_A receptor function was assessed by measuring muscimol and THIP stimulation, as well as etomidate potentiation of muscimol-stimulated, $^{36}\text{Cl}^-$ flux in *fyn*-deficient and wild-type mice. GABA_A receptor binding using [^3H]flunitrazepam was also performed in *fyn*-null mutant and wild-type mice.

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol.

Materials and Methods

Animals. *Fyn*-kinase null mutant (B6;129S-*Fyn*^{tm1Sor}) and control mice (B6129SF2/J) were obtained from The Jackson Laboratories (Bar Harbor, ME) and bred to generate the mice used in the present studies. Stein et al. (1992) originally developed these mice. Heterozygote mating pairs were maintained to produce wild-type, heterozygous, and homozygous littermates. To minimize the possible effect of genetic background, littermates were used in the present studies. For most experiments, both male and female mice were tested. However, due to availability only female mice were tested in several experiments. Mice were housed in same-sex groups, four to five to a cage. Food and water were available ad libitum. The vivarium was maintained on a 12:12-h light/dark cycle with lights on at 7:00 AM. The temperature and humidity of the room were maintained at 20°C and 50%, respectively. All experiments were performed during the light phase of the light/dark cycle. Behavioral testing began when the mice were at least 3 months and not more than 6 months old.

Drugs. THIP and alfaxalone were obtained from Sigma-Aldrich (St. Louis, MO). Flurazepam and diazepam were purchased from Hoffman-LaRoche (Nutley, NJ), pentobarbital from Sigma/RBI (Natick, MA), and etomidate from Tocris Cookson Inc. (Ellisville, MO). Zolpidem was a generous gift from Dr. J. S. Vedo (Pharmacia, Skokie, IL). For behavioral experiments, THIP and pentobarbital were dissolved in 0.9% saline (20% v/v). Flurazepam, zolpidem, and etomidate were suspended in 3 to 4 drops of Tween 80 (Sigma-Aldrich) before dissolving in saline. Alfaxalone was dissolved in 15% 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich). For the $^{36}\text{Cl}^-$ flux and binding assays, THIP, etomidate, and diazepam were dissolved in assay buffer (see below).

Loss of Righting Reflex. Animals were injected with THIP (30 or 50 mg/kg, s.c.), and the length of ethanol-induced loss of righting reflex (sleep-time) was measured. Upon loss of the righting reflex, mice were placed on their backs in a sleep trough (~90° angle), and the time to regain the righting reflex was measured. Loss of righting reflex was defined as the inability of a mouse to right itself within 30 s. Return of the righting response was defined as the ability of a mouse to right itself twice in 1 min. Sleep-time, or duration of loss of righting reflex, was defined as the time between loss and return of the righting response. Sensitivity to the hypnotic effects of pentobarbital (50 mg/kg, i.p.), flurazepam (180 mg/kg, i.p.), alfaxalone (70 mg/kg, i.p.), zolpidem (45 mg/kg, i.p.), and etomidate (20 mg/kg, i.p.) was also assessed using similar procedures.

Because regain of the righting reflex is measured at long intervals (in some cases as long as several hours) after drug administration, it is possible that acute tolerance, and not sensitivity per se, can produce genotypic differences. Thus, we assessed initial sensitivity to etomidate-induced loss of righting reflex using the up-and-down method described by Dixon (1965). Each mouse was injected with a given drug dose and placed in the V-shaped troughs used above. For this test, loss of righting reflex was defined as an inability to right for 1 min within 5 min of drug administration. The hypnotic effect of the given dose determined the drug dose that the next animal received (i.e., if the mouse was unable to right itself then the dose of etomidate was decreased by a log interval, or if the mouse successfully righted itself the etomidate dose was increased by a log interval). ED_{50} values were determined by the following equation: 95% confidence interval = dosing increment $\times \sqrt{2/n} \times 1.96$, in which n = the last n trials and 1.96 reflects the 0.05 α level (Dixon and Massey, 1969).

GABA_A Receptor Functional Assay. Isolated cortical or cerebellar membrane vesicles (microsacs) were prepared, and muscimol- or THIP-mediated $^{36}\text{Cl}^-$ uptake was assayed (Harris and Allan, 1985). The tissue was homogenized in 4.5 ml of ice-cold assay buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM D-glucose, 10 mM Hepes, adjusted to pH 7.5 with Tris base). The final pellet was suspended in assay buffer and tissue aliquots (0.6–1.5 mg of protein) were incubated in a 34°C water bath for 5 min.

Uptake was initiated by adding 200 μl of $^{36}\text{Cl}^-$ solution (2 $\mu\text{Ci}/\text{ml}$ of assay buffer: 1 μCi = 37 kBq; MP Biomedicals, Irvine, CA) containing the drugs to be tested. Three seconds after the addition of $^{36}\text{Cl}^-$, influx was terminated by adding 4 ml of ice-cold quench buffer (assay buffer with 100 μM picrotoxin) followed by rapid filtration through a GB100R filter (Advantec MFS, Dublin, CA), and subsequent washing with 8 ml of quench buffer. Filters were incubated overnight in 4 ml of Biosafe II scintillation liquid (Research Products International, Mount Prospect, IL) before analysis in a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). The amount of $^{36}\text{Cl}^-$ bound to the filters in the absence of membranes (no-tissue blank) was subtracted from all values. Muscimol- or THIP-dependent influx was defined as the amount of $^{36}\text{Cl}^-$ taken up when agonist was present in the assay medium (total uptake) minus the amount of $^{36}\text{Cl}^-$ uptake when agonist was not present (muscimol- or THIP-independent or nonspecific uptake). Etomidate potentiation of muscimol-stimulated $^{36}\text{Cl}^-$ flux was also assessed. Etomidate concentrations were 1, 3, and 10 μM . These concentrations were chosen based on a pilot study and because they are very near the ED_{50} for etomidate's anesthetic effects in vivo (Tomlin et al., 1998).

[³H]Flunitrazepam Binding. Cortical or cerebellar tissue was harvested from female *fyn*-deficient and wild-type mice, and GABA_A receptor binding was performed using [³H]flunitrazepam (PerkinElmer Life and Analytical Sciences, Boston, MA). Tissue was homogenized in 25 ml of ice-cold assay buffer (50 mM Tris, 25 mM Hepes, pH 7.4) and centrifuged twice at 20,000 rpm for 10 min (4°C). Final pellets were suspended in ice-cold assay buffer, and binding was initiated by adding 200- μl aliquots of cortical tissue (100–200 μg of protein) to a reaction mixture containing 200 μl of ice-cold assay buffer, 50 μl of [³H]flunitrazepam (84.5 Ci/mmol; 1, 3, 10, 30, or 100 nM), and 50 μl of additional ice-cold assay buffer (nonspecific binding) or 100 μM diazepam (total binding). The reaction mixture was allowed to incubate for 60 min at 4°C and was terminated by the addition of 2 ml of ice-cold assay buffer followed by rapid filtration through a GB100R filter (Advantec MFS) and subsequent washing with ice-cold assay buffer. Filters were incubated overnight in 4 ml of Biosafe II scintillation liquid (Research Products International) before analysis in a Beckman LS 6500 scintillation counter (Beckman Coulter). Specific binding was calculated by subtracting nonspecific binding from total binding. K_d and B_{max} values were calculated using the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

Statistical Analysis. Preliminary analyses (analysis of variance) performed for data sets that included both males and females did not indicate significant interactions of gender with any other factor. Thus, male and female data were combined for experiments in which both genders were tested. Data are reported as mean \pm S.E.M. (except initial sensitivity to loss of righting reflex data, which is reported as $\text{ED}_{50} \pm 95\%$ confidence interval), and were analyzed using two-tailed t test or two-way analysis of variance. Post hoc comparisons were made where appropriate.

Results

Loss of Righting Reflex. To assess whether sensitivity to GABAergic drugs was altered by null mutation of the *fyn*-kinase gene, duration of loss of righting reflex was measured following administration of THIP (GABA_A receptor partial agonist) and the nonsubunit-selective allosteric modulators pentobarbital, flurazepam, and alfaxalone. *Fyn*-null mutant mice were less sensitive to the hypnotic effects of THIP as they exhibited a shorter duration of loss of righting reflex compared with their wild-type counterparts (Fig. 1). However, the genotypes did not significantly differ in sensitivity to the hypnotic effects of pentobarbital, flurazepam, or alfaxalone (Table 1).

We next wanted to establish whether the reduced sensitiv-

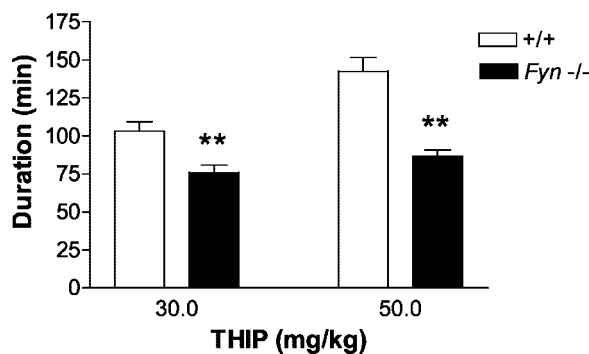


Fig. 1. Sensitivity to the hypnotic effects of THIP in female *fyn*-kinase-null mutant and wild-type mice. THIP (30 or 50 mg/kg; i.p.) was injected, and loss of the righting reflex was assessed. Values represent mean \pm S.E.M. *Fyn*-deficient mice were less sensitive to the hypnotic effects of THIP (interaction of genotype and treatment, $F[1,28] = 5.5$, $p < 0.03$). Null mutants exhibited shorter durations of loss of the righting reflex following both THIP doses (Bonferroni post-tests, p values < 0.01 ; $n = 7-9$ per genotype and treatment).

TABLE 1

Sensitivity to the hypnotic effects of several other GABAergic drugs in *fyn*-kinase-null mutant and wild-type mice

	Wild-Type	Null Mutant
Pentobarbital 50 mg/kg	83.9 \pm 6.1	93.4 \pm 8.6
Flurazepam 180 mg/kg	98.8 \pm 13.8	96.1 \pm 10.6
Alfaxalone 70 mg/kg	67.4 \pm 7.7	59.9 \pm 4.2

Values represent duration of loss of righting reflex (min) and are given as mean \pm S.E.M. ($n = 10-18$ per genotype).

ity to THIP in *fyn*-null mutant mice might be subunit-specific. We measured hypnotic sensitivity to the $\alpha 1$ -selective drug, zolpidem, and the $\beta 2/\beta 3$ -selective drug, etomidate. *Fyn*-null mutant and wild-type mice did not differ in sensitivity to zolpidem (Fig. 2A). However, *fyn*-deficient mice were less sensitive to the hypnotic effects of etomidate (Fig. 2B). These results suggest that $\beta 2$ and/or $\beta 3$ receptor subunits are important mediators of the effects of *fyn* gene deletion on GABAergic systems.

Because the intervals between drug injection and test endpoint render the traditional regaining of righting reflex incapable of separating the roles of initial sensitivity and acute tolerance, we determined the ED_{50} for etomidate-induced loss of righting reflex. Initial sensitivity (ED_{50}) was defined as loss of righting reflex within 5 min of etomidate injection. *Fyn*-null mutant and wild-type mice exhibited similar ED_{50} values for etomidate-induced loss of righting reflex (8.9 ± 1.1 and 9.3 ± 1.1 mg/kg for null mutants and wild-types, respectively), suggesting that null mutants do not differ in initial sensitivity but may instead develop greater acute tolerance to etomidate.

GABA_A Receptor Functional Assay. Our behavioral data suggested that GABA_A receptor function may have been altered by null mutation of the *fyn*-kinase gene in mice. *Fyn*-deficient mice were less sensitive to muscimol-stimulated $^{36}Cl^-$ flux in cerebellar microsacs ($EC_{50} \pm 95\%$ confidence interval, 1.2 ± 0.2 and 0.9 ± 0.2 μM for null mutant and wild-type, respectively; $E_{max} \pm$ S.E.M., 6.2 ± 0.5 and 8.5 ± 0.6 nmol/mg protein for null mutants and wild-types, respectively) (Fig. 3B). However, *fyn*-null mutant and wild-type mice did not differ in sensitivity to muscimol stimula-

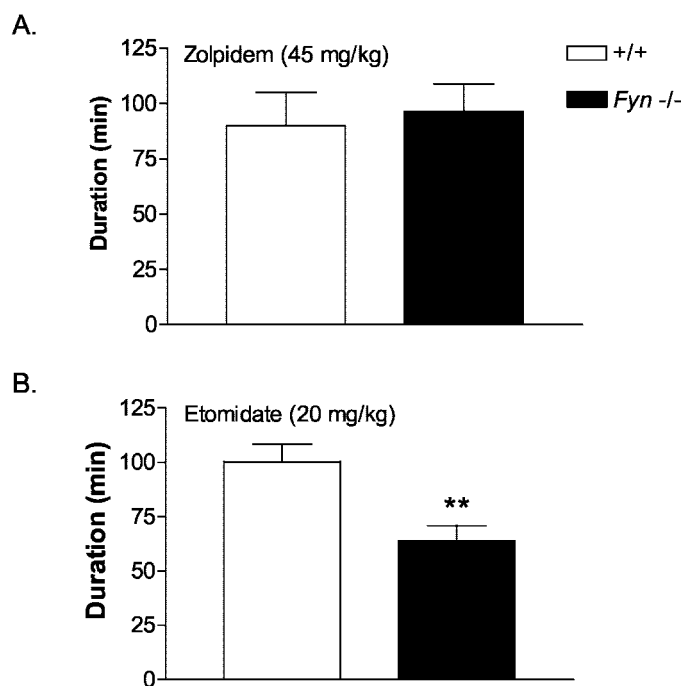


Fig. 2. Sensitivity to the hypnotic effects of zolpidem ($\alpha 1$ -selective) and etomidate ($\beta 2/\beta 3$ -selective). *fyn*-Kinase-null mutants were injected (i.p.) with 45 mg/kg zolpidem or 20 mg/kg etomidate, and loss of the righting reflex was measured ($n = 10$ per genotype). Values represent mean \pm S.E.M. A, *fyn*-null mutant and wild-type mice did not differ in sensitivity to the hypnotic effects of zolpidem. B, the duration of loss of the righting reflex was reduced in *fyn*-null mutant mice following administration of the $\beta 2/\beta 3$ -selective drug, etomidate ($t[17] = 3.4$, $p < 0.01$; $n = 9-10$ per genotype).

tion of $^{36}Cl^-$ flux in cortical microsacs ($EC_{50} \pm 95\%$ confidence interval, 8.0 ± 1.1 and 7.3 ± 1.2 μM for null mutant and wild-type, respectively; $E_{max} \pm$ S.E.M., 21.5 ± 1.7 and 21.9 ± 2.4 nmol/mg protein for null mutant and wild-type, respectively) (Fig. 3A).

Similar results were obtained with the GABA_A agonist, THIP. *Fyn*-null mutant and wild-type mice also did not differ in sensitivity to THIP-stimulated $^{36}Cl^-$ flux in cortical microsacs (data not shown). However, when the actions of low and maximal concentrations of THIP (0.1 and 3 mM) were tested in cerebellar membranes, *fyn*-null mutants exhibited reduced THIP-stimulated $^{36}Cl^-$ flux (main effect of genotype, $F[1,64] = 15.9$, $p < 0.001$; $n = 10$ per genotype). Values were 3.2 ± 0.2 and 4.2 ± 0.7 nmol/mg protein following application of 0.1 mM THIP, and 7.3 ± 0.5 and 9.5 ± 0.8 nmol/mg protein following application of 3 mM THIP, for null mutant and wild-type mice, respectively. Although the interaction just missed statistical significance ($p = 0.07$), follow-up tests showed that the actions of THIP were reduced following application of 3 mM THIP ($t[18] = 2.4$, $p < 0.03$).

The above functional results suggest that deletion of the *fyn*-kinase gene altered GABA_A receptor function. Furthermore, our behavioral data suggest that the alteration may have involved the $\beta 2$ and/or $\beta 3$ receptor subunits. Thus, we next wanted to assess the actions of etomidate on GABA_A receptor function in *fyn*-null mutant and wild-type mice. The actions of etomidate were reduced at concentrations at or below the ED_{50} for its anesthetic effects in the cerebellar microsacs of *fyn*-null mutant mice (Fig. 4B). However, similar to that seen after muscimol and THIP application, etomi-

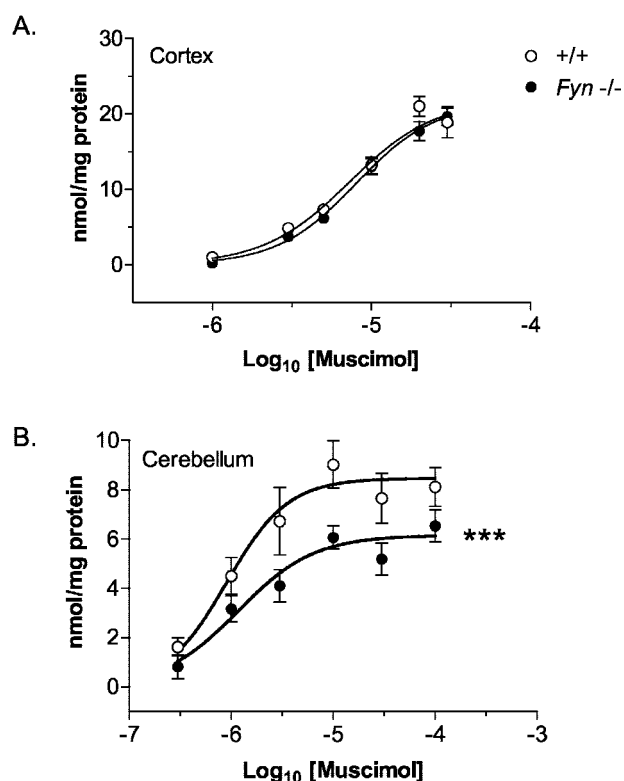


Fig. 3. Muscimol-stimulated $^{36}\text{Cl}^-$ flux in *fyn*-kinase-null mutant and wild-type mice. A, muscimol-stimulated flux in cortical membranes did not differ between *fyn*-null mutant and wild-type mice. B, *fyn* gene deletion reduced muscimol-stimulated $^{36}\text{Cl}^-$ flux in cerebellar membranes (main effect of genotype, $F[1,64] = 15.9$, $p < 0.001$; $n = 11$ per genotype).

date did not differentially alter muscimol-stimulated chloride flux in cortical microsacs (Fig. 4A). These results are in agreement with our behavioral data and strongly implicate the $\beta 2$ and/or $\beta 3$ receptor subunits in the interaction between *fyn*-kinase and GABAergic systems.

[^3H]Flunitrazepam Binding. Because cerebellar GABA_A receptor function was altered, we measured [^3H]flunitrazepam binding to cerebellar ($n = 7-8$) and cortical ($n = 3$) membranes from *fyn*-null mutant and wild-type mice (Fig. 5). Null mutants exhibited reduced cerebellar [^3H]flunitrazepam binding. Cerebellar B_{max} values were lower (1094 ± 80 and 1588 ± 113 fmol/mg for null mutants and wild-types, respectively), but K_d values did not differ between the genotypes (6.7 ± 1.9 and 5.3 ± 1.5 nM for null mutants and wild-types, respectively). The cortical binding curves were similar for both genotypes (K_d , 6.8 ± 1.5 and 5.9 ± 1.4 nM for null mutants and wild-types, respectively; B_{max} , 1534 ± 90 and 1414 ± 87 fmol/mg for null mutants and wild-types, respectively).

Discussion

The goal of the present studies was to determine whether *fyn* gene deletion altered the behavioral and functional actions of drugs that act at GABA_A receptors. To this end, we obtained our own colony of *fyn*-null mutant and wild-type mice. Similar to the results of Miyakawa et al. (1997) using mice that were developed by a different research group, our null mutants were more sensitive to the hypnotic effects of ethanol (Boehm et al., 2003), a compound known to enhance GABA_A receptor function. Moreover, although the genotypes

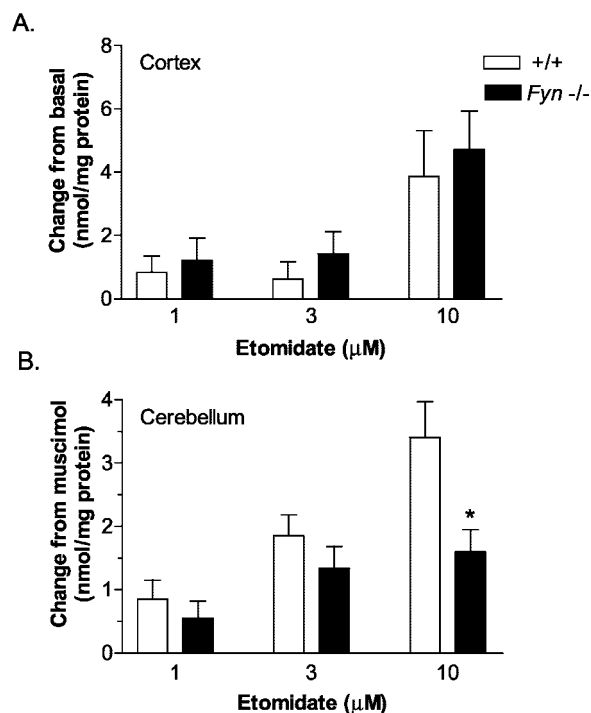


Fig. 4. Etomidate potentiation of muscimol-stimulated $^{36}\text{Cl}^-$ flux in *fyn*-kinase-null mutant and wild-type mice. The actions of 1, 3, and 10 μM etomidate on 0.2 or 0.5 μM muscimol concentrations were assessed. The above etomidate concentrations were chosen based on pilot studies and because they are at or below the ED_{50} for etomidate's in vivo anesthetic effects. The above muscimol concentrations were chosen based on pilot studies; they represent the most effective concentrations for detecting the potentiating effects of etomidate in cortical and cerebellar tissue, respectively. A, *fyn*-null mutant and wild-type mice did not differ in etomidate potentiation of 0.2 μM muscimol-stimulated chloride flux in cortical microsacs (11–12 per genotype). B, deletion of the *fyn*-kinase gene reduced etomidate potentiation of 0.5 μM muscimol-stimulated chloride flux in cerebellar microsacs (main effect of genotype, $F[1,42] = 7.8$, $p < 0.01$; $n = 10$ per genotype). Although the interaction was not significant, individual t tests revealed that *fyn* gene deletion resulted in reduced 10 μM etomidate potentiation of muscimol-stimulated flux in cerebellum ($t[6] = 2.7$, $p < 0.05$).

did not differ in hypnotic sensitivity to the positive allosteric modulators pentobarbital, flurazepam, and alfaxalone, our *fyn*-deficient mice exhibited a shorter duration of loss of righting reflex following administration of the GABA_A receptor agonist, THIP.

The above results were consistent with those of Kitazawa et al. (1998) and suggested that *fyn* gene deletion altered GABA_A receptor function, so we next assessed the actions of THIP using a functional assay. THIP-stimulated $^{36}\text{Cl}^-$ flux was reduced in the cerebellar microsacs of *fyn*-null mutant mice, consistent with their reduced hypnotic sensitivity. Moreover, the actions of another GABA_A receptor agonist, muscimol, were also reduced. No such changes were observed in cortical membranes.

Our results suggest that *fyn*-kinase may phosphorylate GABA_A receptors in the cerebellum, altering their function. However, there is currently no direct evidence indicating that *fyn* interacts with this receptor or any another protein important for GABAergic function. Indeed, it is possible that the resulting GABA-related phenotypes may have resulted from *fyn*'s absence during neuronal development (Yagi, 1999); *fyn*-kinase is expressed during neuronal cell migration

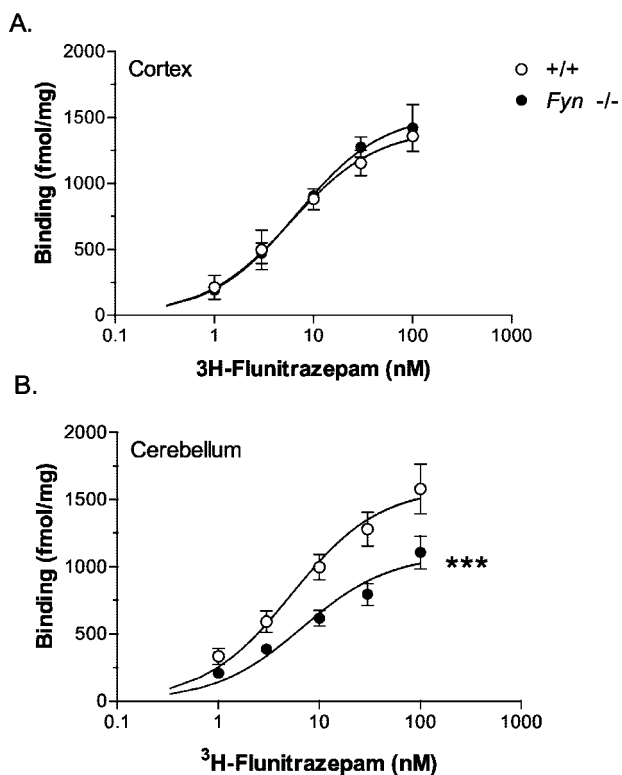


Fig. 5. [^3H]Flunitrazepam binding in *fyn*-null mutant and wild-type mice. A, [^3H]flunitrazepam in cortical membranes did not differ between female *fyn*-null mutant and wild-type mice. B, *fyn* gene deletion reduced [^3H]flunitrazepam binding in the cerebellar membranes of female mice (main effect of genotype, $F[1,65] = 27.0, p < 0.001; n = 7-8$ per genotype).

and differentiation, axon guidance, target recognition, and synaptogenesis (Yagi, 1994).

One way to distinguish between these possibilities is to assess sensitivity to subunit specific drugs. We reasoned that if *fyn* was necessary for the normal development of GABA_A receptor systems, then differences in GABAergic sensitivity observed between *fyn*-deficient and wild-type mice would likely not exhibit subunit selectivity. We initially chose to assess behavioral sensitivity to two different GABA_A receptor drugs, the $\alpha 1$ -selective benzodiazepine, zolpidem, and the $\beta 2/\beta 3$ -selective agonist, etomidate. The genotypes did not differ in sensitivity to zolpidem. However, *fyn*-null mutants were less sensitive to the hypnotic effects of etomidate. These results indicate that *fyn*-kinase alters GABAergic function and that $\beta 2$ and/or $\beta 3$ receptor subunits are important for this effect. Moreover, the genotypes did not differ in initial sensitivity to etomidate's hypnotic effects. This observation suggests that *fyn*-null mutants may develop greater acute tolerance to etomidate, a process that likely involves phosphorylation of GABA_A receptors.

If *fyn*-kinase phosphorylates the $\beta 2$ and/or $\beta 3$ receptor subunits, then one would predict an associated change in GABA_A receptor function. *Fyn*-null mutants were less sensitive to the actions of the GABA_A receptor agonists, muscimol and THIP. However, these studies do not necessarily implicate $\beta 2$ and/or $\beta 3$ receptor subunits, so we measured the actions of etomidate. Etomidate potentiation of muscimol-stimulated $^{36}\text{Cl}^-$ flux was reduced in the cerebellar microsacs of *fyn*-deficient mice. These results are consistent with the reduced hypnotic sensitivity to etomidate seen in *fyn*-null

mutants, and strongly suggest that $\beta 2$ and/or $\beta 3$ receptor subunits are important in *fyn*'s effects on GABAergic function. Moreover, they support a scenario in which *fyn*-kinase phosphorylates the $\beta 2$ and/or $\beta 3$ receptor subunits. There is precedence for tyrosine kinase phosphorylation of specific GABA_A receptor subunits. Indeed, *src* tyrosine kinase (related to *fyn*) phosphorylates the GABA_A $\gamma 2$ (Valenzuela et al., 1995; Brandon et al., 2001) and β (Valenzuela et al., 1995; Wan et al., 1997; Brandon et al., 2001) subunits. Studies aimed at determining whether *fyn*-kinase phosphorylates $\beta 2$ and/or $\beta 3$ receptor subunits are underway in our laboratory.

Behavioral data suggest that $\beta 3$ receptor subunits may be more important targets for *fyn* than $\beta 2$ receptor subunits. $\beta 3$ -null mutant mice were less sensitive to pentobarbital (Quinlan et al., 1998), and mice in whom a point mutation was introduced into the $\beta 3$ subunit gene were much less sensitive to the hypnotic effects of etomidate but did not differ in hypnotic sensitivity to alfaxalone (Jurd et al., 2003). In contrast, mice with the same mutation in the $\beta 2$ subunit gene differed in sensitivity to the sedative but not hypnotic effects of etomidate (Reynolds et al., 2003), and Blednov et al. (2003) showed that $\beta 2$ -null mutant mice were less sensitive to the hypnotic effects of flurazepam, THIP, etomidate, and zolpidem, but not pentobarbital. Thus, our behavioral data are most consistent with that from $\beta 3$ mutant mice.

A potential problem in attributing our findings to *fyn*'s actions at $\beta 3$ receptor subunits is that muscimol and THIP do not appear to exhibit β subunit specificity. However, Ebert et al. (1997) reported that muscimol exhibited greater efficacy at $\alpha 6$ -containing receptors ($\alpha 6 > \alpha 5 = \alpha 2 > \alpha 1 > \alpha 3$), and other studies demonstrated that THIP has a greater efficacy than GABA at receptors composed of $\alpha 6\beta x\gamma 2s$, that it is a full agonist at receptors composed of $\alpha 5\beta x\gamma 2s$, and that it is only a partial agonist at receptors composed of $\alpha 1-4\beta x\gamma 2s$ (Ebert et al., 1994; Wafford et al., 1996). Here we report that *fyn*-kinase-null mutants were less sensitive to the functional actions of muscimol and less sensitive to the behavioral and functional actions of THIP. Considered along with evidence that cerebellar GABA_A receptors are composed of $\alpha 1\beta x\gamma 2$, $\alpha 6\beta x\gamma 2$, and $\alpha 6\beta x\delta$ containing receptors (Saxena and Macdonald, 1996; Pörtl et al., 2003), that *fyn*-null mutants do not differ in hypnotic sensitivity to the $\alpha 1$ -selective benzodiazepine, zolpidem (current study), and that [^3H]muscimol-labeled cerebellar GABA_A receptors are primarily composed of $\alpha 6\beta x\delta$ (Quirk et al., 1994), we speculate that *fyn*-kinase interacts with $\beta 3$ -containing GABA_A receptors that also possess $\alpha 6$ and δ subunits. If true, it might explain how sensitivity to compounds that lack β subunit specificity might also have been altered in *fyn*-kinase-null mutant mice; mutated *fyn* was incapable of phosphorylating receptors composed of these subunits, resulting in the reduced actions of muscimol and THIP. Interestingly, $\alpha 6\beta 3\delta$ -containing GABA_A receptors were recently implicated as being highly sensitive to the enhancing effects of ethanol (Wallner et al., 2003).

Despite the reduced number of benzodiazepine ([^3H]flunitrazepam) binding sites in the cerebellum of *fyn*-null mutant mice, we did not observe changes in hypnotic sensitivity to zolpidem or flurazepam. Although puzzling, the subunit selectivity of these drugs might offer an explanation (Smith, 2001). For example, zolpidem preferentially binds GABA_A receptors that possess $\alpha 1$ subunits. We hypothesize that *fyn*-

kinase interacts with GABA_A receptors that possess $\alpha 6$ and not $\alpha 1$ subunits. Therefore, we might not expect to see genotypic differences in hypnotic sensitivity to zolpidem. However, it is currently unknown whether flunitrazepam or flurazepam exhibit any subunit selectivity.

An intriguing finding from our functional studies was that the actions of muscimol, THIP, and etomidate were reduced in cerebellar but not cortical membranes. Insofar as the righting reflex involves motor control, this finding was not surprising. Cerebellar nuclei have long been known to mediate motor functions (Miall, 1998). Moreover, the available data suggest that etomidate's hypnotic effects are likely mediated by $\beta 3$ -containing GABA_A receptors (Jurd et al., 2003) and that these subunits are more abundant in cerebellum compared with cortex (Fritschy and Mohler, 1995). Nevertheless, $\beta 3$ receptor subunits are found in cortex, and it is not clear why we did not detect changes in GABA_A receptor function in this brain region. It is interesting to note, however, that we also did not detect genotypic differences in cortical [³H]flunitrazepam binding.

It is tempting to speculate as to which cerebellar cell types might be important for imparting fyn's effects on GABAergic function. An early paper reported that ethanol depression of Purkinje cell activity correlated with enhanced hypnotic sensitivity to ethanol using the loss of righting reflex test (Johnson et al., 1985). Indeed, fyn-kinase has been localized to cerebellar Purkinje cells (Seykora et al., 2002), and mRNA coding for $\beta 3$ subunits have also been localized to these neurons (Laurie et al., 1992; Zdilar et al., 1992). However, more recent studies examining protein expression have localized $\beta 3$ subunits to cerebellar granule cells and not Purkinje cells (Fritschy and Mohler, 1995; Pirker et al., 2000). Thus, the literature is not in agreement on the precise localization of cerebellar $\beta 3$ receptor subunits. Nevertheless, as fyn-kinase has also been localized to cerebellar granule cells, it could presumably interact with $\beta 3$ subunits to alter GABAergic function in either cell type.

In conclusion, deletion of the fyn-kinase gene in mice appears to have altered sensitivity to several GABAergic drugs, likely by a mechanism that includes $\beta 2$ and/or the $\beta 3$ receptor subunits. Although our behavioral and functional data do not disprove the alternative hypothesis that development of GABAergic systems was disrupted in the mutant mice, they support the view that fyn-kinase phosphorylates GABA_A receptors via direct or indirect actions at $\beta 3$ receptor subunits. Ongoing studies in our laboratory continue to probe this possibility.

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References

- Blednov YA, Jung S, Alva H, Wallace D, Rosahl T, Whiting PJ, and Harris RA (2003) Deletion of the $\alpha 1$ or $\beta 2$ subunit of GABA_A receptors reduces actions of alcohol and other drugs. *J Pharmacol Exp Ther* **304**:30–36.
- Boehm SL 2nd, Peden L, Chang R, Harris RA, and Blednov YA (2003) Deletion of the fyn-kinase gene alters behavioral sensitivity to alcohol. *Alcohol Clin Exp Res* **27**:1033–1040.
- Brandon NJ, Delmas P, Hill J, Smart TG, and Moss SJ (2001) Constitutive tyrosine phosphorylation of the GABA_A receptor $\gamma 2$ subunit in rat brain. *Neuropharmacology* **41**:745–752.
- Dixon WJ (1965) The up-and-down method for small samples. *J Am Stat Assoc* **60**:967–978.
- Dixon WJ and Massey FJ (1969) Sensitivity experiments, in *Introduction to Statistical Analysis*, pp 377–394, McGraw Hill, New York.

- Ebert B, Thompson SA, Saounatsou K, McKernan R, Krogsgaard-Larsen P, and Wafford KA (1997) Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human γ -aminobutyric acid type A receptors. *Mol Pharmacol* **52**:1150–1156.
- Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P, and Kemp JA (1994) Molecular pharmacology of γ -aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α , β and γ receptor subunit combinations. *Mol Pharmacol* **46**:957–963.
- Fritschy JM and Mohler H (1995) GABA_A-receptor heterogeneity in adult rat brain: Differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359**:154–194.
- Harris RA and Allan AM (1985) Functional coupling of γ -aminobutyric acid receptors to chloride channels in brain membranes. *Science (Wash DC)* **228**:1108–1110.
- Johnson SW, Hoffer BJ, Baker R, and Freedman R (1985) Correlation of Purkinje neuron depression and hypnotic effects of ethanol in inbred strains of rats. *Alcohol Clin Exp Res* **9**:56–58.
- Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, et al. (2003) General anesthetic actions in vivo strongly attenuated by a point mutation in the GABA_A receptor beta3 subunit. *FASEB J* **17**:250–252.
- Kitazawa H, Yagi T, Miyakawa T, Niki H, and Kawai N (1998) Abnormal synaptic transmission in the olfactory bulb of fyn-kinase-deficient mice. *J Neurophysiol* **79**:137–142.
- Laurie DJ, Wisden W, and Seeburg PH (1992) The distribution of thirteen GABA_A receptor subunit mRNAs in rat brain. III. Embryonic and postnatal development. *J Neurosci* **12**:4151–4172.
- Miall RC (1998) The cerebellum, predictive control and motor coordination. *Novartis Found Symp* **218**:272–290.
- Miyakawa T, Yagi T, Kitazawa H, Yasuda M, Kawai N, Tsuboi K, and Niki H (1997) Fyn-kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. *Science (Wash DC)* **278**:698–701.
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, and Sperk G (2000) GABA_A receptors: immunocytochemical distribution of 13 subunits in adult rat brain. *Neuroscience* **101**:815–850.
- Pörtl A, Hauer B, Fuchs K, Tretter V, and Sieghart W (2003) Subunit composition and quantitative importance of GABA_A receptor subtypes in the cerebellum of mouse and rat. *J Neurochem* **87**:1444–1455.
- Quinlan JJ, Homanics GE, and Firestone LL (1998) Anesthesia sensitivity in mice that lack the $\beta 3$ subunit of the γ -aminobutyric acid type A receptor. *Anesthesiology* **88**:775–780.
- Quirk K, Gillard NP, Ragan CI, Whiting PJ, and McKernan RM (1994) Model of subunit composition of γ -aminobutyric acid type A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. *J Biol Chem* **269**:16020–16028.
- Reynolds DS, Rosahl TW, Cirone J, O'Meara GF, Haythornthwaite A, Newman RJ, Myers J, Sur C, Howell O, Rutter AR, et al. (2003) Sedation and anesthesia mediated by distinct GABA_A receptor isoforms. *J Neurosci* **23**:8608–8617.
- Saxena NC and Macdonald RL (1996) Properties of putative cerebellar γ -aminobutyric acid A receptor isoforms. *Mol Pharmacol* **49**:567–579.
- Seykora JT, Mei L, Dotto GP, and Stein PL (2002) Srcasm: a novel src activating and signaling molecule. *J Biol Chem* **277**:2812–2822.
- Smith TA (2001) Type A gamma-aminobutyric acid (GABA_A) receptor subunits and benzodiazepine binding: significance to clinical syndromes and their treatment. *Br J Biomed Sci* **58**:111–121.
- Stein PL, Lee HM, Rich S, and Soriano P (1992) pp59^{lyn} mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* **70**:741–750.
- Suzuki T and Okumura-Noji K (1995) NMDA receptor subunits $\epsilon 1$ (NR2A) and $\epsilon 2$ (NR2B) are substrates for Fyn in the postsynaptic density fraction isolated from the rat brain. *Biochem Biophys Res Commun* **216**:582–588.
- Tomlin SL, Jenkins A, Lieb WR, and Franks NP (1998) Stereoselective effects of etomidate optical isomers on γ -aminobutyric acid type A receptors and animals. *Anesthesiology* **88**:708–717.
- Valenzuela JL, Machu TK, McKernan RM, Whiting P, VanRenterghem BB, McManaman CJ, Brozowski SJ, Smith GB, Olsen RW, and Harris RA (1995) Tyrosine kinase phosphorylation of GABA_A receptors. *Mol Brain Res* **31**:165–172.
- Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS, and Whiting PJ (1996) Functional characterization of human γ -aminobutyric acid A receptors containing the $\alpha 4$ subunit. *Mol Pharmacol* **50**:670–678.
- Wallner M, Hancher HJ, and Olsen RW (2004) Ethanol enhances $\alpha 4\beta 3\delta$ and $\alpha 6\beta 3\delta$ γ -aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci USA* **100**:15218–15223.
- Wan Q, Man HY, Brauntun J, Wang W, Salter MW, Becker L, and Wang YT (1997) Modulation of GABA_A receptor function by tyrosine phosphorylation of β subunits. *J Neurosci* **17**:5062–5069.
- Yagi T (1994) Src family kinases control neural development and function. *Dev Growth Differ* **36**:543–550.
- Yagi T (1999) Molecular mechanisms of fyn-tyrosine kinase for regulating mammalian behaviors and ethanol sensitivity. *Biochem Pharmacol* **57**:845–850.
- Yagi T, Shigetani Y, Okado N, Tokunaga T, Ikawa Y, and Aizawa S (1993) Regional localization of fyn in adult brain; studies with mice in which the fyn gene was replaced by lac Z. *Oncogene* **8**:3343–3351.
- Yaka R, Phamluong K, and Ron D (2003) Scaffolding of fyn kinase to the NMDA receptor determines brain region sensitivity to ethanol. *J Neurosci* **23**:3623–3632.
- Zdilar D, Luntz-Leybman V, Frosthalm A, and Rotter A (1992) Differential expression of GABA_A/benzodiazepine receptor $\beta 1$, $\beta 2$ and $\beta 3$ subunit mRNAs in the developing mouse cerebellum. *J Comp Neurol* **326**:580–594.

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