

Fluoxetine Increases GABA_A Receptor Activity through a Novel Modulatory Site

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ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor used widely in the treatment of depression. In contrast to the proconvulsant effect of many antidepressants, fluoxetine has anticonvulsant activity. This property may be due in part to positive modulation of the GABA_A receptors (GABARs), which mediate most fast inhibitory neurotransmission in the mammalian brain. We examined the effect of fluoxetine on the activity of recombinant GABARs transiently expressed in mammalian cells. Fluoxetine increased the response of the receptor to submaximal GABA concentrations but did not alter the maximum current amplitude. Sensitivity did not depend upon the β -

or γ -subtype composition of the receptor when coexpressed with the α_1 subunit. Among the six α subtypes, only the α_5 subunit conferred reduced sensitivity to fluoxetine. The metabolite norfluoxetine was even more potent than fluoxetine. Mutations at residues in the α_5 subunit that alter its sensitivity to zinc or selective benzodiazepine derivatives did not affect potentiation by fluoxetine. This suggests that fluoxetine acts through a novel modulatory site on the GABAR. The direct positive modulation of GABARs by fluoxetine may be a factor in its anticonvulsant activity.

Fluoxetine (Prozac) is widely used in the treatment of anxiety-related symptoms through its ability to inhibit the transporter for the neurotransmitter serotonin (5-hydroxytryptamine). Whereas many antidepressants are considered proconvulsant, and therefore are not recommended for use by patients with epilepsy (Rosenstein et al., 1993), fluoxetine has commonly been reported to exhibit anticonvulsant activity. In human studies, adjunctive treatment of epileptic patients with fluoxetine was found to reduce or eliminate the occurrence of seizures (Favale et al., 1995). In animal studies, fluoxetine increased the effectiveness of several antiepileptic drugs (Leander, 1992) and was shown to suppress seizure activity and reduce convulsion intensity in both normal and epilepsy-prone rats (Dailey et al., 1992; Pasini et al., 1992; Prendiville and Gale, 1993). The mechanism underlying this anticonvulsant activity is not known, but it has been suggested that it may occur through modulation of neurotransmitter systems beyond direct effects on serotonin signaling. One possibility is that it reduces neuronal activity by enhancing GABAergic transmission.

Most fast inhibitory neurotransmission in the mammalian central nervous system is mediated through the GABA_A re-

ceptors (GABARs), which contain an intrinsic, chloride-permeable ion channel. Many drugs used clinically as anticonvulsants act by increasing GABAR activity (Korpi et al., 2002). The GABARs have a very complex structure, with 7 different subunit families and 16 subunit subtypes [$\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , π , and θ]. The pharmacological properties of GABARs are determined in large part by their subunit composition (Korpi et al., 2002). Previous studies have suggested both positive and negative regulation of GABAR activity by fluoxetine (Tunnicliff et al., 1999; Matsubara et al., 2000). However, there is little current evidence of a direct effect on the GABAR by fluoxetine or of the possible role of GABAR subunit composition in this modulation.

We examined the effect of fluoxetine and its metabolite norfluoxetine on the activity of recombinant GABARs in a mammalian expression system and found that they are positive modulators at most GABARs. We also examined the subunit subtype dependence and the effect of GABA concentration and membrane voltage on the interaction of fluoxetine with the GABAR.

Materials and Methods

Transfection of Mammalian Cells. Full-length wild-type cDNAs in the pCMVNeo (Dr. Robert Macdonald, Vanderbilt University) or pcDNA1.1/Amp (Invitrogen, Carlsbad CA) expression vectors were transfected into the mouse fibroblast cell line L929 (American Type Culture Collection, Manassas, VA) or the human embryonic

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ABBREVIATION: GABAR, GABA_A receptor.

kidney cell line HEK-293T (GenHunter, Nashville, TN). The results shown were combined from experiments using each of these cell types. Both these lines are widely used to study recombinant GABARs. Studies of other pharmacological and functional properties in our laboratory have shown no differences among GABARs expressed in these two lines. For selection of transfected cells, the plasmid pHook-1 (Invitrogen) containing cDNA encoding the surface antibody sFv was also transfected into the cells. Cells were maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum (L929) or fetal bovine serum (HEK-293T), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. Cells were passaged by a 5-min. incubation with 0.05% trypsin/0.02% EDTA solution in phosphate-buffered saline (10 mM Na₂HPO₄, 150 mM NaCl, pH 7.3).

The cells were transfected using a calcium phosphate method optimized for the L929 cells (Angelotti et al., 1993). Four micrograms of plasmid encoding each of the GABAR subunit cDNAs were added to the cells along with 2 μ g of the plasmid encoding sFv. After a 4-h incubation at 3% CO₂, the cells were treated with a 15% glycerol solution in 50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄ for 20 s. The selection procedure for sFv antibody expression was performed 20 to 28 h later as described by Greenfield et al. (1997). The cells were passaged and mixed with 3 to 5 μ l of magnetic beads coated with hapten (approximately 7.5×10^5 beads) (Chesnut et al., 1996). After a 30- to 60-min. incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into Dulbecco's modified Eagle's medium, plated onto poly(lysine)- and collagen-coated glass coverslips, and used for recording 18 to 28 h later.

Electrophysiological Recording Solutions and Techniques.

For whole-cell recordings the external solution consisted of 142 mM NaCl, 8.1 mM KCl, 6 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4, and osmolarity adjusted to 295 to 305 mOsm. Recording electrodes were filled with an internal solution of 153 mM KCl, 1 mM MgCl₂, 5 mM K-EGTA, 2 mM MgATP, and 10 mM HEPES, pH 7.4, and osmolarity adjusted to 295 to 305 mOsm. These solutions provided a chloride equilibrium potential near 0 mV. GABA and low concentrations of fluoxetine and norfluoxetine (≤ 100 μ M) were diluted into external solution from stocks frozen in water. High concentrations of fluoxetine (>100 μ M) were diluted from freshly made stocks in dimethyl sulfoxide. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota FL) on a two-stage puller (Narishige, Tokyo, Japan) to a resistance of 5 to 10 M Ω . Drugs were applied to cells using a stepper solution exchanger with a complete exchange time of <50 ms (open tip, SF-77B; Warner Instruments, Hamden, CT). There was a continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B (Axon Instruments, Inc., Union City, CA) patch-clamp amplifier and stored on a hard drive for off-line analysis. All experiments were performed at room temperature (near 25°C).

Construction of Mutated Subunit cDNAs. Point mutations were generated using the QuikChange mutagenesis procedure and products (Stratagene, La Jolla, CA). Oligonucleotide primers were synthesized by the University of South Carolina DNA core facility (Columbia, SC). Single amino acid changes were created using two nucleotide primers complementary to one another and encoding the desired amino acid mutation. Incorporation of the mutation was verified with DNA sequencing (University of South Carolina DNA core).

Analysis of Whole-Cell Currents. Whole-cell currents were analyzed using the programs Clampfit (pClamp8 suite; Axon Instruments) and Prism (GraphPad Software Inc., San Diego, CA). Concentration-response data were fit with a four-parameter logistic equation (current = [minimum current + (maximum current - minimum current)] / (1 + (10^{log EC₅₀ - log [GABA])ⁿ)), where *n* represents the Hill number. All fits were made to normalized data with current expressed as a percentage of the response to GABA alone.}

Paired *t* tests and Tukey-Kramer multiple comparisons statistical tests were performed using the InStat program (GraphPad) with a significance level of *p* < 0.05.

Results

Fluoxetine Potentiates GABAR Activity. To determine whether fluoxetine altered the activity of recombinant GABARs, 1 μ M to 1 mM fluoxetine was coapplied for 5 s with 10 μ M GABA to cells expressing the $\alpha 1\beta 3\gamma 2L$ isoform (Fig. 1). Fluoxetine increased the response to GABA in a concentration-dependent manner, with an average EC₅₀ of 134.3 ± 35.1 μ M (*n* = 4). At lower fluoxetine concentrations, the current often continued to increase throughout the 5-s application. Because the peak current was likely not reached in these cases, the measured EC₅₀ for fluoxetine represents an estimation of the actual EC₅₀. The potentiation by 1 mM fluoxetine was not readily reversible. Fluoxetine did not act as an agonist at the GABAR, as application of 100 μ M fluoxetine in the absence of GABA produced an average peak current of 4.0 ± 0.3 pA at -50 mV (*n* = 3), comparable with the variation produced by baseline noise. The average response to 10 μ M GABA in these cells was 867.0 ± 363 pA.

Fluoxetine Potentiation Depends upon the α Subunit Subtype. The activity of many GABAR modulators depends upon the subunit composition of the receptor (for review, see Korpi et al., 2002). The α subunit family is the most diverse of the GABAR families, with six different subtypes (α_1 - α_6). To determine whether fluoxetine sensitivity is affected by the α subtype, we examined receptors containing each of the α subtypes coexpressed with the same β (β_3) and γ (γ_{2L}) subunits (Fig. 2). The GABA concentration was submaximal (EC₂₀₋₃₀) for each isoform. With the exception of the α_5 -containing receptors, enhancement of the GABA-activated current by 100 μ M fluoxetine was similar for all these combinations (*p* > 0.05 compared among isoforms). Only the $\alpha 5\beta 3\gamma 2L$ receptor isoform was not significantly potentiated by 100 μ M fluoxetine (*p* < 0.001 compared with the $\alpha 1\beta 3\gamma 2L$ receptor).

Fluoxetine Potentiation Does Not Depend upon the β or γ Subunit Subtype. Three different GABAR β (β_1 - β_3) and γ (γ_1 - γ_3) subtypes have been described in mammalian species. We examined the sensitivity to fluoxetine of receptors containing each of the β subtypes in combination with α_1 and γ_{2L} (Fig. 2). The responses of the $\alpha 1\beta 1\gamma 2L$, $\alpha 1\beta 2\gamma 2L$, and $\alpha 1\beta 3\gamma 2L$ isoforms to 100 μ M fluoxetine were not significantly different from one another (*p* > 0.6).

To determine whether the γ subunit altered the response to fluoxetine, we examined receptors containing one of the three γ subtypes or the δ subunit in combination with α_1 and β_3 . We also examined the $\alpha 1\beta 3$ isoform, to determine whether the presence of a γ or δ subunit was important for the response (Fig. 2). Potentiation by fluoxetine did not show dependence on the γ subtype, and a γ subunit was not required for sensitivity. The degree of potentiation by 100 μ M fluoxetine was not significantly different among these isoforms (*p* > 0.6).

Fluoxetine Potentiation Is GABA Concentration-Dependent. To determine whether fluoxetine could increase the maximal response to GABA concentration, we coapplied 100 μ M fluoxetine with 100 μ M or 1 mM GABA (Fig. 3). The amount of potentiation decreased with increasing GABA con-

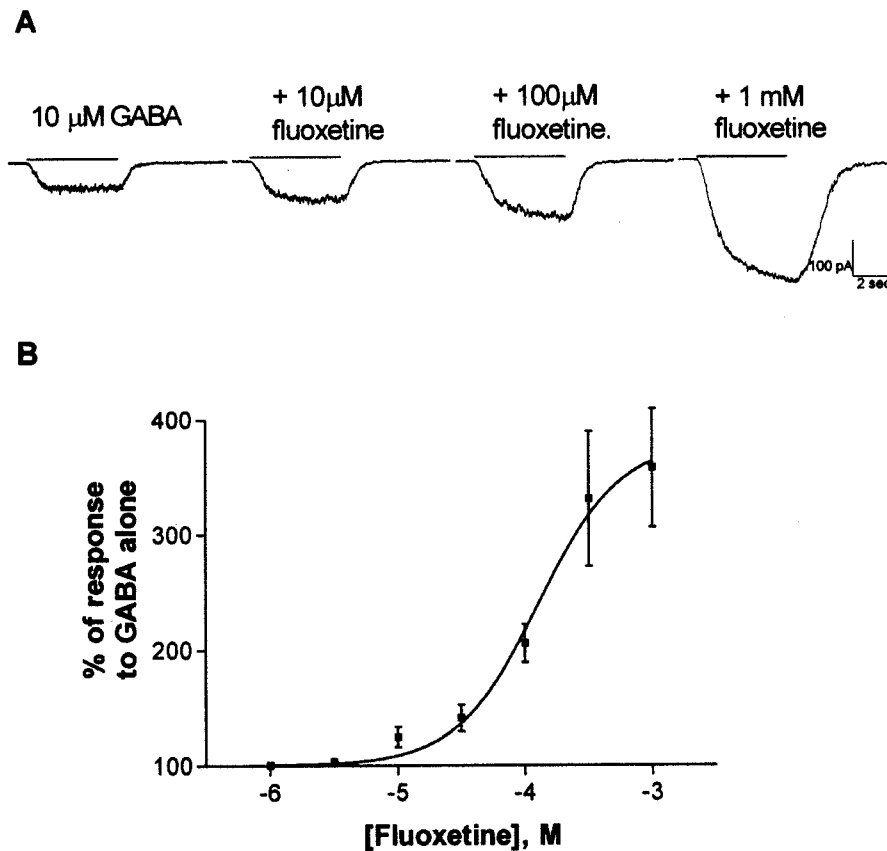


Fig. 1. Fluoxetine enhances the response to GABA. **A**, 10 μM GABA or 10 μM GABA + fluoxetine was applied for 5 s (bar) to cells expressing the $\alpha 1\beta 3\gamma 2\text{L}$ receptor isoform. Cells were voltage-clamped at -50 mV in the whole-cell configuration. All traces shown are from the same cell. **B**, the concentration-response relationship was constructed by measuring the peak current with coapplication of fluoxetine as a percentage of the response to 10 μM GABA alone. Symbols indicate mean \pm S.E.M. from four cells and were fit with a four-parameter logistic equation (solid line). The EC_{50} from the fit of the averaged data is 128.1 μM with a Hill number of 1.4.

centrations and fluoxetine did not significantly enhance the response to a maximal concentration (1 mM GABA). This suggests that, like the benzodiazepines, fluoxetine acts by increasing the sensitivity of the receptor for GABA without affecting the peak current (see Möhler et al., 2002).

Effect of Voltage on Potentiation by Fluoxetine. The lipid solubility of fluoxetine and the gradual increase in potentiation seen throughout the 5-s application period suggest that it may act at a site within the membrane. Therefore, we examined the voltage sensitivity of its action by comparing the response at holding potentials of $+50$ and -50 mV. The $\alpha 1\beta 3\gamma 2\text{L}$ receptor exhibits outward rectification in its response to GABA, due to a voltage-dependent shift in sensitivity to GABA (Fisher, 2002a). Whereas significant potentiation by 100 μM fluoxetine was still observed at $+50$ mV, the amount of potentiation was decreased compared with -50 mV (Fig. 4). This is consistent with the finding that the amount of potentiation depends upon the GABA concentration. Since GABA sensitivity is increased at positive potentials, 10 μM GABA represents a higher effective concentration ($\sim\text{EC}_{50}$) at $+50$ mV than at -50 mV ($\sim\text{EC}_{20-30}$). Therefore, the potentiation by fluoxetine does not appear to be altered by membrane voltage.

The Active Metabolite Norfluoxetine Is a More Potent Modulator Than Fluoxetine. Fluoxetine is metabolized to produce norfluoxetine, which is an even more potent inhibitor of the serotonin transporter (Sanchez and Hyttel,

1999) and voltage-gated K^+ channels (Choi et al., 2001) than fluoxetine. Plasma levels of norfluoxetine and fluoxetine are similar to those in patients treated with fluoxetine (Orsulak et al., 1988; Pato et al., 1991). Therefore, we determined whether this active metabolite also affected the activity of GABARs.

Norfluoxetine increased the response of the $\alpha 1\beta 3\gamma 2\text{L}$ receptor to GABA in a concentration-dependent manner, with an average EC_{50} of 0.6 ± 0.3 μM ($n = 3$) (Fig. 5). However, the maximum potentiation was significantly smaller than that seen with fluoxetine, with an average peak enhancement of $227.5 \pm 15.8\%$ compared with $358.1 \pm 51.5\%$ ($n = 4$) for fluoxetine.

Structural Determinants of Fluoxetine Sensitivity. The $\alpha 5$ subunit appears to confer a unique response in its lower sensitivity to fluoxetine. To begin to elucidate the structures within the $\alpha 5$ subunit responsible for this property, we examined amino acid residues previously shown to be important in determining its pharmacological properties. The $\alpha 5$ subunit is also distinctive in its higher sensitivity to positive modulation by several benzodiazepine derivatives (Liu et al., 1996; Quirk et al., 1996; Strakhova et al., 2000), and an isoleucine residue (I215) in the extracellular domain was identified as important for this characteristic (Strakhova et al., 2000; Casula et al., 2001). This subunit also has a high sensitivity to inhibition by zinc, a property conferred by a unique histidine residue (H195) found only in the $\alpha 5$ subunit

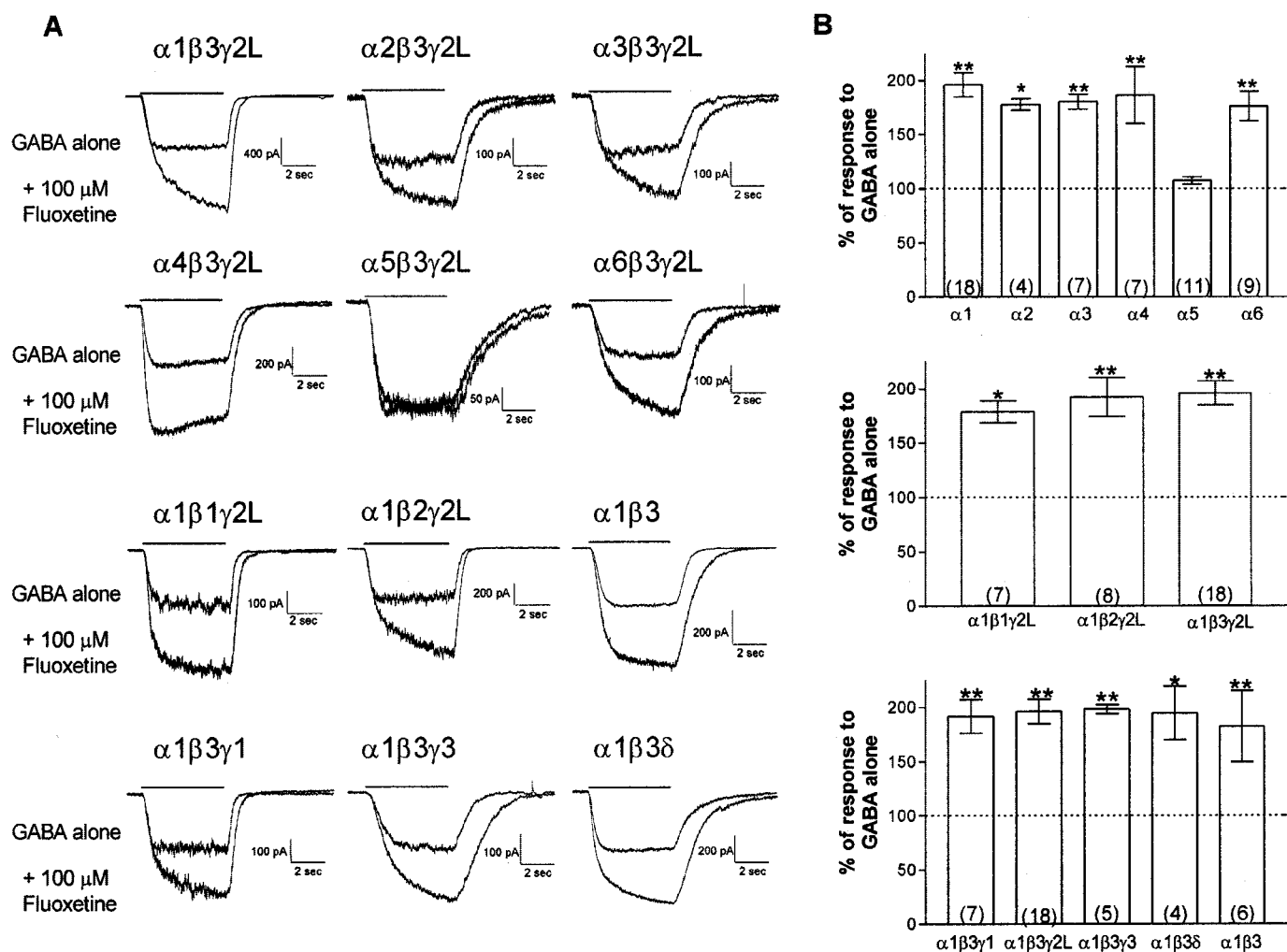


Fig. 2. Only the α_5 subunit confers a reduced sensitivity to fluoxetine. A, representative whole-cell current traces from cells transfected with different combinations of GABA_A subunits in response to GABA and GABA plus 100 μ M fluoxetine. Drugs were applied for 5 s (solid line). The current in response to GABA alone is the smaller in all cases. Cells were voltage-clamped at -50 mV. B, average potentiation of the response to GABA by 100 μ M fluoxetine. α subunits were coexpressed with β_3 and γ_{2L} . The current in response to GABA + fluoxetine was normalized to the response to GABA alone. Bars show the mean \pm S.E.M., and n is given by the number in parentheses. The dotted line represents the response to GABA alone. ** ($p < 0.01$) or * ($p < 0.05$) indicates a significant difference from the response to GABA alone for each isoform (paired t test).

(Fisher, 2002b). Since these residues have been shown to contribute to properties associated with the α_5 subunit, we examined whether they are also important in fluoxetine sensitivity.

The wild-type α_5 residues were exchanged for the residues found in the homologous sites of the α_1 subunit with histidine195 changed to aspartate and isoleucine215 changed to valine. The mutated α_5 subunits were coexpressed with wild-type β_3 and γ_{2L} subunits and their sensitivity to 100 μ M fluoxetine was determined (Fig. 6). Both the $\alpha 5_{(H195D)}\beta_3\gamma_{2L}$ and the $\alpha 5_{(I215V)}\beta_3\gamma_{2L}$ receptors were similar to wild-type in their lack of sensitivity to 100 μ M fluoxetine ($p > 0.8$), suggesting that these residues do not play a role in regulating the response to fluoxetine.

Discussion

We have demonstrated that the antidepressant fluoxetine directly potentiates the activity of recombinant GABA_A receptors. The response to submaximal GABA concentrations was increased, but the maximal current response was unaffected.

Fluoxetine showed an interesting and unique dependence on the GABA_A subunit composition. Only receptors containing an α_5 subtype were insensitive to potentiation. Receptors containing any of the other six α subtypes were equally enhanced. The nature of the β or γ subtype had no effect on sensitivity, and a γ subunit was unnecessary for responsiveness, because $\alpha\beta\delta$ and $\alpha\beta$ receptors were also potentiated. Fluoxetine did not directly activate the GABA_A receptor, and its effect was not altered by membrane voltage. Norfluoxetine, a metabolic product of fluoxetine, was even more potent in its enhancement of GABA_A activity. Mutations of amino acid residues in the α_5 subunit that regulate its sensitivity to modulation by zinc and benzodiazepines did not alter its insensitivity to fluoxetine.

Many other allosteric sites have been described for the GABA_A receptor, and the affinity or efficacy of most of these depends upon the subunit composition of the receptor (for review, see Korpi et al., 2002). Although several benzodiazepine derivatives selectively enhance α_5 -containing receptors, fluoxetine is the first positive modulator reported for which the α_5 subunit is selectively insensitive. In addition, the activity of

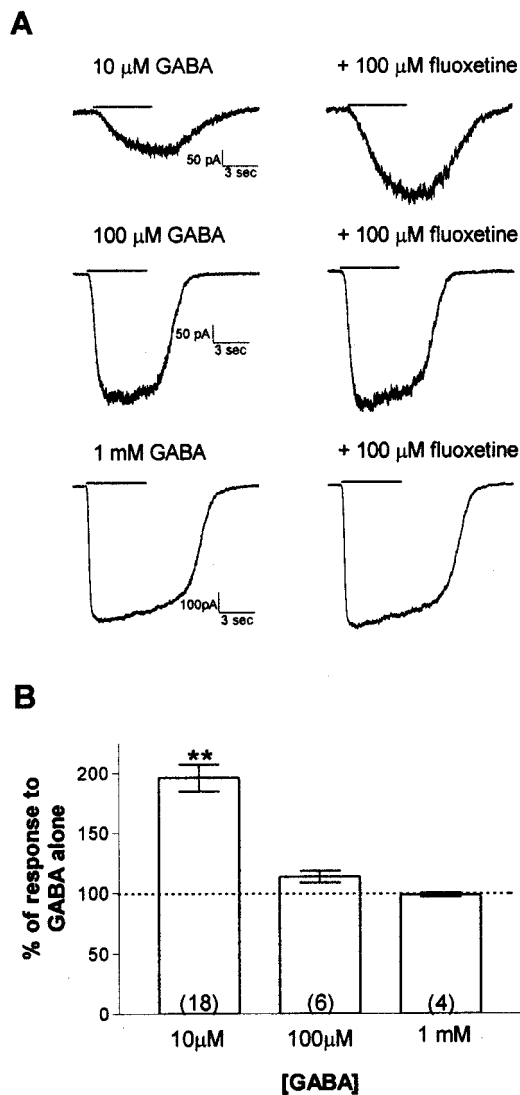


Fig. 3. Effect of GABA concentration on potentiation by fluoxetine. **A**, representative traces from cells expressing the $\alpha 1\beta 3\gamma 2\text{L}$ isoform in response to varying GABA concentrations alone or with 100 μM fluoxetine. Drugs were applied for 5 s as indicated by the solid line. Cells were voltage-clamped at -50mV . **B**, effect of 100 μM fluoxetine on the peak current response to different GABA concentrations. Bars indicate mean \pm S.E.M., and n is given by the number in parentheses. The dotted line represents the response to GABA alone. ** ($p < 0.01$) indicates a significant difference from the response to GABA alone for each GABA concentration (paired t test).

the benzodiazepines also depends upon the nature of the γ subunit, whereas the activity of fluoxetine does not. Other modulators that do not require a γ subunit for activity, such as the barbiturates or loreclezole, do not share this α -subtype dependence. This suggests that fluoxetine acts through a unique site on the GABAR.

The α_5 subunit apparently confers a unique lack of sensitivity to fluoxetine modulation. Therefore, this modulatory site may be exploited for the development of drugs designed to spare this population of receptors. The α_5 subunit is expressed primarily in the CA1 and CA3 regions of the hippocampus, and its production decreases with development (Laurie et al., 1992; Wisden et al., 1992). The physiological importance of these subunits in hippocampal function is unknown. However, changes in expression of the α_5 subunit

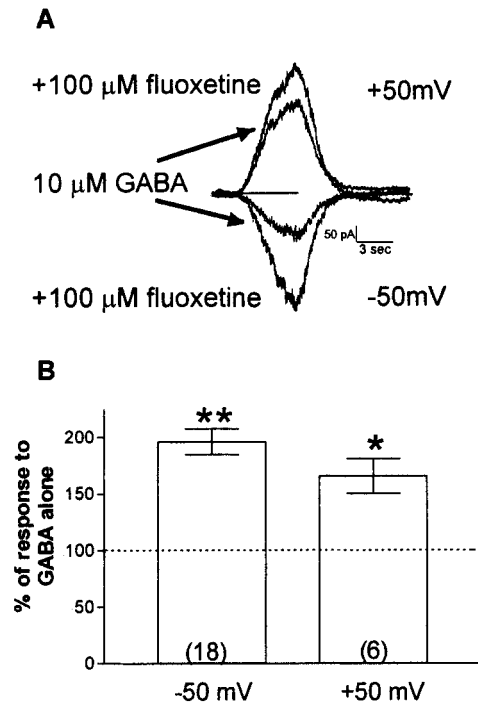


Fig. 4. Effect of membrane voltage on potentiation by fluoxetine. **A**, representative whole-cell traces from cells expressing the $\alpha 1\beta 3\gamma 2\text{L}$ isoform at membrane potentials of -50mV or $+50\text{mV}$. GABA (10 μM) or GABA + 100 μM fluoxetine was applied for 5 s as indicated by the solid line. All traces are from the same cell. **B**, effect of 100 μM fluoxetine on the peak current response at different holding potentials. Bars indicate mean \pm S.E.M., and n is given by the number in parentheses. The dotted line represents the response to GABA alone. ** ($p < 0.01$) or * ($p < 0.05$) indicates a significant difference from the response to GABA alone at each potential (paired t test).

have been suggested to play a role in learning and memory (Collinson et al., 2002), seizure development (Houser and Esclapez, 1996; Rice et al., 1996; Fritschy et al., 1999), benzodiazepine tolerance (Li et al., 2000), and reward conditioning (June et al., 2001).

In addition to the direct effect shown here, fluoxetine may also increase GABAR activity indirectly, by enhancing the synthesis of neuroactive steroids that positively modulate these receptors. Fluoxetine treatment increases the concentration of allopregnanalone (Uzunova et al., 1998), likely through a direct effect on the activity of the synthetic enzymes responsible for its production (Griffin and Mellon, 1999).

The modulation of the GABAR we observed may not be particularly surprising because fluoxetine and its metabolites have been found to have direct effects on a wide variety of channels. These include the structurally related nicotinic acetylcholine (García-Colunga et al., 1997) and 5-hydroxytryptamine₃ serotonin receptors (Fan, 1994; Breitingner et al., 2001) as well as several Cl^- channels (Maertens et al., 1999) and voltage-gated Ca^{2+} and K^+ channels (Tytgat et al., 1997; Deák et al., 2000; Choi et al., 2001; Thomas et al., 2002). These effects generally occur within a concentration range of 1 to 10 μM . In contrast to the GABAR, however, fluoxetine was found to inhibit channel activity in all these cases. At both the nAChR and 5-hydroxytryptamine₃ receptors fluoxetine reduced the response in a noncompetitive manner. For the nAChR, the inhibition was voltage-dependent and consistent with an open channel block mechanism.

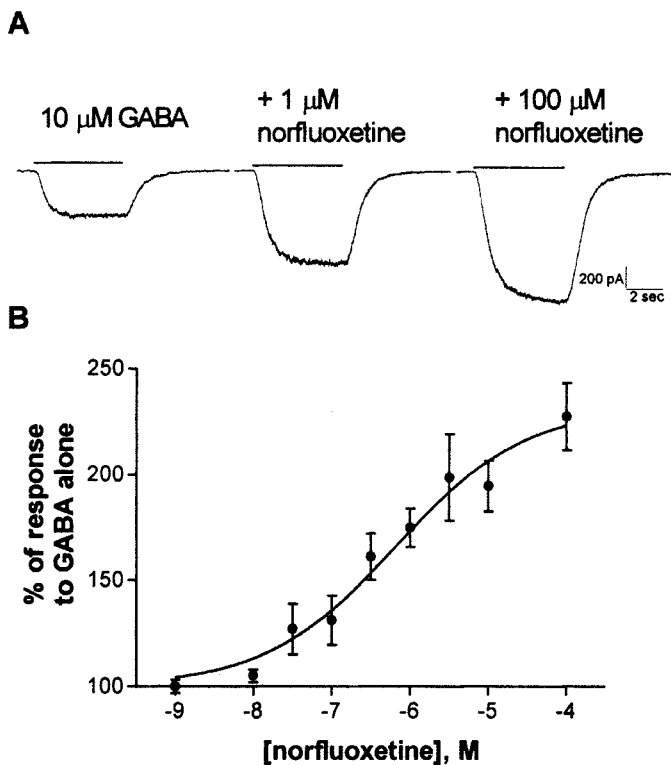


Fig. 5. Norfluoxetine is a more potent modulator of GABA_A activity. A, 10 μM GABA or 10 μM GABA + norfluoxetine was applied for 5 s (bar) to cells expressing the $\alpha 1\beta 3\gamma 2L$ receptor isoform. Cells were voltage-clamped at -50 mV in the whole-cell configuration. All traces shown are from the same cell. B, the concentration-response relationship was constructed by measuring the peak current with coapplication of norfluoxetine as a percentage of the response to 10 μM GABA alone. Symbols indicate mean \pm S.E.M. from three cells and were fit with a four-parameter logistic equation (solid line). The EC₅₀ from the fit of the averaged data is 0.7 μM with a Hill number of 0.5.

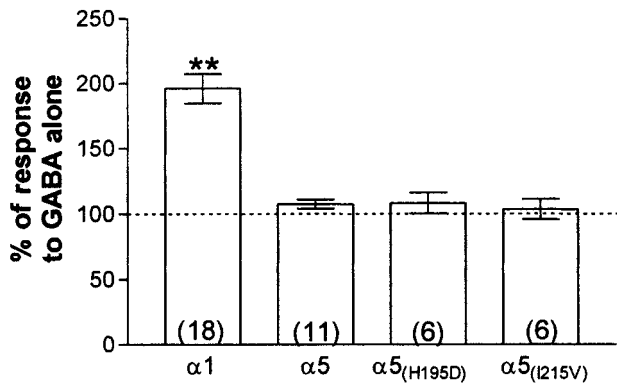


Fig. 6. Histidine195 and isoleucine215 of the $\alpha 5$ subunit do not influence fluoxetine sensitivity. Cells were transfected with mutated $\alpha 5_{(H195D)}$ or $\alpha 5_{(I215V)}$ subunits along with wild-type $\beta 3$ and $\gamma 2L$, and the peak current response to GABA and GABA + 100 μM fluoxetine was measured. Bars indicate mean \pm S.E.M., and n is given by the number in parentheses. The dotted line represents the response to GABA alone. ** ($p < 0.01$) indicates a significant difference from the response to GABA alone for each receptor isoform (paired t test).

Plasma fluoxetine and norfluoxetine concentrations in patients are typically reported near 1 μM (Orsulak et al., 1988; Pato et al., 1991). Although this level of fluoxetine would have little effect on the GABA_A isoforms studied here, accumulation of fluoxetine in the brains of chronically treated patients has been reported to increase the concentration

nearly 20-fold compared with plasma levels (Karson et al., 1993). Therefore, in the central nervous system, levels can be reached that might enhance the activity of most GABA_ARs. In addition, the more potent metabolite norfluoxetine would effectively modulate GABA_ARs at these concentrations. It may be that much of the anti-convulsant effect of fluoxetine treatment is mediated through this metabolic product.

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