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Deletion of the *fyn*-Kinase Gene Alters Sensitivity to GABAergic Drugs: Dependence on $\beta2/\beta3$ $GABA_A$ 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 $\beta2$ and/or $\beta3$ receptor subunits. The genotypes did not differ in sensitivity to zolpidem, an $\alpha1$-selective GABAergic drug. $GABA_A$ receptor functional assays ($^{36}Cl^-$ 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 [^{3}H]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 $\beta2$ and/or $\beta3$ 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*-deficient 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 $\gamma2$ (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 $\alpha1$-selective drug, zolpidem, and the $\beta2/\beta3$-selective drug, etomidate. $GABA_A$ receptor function was assessed by measuring muscimol and THIP stimulation, as well as etomidate potentiation of muscimol-stimulated, $^{36}Cl^-$ flux in *fyn*-deficient and wild-type mice. $GABA_A$ receptor binding using [^{3}H]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 $^{36}Cl^-$ 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 g/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 $^{36}Cl^-$ 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 $^{36}Cl^-$ solution (2 μCi/ml of assay buffer: 1 μCi = 37 kBq; MP Biomedicals, Irvine, CA) containing the drugs to be tested. Three seconds after the addition of $^{36}Cl^-$, 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 $^{36}Cl^-$ 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 $^{36}Cl^-$ taken up when agonist was present in the assay medium (total uptake) minus the amount of $^{36}Cl^-$ uptake when agonist was not present (muscimol- or THIP-independent or nonspecific uptake). Etomidate potentiation of muscimol-stimulated $^{36}Cl^-$ 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).

[^{3}H]Flunitrazepam Binding. Cortical or cerebellar tissue was harvested from female *fyn*-deficient and wild-type mice, and $GABA_A$ receptor binding was performed using [^{3}H]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 [^{3}H]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 ± S.E.M. (except initial sensitivity to loss of righting reflex data, which is reported as ED_{50} ± 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-

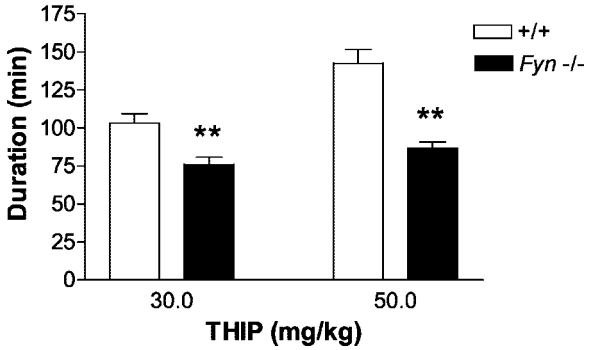

Fig. 1. Sensitivity to the hypnotic effects of THIP in female *fyn*-kinase-null mutant and wild-type mice. THIP (30 or 50 mg/kg; i.p.) was injected, and loss of the righting reflex was assessed. Values represent mean ± S.E.M. *Fyn*-deficient mice were less sensitive to the hypnotic effects of THIP (interaction 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 ± 6.1	93.4 ± 8.6
Flurazepam 180 mg/kg	98.8 ± 13.8	96.1 ± 10.6
Alfaxalone 70 mg/kg	67.4 ± 7.7	59.9 ± 4.2

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

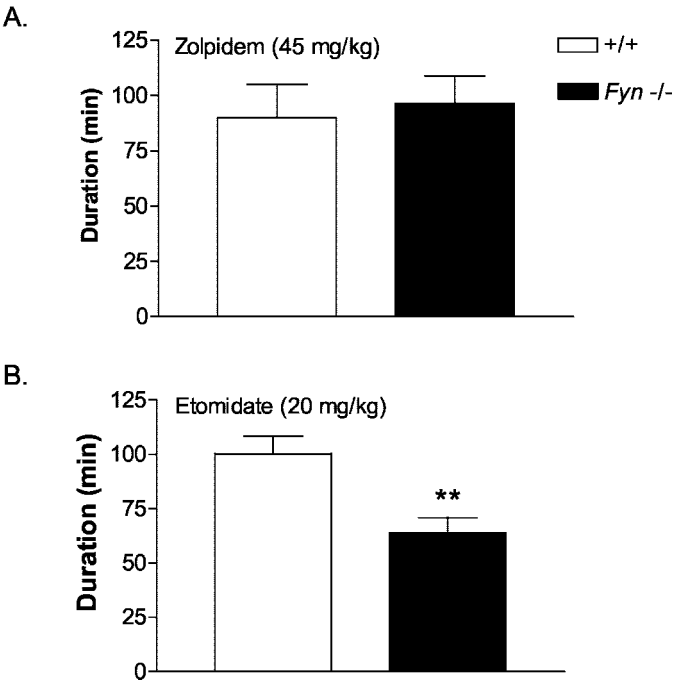

Fig. 2. Sensitivity to the hypnotic effects of zolpidem (α1-selective) and etomidate (β2/β3-selective). *fyn*-Kinase-null mutants were injected (i.p.) with 45 mg/kg zolpidem or 20 mg/kg etomidate, and loss of the righting reflex was measured ($n = 10$ per genotype). Values represent mean ± S.E.M. A, *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 β2/β3-selective drug, etomidate ($t[17] = 3.4$, $p < 0.01$; $n = 9–10$ per genotype).

ity to THIP in *fyn*-null mutant mice might be subunit-specific. We measured hypnotic sensitivity to the α1-selective drug, zolpidem, and the β2/β3-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 β2 and/or β3 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} ± 95% confidence interval, 1.2 ± 0.2 and 0.9 ± 0.2 μM for null mutant and wild-type, respectively; E_{max} ± 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 stimulation of $^{36}Cl^-$ flux in cortical microsacs (EC_{50} ± 95% confidence interval, 8.0 ± 1.1 and 7.3 ± 1.2 μM for null mutant and wild-type, respectively; E_{max} ± 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 β2 and/or β3 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-

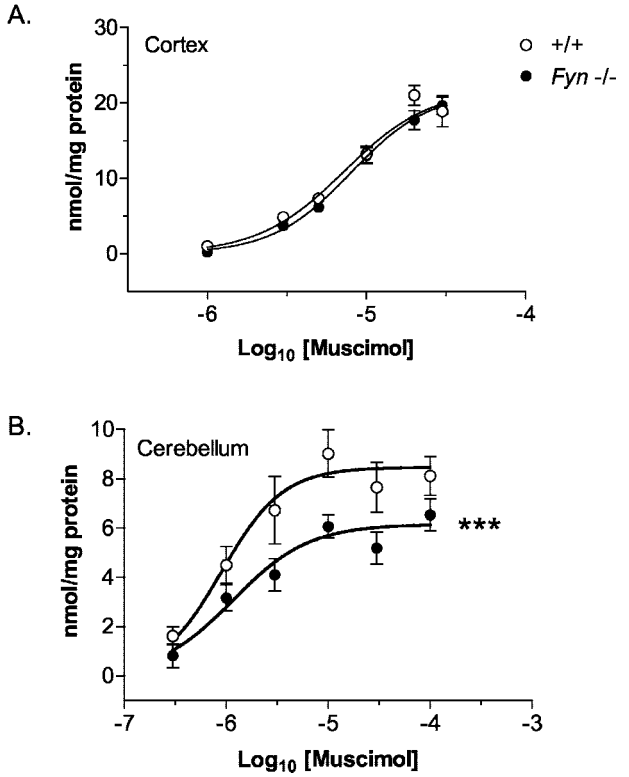

Fig. 3. Muscimol-stimulated $^{36}Cl^-$ flux in *fyn*-kinase-null mutant and wild-type mice. A, muscimol-stimulated flux in cortical membranes did not differ between *fyn*-null mutant and wild-type mice. B, *fyn* gene deletion reduced muscimol-stimulated $^{36}Cl^-$ flux in cerebellar membranes (main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

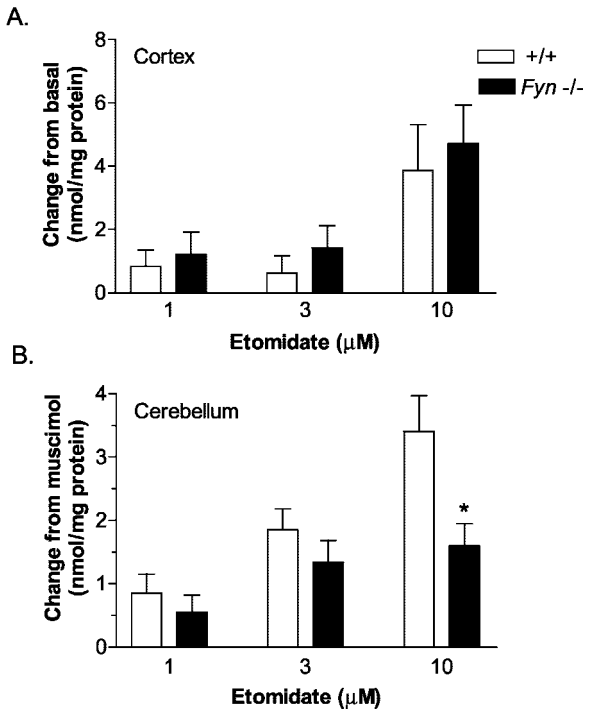

Fig. 4. Etomidate potentiation of muscimol-stimulated $^{36}Cl^-$ flux in *fyn*-kinase-null mutant and wild-type mice. The actions of 1, 3, and 10 μM etomidate on 0.2 or 0.5 μM muscimol concentrations were assessed. The above etomidate concentrations were chosen based on pilot studies and because they 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 ($t[6] = 2.7$, $p < 0.05$).

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 β2 and/or β3 receptor subunits in the interaction between fyn-kinase and GABAergic systems.

[^{3}H]Flunitrazepam Binding. Because cerebellar $GABA_A$ receptor function was altered, we measured [^{3}H]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 [^{3}H]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 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 $^{36}Cl^-$ 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

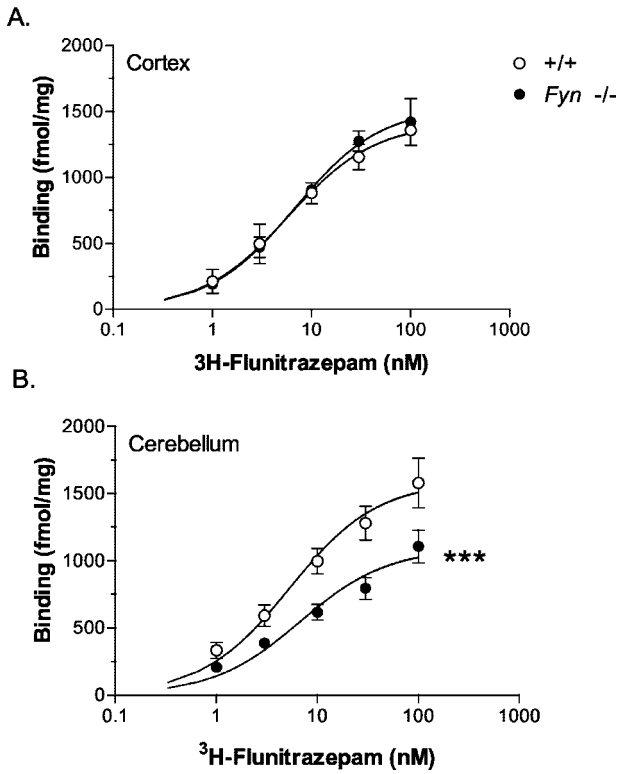

Fig. 5. [^{3}H]Flunitrazepam binding in *fyn*-null mutant and wild-type mice. A, [^{3}H]flunitrazepam in cortical membranes did not differ between female *fyn*-null mutant and wild-type mice. B, *fyn* gene deletion reduced [^{3}H]flunitrazepam binding in the cerebellar membranes of female mice (main 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 α1-selective benzodiazepine, zolpidem, and the β2/β3-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 β2 and/or β3 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 β2 and/or β3 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 β2 and/or β3 receptor subunits, so we measured the actions of etomidate. Etomidate potentiation of muscimol-stimulated $^{36}Cl^-$ 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 β2 and/or β3 receptor subunits are important in fyn's effects on GABAergic function. Moreover, they support a scenario in which fyn-kinase phosphorylates the β2 and/or β3 receptor subunits. There is precedence for tyrosine kinase phosphorylation of specific $GABA_A$ receptor subunits. Indeed, *src* tyrosine kinase (related to fyn) phosphorylates the $GABA_A$ γ2 (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 β2 and/or β3 receptor subunits are underway in our laboratory.

Behavioral data suggest that β3 receptor subunits may be more important targets for fyn than β2 receptor subunits. β3-null mutant mice were less sensitive to etomidate and exhibited equal sensitivity to pentobarbital (Quinlan et al., 1998), and mice in whom a point mutation was introduced into the β3 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 β2 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 β2-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 β3 mutant mice.

A potential problem in attributing our findings to fyn's actions at β3 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 α6-containing receptors ($\alpha6 > \alpha5 = \alpha2 > \alpha1 > \alpha3$), and other studies demonstrated that THIP has a greater efficacy than GABA at receptors composed of $\alpha6\beta x\gamma2$s, that it is a full agonist at receptors composed of $\alpha5\beta x\gamma2$s, and that it is only a partial agonist at receptors composed of $\alpha1\text{-}4\beta x\gamma2$s (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 $\alpha1\beta x\gamma2$, $\alpha6\beta x\gamma2$, and $\alpha6\beta x\delta$ containing receptors (Saxena and Macdonald, 1996; Pöltl et al., 2003), that *fyn*-null mutants do not differ in hypnotic sensitivity to the α1-selective benzodiazepine, zolpidem (current study), and that [^{3}H]muscimol-labeled cerebellar $GABA_A$ receptors are primarily composed of $\alpha6\beta x\delta$ (Quirk et al., 1994), we speculate that fyn-kinase interacts with β3-containing $GABA_A$ receptors that also possess α6 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, $\alpha6\beta3\delta$-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 ([^{3}H]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 α1 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 [^{3}H]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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