DELETION OF THE FYN-KINASE GENE ALTERS SENSITIVITY TO GABAERGIC DRUGS: DEPENDENCE ON β2/β3 GABA_A RECEPTOR SUBUNITS

Stephen L. Boehm II, Laura Peden, R. Adron Harris, and Yuri A. Blednov

Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, Austin, Texas 78712
Running Title: *Fyn* null mutant mice and GABAergic function

Direct correspondence to: Stephen Boehm II, University of Texas at Austin, Waggoner Center for Alcohol and Addiction Research, 1 University Station A4800, Austin TX, 78712-0159, phone: 512-232-2487, fax: 512-232-2525, email: slboehm@mail.utexas.edu

Number of text pages: 28

Number of tables: 1

Number of figures: 5

Number of references: 34

Number of words in Abstract: 216

Number of words in Introduction: 369

Number of words in Discussion: 1474

Nonstandard Abbreviations: THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

Assignment Section: behavioral Pharmacology
Abstract

Tyrosine phosphorylation can modulate GABA<sub>A</sub> receptor function, and deletion of the <i>fyn</i> kinase gene alters GABAergic function in olfactory bulb neurons (Kitazawa et al., 1998). Our goal was to determine whether <i>fyn</i> gene deletion altered behavioral and functional actions of compounds that act on GABA<sub>A</sub> receptors. Such evidence might suggest a role for <i>fyn</i>-kinase in modulating GABA<sub>A</sub> 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, a GABA<sub>A</sub> 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 β2 and/or β3 receptor subunits. The genotypes did not differ in sensitivity to zolpidem, an α1-selective GABAergic drug. GABA<sub>A</sub> receptor functional assays (³⁶Cl⁻ influx) supported our behavioral results; the actions of the GABA<sub>A</sub> agonists, THIP and muscimol, were reduced in the cerebellar membranes of <i>fyn</i> null mutant mice. Importantly, similar results were seen with etomidate. Binding of <sup>3</sup>H-flunitrazepam supported the idea that this is due to a decrease in functional GABA<sub>A</sub> receptor density. These data suggest that <i>fyn</i>-kinase may alter the function of GABA<sub>A</sub> receptors, perhaps via actions on β2 and/or β3 receptor subunits.
Fyn-kinase, a member of the src family of non-receptor tyrosine kinases, is localized at the growth cones and post-synaptic 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 and colleagues (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<sub>A</sub> 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<sub>A</sub> receptor (Kitazawa et al., 1998). Moreover, several studies have shown that src tyrosine kinase, closely related to fyn-kinase, interacts with or phosphorylates the γ2 (Brandon et al., 2001; Valenzuela et al., 1995) and β (Brandon et al., 2001; Valenzuela et al., 1995; Wan et al., 1997) subunits of the GABA<sub>A</sub> receptor. Thus, the available evidence suggests that deletion of the fyn-kinase gene may, in addition to the NMDA receptor, interact with the GABA<sub>A</sub> receptor.

The present studies examined the potential interaction between fyn-kinase and GABA<sub>A</sub> receptors in fyn null mutant and wild-type mice. We assessed hypnotic sensitivity to the GABA<sub>A</sub> 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 $^3\text{H}$-flunitrazepam was also performed in fyn null mutant and wild-type mice.
Materials and Methods

Animals. Fyn-kinase null mutant (B6;129S-Fyn<sup>tm1Sor</sup>) and control mice (B6129SF2/J) were obtained from Jackson Laboratories (Bar Harbor, ME), and bred to generate the mice used in the present studies. These mice were originally developed by Stein et al. (1992). 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-hr 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 three months and not more than six months old.

Drugs. THIP and alfaxalone were obtained from Sigma (St. Louis, MO). Flurazepam and diazepam were purchased from Hoffman-LaRoche (Nutley, NJ), pentobarbital from Sigma/RBI (Natick, MA), etomidate from Tocris (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 3–4 drops of Tween 80 (Sigma, St. Louis, MO) before dissolving in saline. Alfaxalone was dissolved in 15% 2-
hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO). 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 30s. 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 (50mg/kg, i.p.), flurazepam (180mg/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 hrs) following 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 one 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% CI = dosing increment $\times \sqrt{\frac{2}{n}} \times 1.96$, in which $n$ = the last $n$ trials and 1.96 reflects the 0.05 $\alpha$ 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 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 protein) were incubated in a 34$^\circ$C water bath for 5 min. Uptake was initiated by adding 200 $\mu$l of $^{36}$Cl$^-$ solution (ICN, Costa Mesa, CA; 2 $\mu$Ci/ml of assay buffer: 1 $\mu$Ci = 37 kBq) 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 $\mu$M picrotoxin) followed by rapid filtration through a GB100R filter (Advantec MFS, Dublin, CA), and subsequent washing with 8 ml quench buffer. Filters were incubated overnight in 4 ml 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 non-specific 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 (Perkin Elmer Life Sciences, Boston, MA). Tissue was homogenized in 25 ml 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 protein) to a reaction mixture containing 200 µl ice-cold assay buffer, 50 µl $^3$H-flunitrazepam (84.5 Ci/mmol; 1, 3, 10, 30, or 100 nM), and 50 µL additional ice-cold assay buffer (non-specific 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 ice-cold assay buffer followed by rapid filtration through a GB100R filter (Advantec MFS, Dublin, CA) and subsequent washing with ice-cold assay buffer. Filters were incubated overnight in 4 ml Biosafe II scintillation liquid (Research Products International, Mount Prospect, IL) before analysis in a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Specific binding was calculated by subtracting non-specific binding from total binding. $K_d$ and $B_{max}$ values were calculated using the Prism 3.0 program (GraphPad Software, San Diego, CA).
**Statistical analysis.** Preliminary analyses (ANOVA) 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 ANOVA. 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 \textit{fyn}-kinase gene, duration of loss of righting reflex was measured following administration of THIP (GABA\textsubscript{A} receptor partial agonist), and the non-subunit selective allosteric modulators pentobarbital, flurazepam, and alfaxalone. \textit{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 to their wild-type counterparts (Figure 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 sensitivity to THIP in \textit{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. \textit{Fyn} null mutant and wild-type mice did not differ in sensitivity to zolpidem (Figure 2A). However, \textit{fyn} deficient mice were less sensitive to the hypnotic effects of etomidate (Figure 2B). These results suggest that \(\beta_{2}\) and/or \(\beta_{3}\) receptor subunits are important mediators of the effects of \textit{fyn} gene deletion on GABAergic systems.

Because the intervals between drug injection and test end point render the traditional regaining of righting reflex incapable of separating the roles of initial sensitivity and acute tolerance, we determined the ED\textsubscript{50} for etomidate-induced loss of righting reflex. Initial sensitivity (ED\textsubscript{50}) was defined as loss of righting reflex within 5 min of etomidate injection. \textit{Fyn} null mutant and wild-type mice exhibited similar ED\textsubscript{50} values for etomidate-induced loss of righting reflex (8.9 ± 1.1 and 9.3 ± 1.1 mg/kg for

This article has not been copyedited and formatted. The final version may differ from this version.
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<sub>A</sub> receptor functional assay.** Our behavioral data suggested that GABA<sub>A</sub> 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<sup>-</sup> flux in cerebellar microsacs (EC<sub>50</sub> ± 95% confidence interval: 1.2 ± 0.2 and 0.9 ± 0.2 µM for null mutant and wild-type, respectively; Emax ± SEM: 6.2 ± 0.5 and 8.5 ± 0.6 nmol/mg protein for null mutants and wild-types, respectively; Figure 3B). However, *fyn* null mutant and wild-type mice did not differ in sensitivity to muscimol-stimulation of $^{36}$Cl<sup>-</sup> flux in cortical microsacs (EC<sub>50</sub> ± 95% confidence interval: 8.0 ± 1.1 and 7.3 ± 1.2 µM for null mutant and wild-type, respectively; Emax ± SEM: 21.5 ± 1.7 and 21.9 ± 2.4 nmols/mg protein for null mutant and wild-type, respectively; Figure 3A).

Similar results were obtained with the GABA<sub>A</sub> agonist, THIP. *Fyn* null mutant and wild-type mice also did not differ in sensitivity to THIP-stimulated $^{36}$Cl<sup>-</sup> 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<sup>-</sup> 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 (Figure 4B). However, similar to that seen following muscimol and THIP application, etomidate did not differentially alter muscimol-stimulated chloride flux in cortical microsacs (Figure 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.

**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 (Figure 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 Kd 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 (Kd: 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 II 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 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\textsubscript{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\textsubscript{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\textsubscript{A} receptors.

If fyn-kinase phosphorylates the $\beta_2$ and/or $\beta_3$ receptor subunits, then one would predict an associated change in GABA\textsubscript{A} receptor function. Fyn null mutants were less sensitive to the actions of the GABA\textsubscript{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 β2 and/or β3 receptor subunits. There is precedence for tyrosine kinase phosphorylation of specific GABA<sub>A</sub> receptor subunits. Indeed, src tyrosine kinase (related to fyn) phosphorylates the GABA<sub>A</sub> γ2 (Brandon et al., 2001; Valenzuela et al., 1995) and β (Brandon et al., 2001; Valenzuela et al., 1995; Wan et al., 1997) 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 which 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 (α6 > α5 = α2 > α1 > α3), and other studies demonstrated that THIP has a greater efficacy than GABA at receptors composed of α6βxyγ2s, that it is a full agonist at receptors composed of α5βxyγ2s, 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 $\text{fyn}$ 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 et al., 1996; Pöltl et al., 2003), that $\text{fyn}$ null mutants do not differ in hypnotic sensitivity to the $\alpha_1$-selective benzodiazepine, zolpidem (current study), and that $^3$H-muscimol labeled cerebellar GABA$_A$ receptors are primarily composed of $\alpha_6\beta x\delta$ (Quirk et al., 1994), we speculate that $\text{fyn}$ kinase interacts with $\beta_3$-containing GABA$_A$ receptors that also possess $\alpha_6$ and $\delta$ subunits. If true, it might explain how sensitivity to compounds that lack $\beta$ subunit specificity might also have been altered in $\text{fyn}$-kinase null mutant mice; mutated $\text{fyn}$ 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 ($^3$H-flunitrazepam) binding sites in the cerebellum of $\text{fyn}$ 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 posses $\alpha_1$ subunits. We hypothesize that $\text{fyn}$-kinase interacts with GABA$_A$ receptors that posses $\alpha_6$, 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. In so far 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 β3-containing GABA_A receptors (Jurd et al., 2003), and that these subunits are more abundant in cerebellum, compared to cortex (Fritschy and Mohler, 1995). Nevertheless, β3 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 β3 subunits have also been localized to these neurons (Zdilar et al., 1992; Laurie et al., 1992). However, more recent studies examining protein expression have localized β3 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 β3 receptor subunits. Nevertheless, as fyn-kinase has also been localized to cerebellar granule cells, it could presumably interact with β3 subunits to alter GABAergic function in either cell type.
In conclusion, deletion of the \textit{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$3 receptor subunits. Although our behavioral and functional data do not disprove the alternative hypothesis that development of GABAergic systems was disrupted in the mutant mice, they support the view that \textit{fyn}-kinase phosphorylates GABA$_A$ receptors via direct or indirect actions at $\beta$3 receptor subunits. Ongoing studies in our laboratory continue to probe this possibility.
Acknowledgments

The authors would like to thank Virginia Bleck and Elizabeth Osterndorff-Kahanek for their excellent technical assistance.
References


Yagi T, Shigetani Y, Okado N, Tokunaga T, Ikawa Y, 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.


Footnotes

These experiments were supported by NIAAA (AA07471, AA13520, AA06399).
Figure Legends

Figure 1. Sensitivity to the hypnotic effects of THIP in female \textit{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 ± SEM. \textit{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 posttests, p’s < 0.01; n = 7-9 per genotype and treatment).

Figure 2. Sensitivity to the hypnotic effects of zolpidem ($\alpha_1$ selective) and etomidate ($\beta_2/\beta_3$ selective). \textit{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 ± SEM. A. \textit{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 \textit{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).

Figure 3. Muscimol-stimulated $^{36}$Cl$^{-}$ flux in \textit{fyn}-kinase null mutant and wild-type mice. A. Muscimol-stimulated flux in cortical membranes did not differ between \textit{fyn} null mutant and wild-type mice. B. \textit{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).
Figure 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, and represent the most effect 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 ($t[6] = 2.7$, $P < 0.05$).

Figure 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).
Table 1. Sensitivity to the hypnotic effects of several other GABAergic drugs in \textit{fyn}-kinase null mutant and wild-type mice.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>wild-type</th>
<th>null mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentobarbital</td>
<td>50 mg/kg</td>
<td>83.9 ± 6.1</td>
<td>93.4 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>180 mg/kg</td>
<td>98.8 ± 13.8</td>
<td>96.1 ± 10.6</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>70 mg/kg</td>
<td>67.4 ± 7.7</td>
<td>59.9 ± 4.2</td>
</tr>
</tbody>
</table>

Values represent duration of loss of righting reflex (min), and are given as mean ± SEM (n's = 10-18 per genotype).
Figure 1.
Figure 2.

A. Zolpidem (45 mg/kg)

B. Etomidate (20 mg/kg)

Duration (min)

/++

Fyn +/-

**
Figure 3.

A. Cortex

B. Cerebellum

nmol/mg protein

Log$_{10}$ [Muscimol]

---

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4.

A. Cortex

<table>
<thead>
<tr>
<th>Etomidate (µM)</th>
<th>+/+</th>
<th>Fyn -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

B. Cerebellum

<table>
<thead>
<tr>
<th>Etomidate (µM)</th>
<th>Change from muscimol (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

* Indicates significant difference.
Figure 5.

A. Cortex

B. Cerebellum

- **+/+**
- **Fyn -/-**