Fluoxetine increases GABA$_A$ receptor activity

through a novel modulatory site

Richard T. Robinson, Brandon C. Drafts and Janet L. Fisher

Department of Pharmacology, Physiology and Neuroscience

University of South Carolina School of Medicine
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Corresponding Author:
Janet L. Fisher
Department of Pharmacology, Physiology and Neuroscience
University of South Carolina School of Medicine
Columbia, South Carolina 29208
Phone number: 803-733-3224
Fax number: 803-733-1523
E-mail: jfisher@med.sc.edu

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Abbreviations - GABA - γ-aminobutyric acid; GABAR - GABA_A receptor; DMEM - Dulbecco’s modified Eagle medium; BES - N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Abstract

Fluoxetine is a selective serotonin reuptake inhibitor used widely in the treatment of depression. In contrast to the pro-convulsant effect of many antidepressants, fluoxetine has anti-convulsant activity. This property may be due in part to positive modulation of the GABA<sub>A</sub> 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 sub-maximal 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 co-expressed with the α1 subunit. Among the six α subtypes only the α5 subunit conferred reduced sensitivity to fluoxetine. The metabolite norfluoxetine was even more potent than fluoxetine. Mutations at residues in the α5 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 anti-convulsant 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). While many antidepressants are considered pro-convulsant, and therefore are not recommended for use by patients with epilepsy (Rosenstein et al., 1993), fluoxetine has commonly been reported to exhibit anti-convulsant 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 anti-epileptic drugs (Leander, 1992) and was shown to suppress seizure activity and reduce convulsion intensity in both normal and epilepsy-prone rats (Pasini et al., 1992; Dailey et al., 1992; Prendiville and Gale, 1993). The mechanism underlying this anti-convulsant 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<sub>A</sub> receptors (GABARs), which contain an intrinsic, chloride permeable ion channel. Many drugs used clinically as anti-convulsants act by increasing GABAR activity (Korpi et al., 2002). The GABARs have a very complex structure, with seven different subunit families and sixteen subunit subtypes (α(1-6), β(1-3), γ(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.
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, Rockville MD) or the human embryonic 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 medium (DMEM) plus 10% heat inactivated horse serum (L929) or fetal bovine serum (HEK-293T), 100 IU/ml penicillin and 100 μg/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). 4 μg of plasmid encoding each of the GABAR subunit cDNAs were added to the cells along with 2 μg of the plasmid encoding sFv. Following a 4 hr. incubation at 3% CO₂, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5mM Na₂HPO₄) for 20 sec. The selection procedure for sFv antibody expression was performed 20-28 hrs. later as described by Greenfield et al. (1997). The cells were passaged and mixed with 3-5 μL of magnetic beads coated with hapten (approximately 7.5 x 10⁵ beads) (Chesnut et al., 1996).
Following a 30-60 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 DMEM, plated onto poly-lysine and/or collagen-coated glass coverslips and used for recording 18-28 hrs. later.

**Electrophysiological recording solutions and techniques**

For whole-cell recordings the external solution consisted of (in mM): 142 NaCl, 8.1 KCl, 6 MgCl₂, 1 CaCl₂, 10 glucose and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295-305 mOsm. Recording electrodes were filled with an internal solution of (in mM): 153 KCl, 1 MgCl₂, 5 K-EGTA (ethylene glycol-bis (β-aminoethyl ether N,N',N''-tetraacetate), 2 MgATP and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295-305 mOsm. These solutions provided a chloride equilibrium potential near 0 mV. GABA and low concentrations of fluoxetine and norfluoxetine (≤100 μM) were diluted into external solution from stocks frozen in water. High concentrations of fluoxetine (>100 μM) were diluted from freshly made stocks in DMSO. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota FL) on a two-stage puller (Narishige, Japan) to a resistance of 5-10 MΩ. Drugs were applied to cells using a stepper solution exchanger with a complete exchange time of <50 msec (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 (Foster City, CA) patch clamp amplifier and stored on 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, Foster City CA) and Prism (Graphpad, San Diego, CA). Concentration-response data was fit with a four-parameter logistic equation (Current = [Minimum current + (Minimum current - Maximum 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 μM -1 mM fluoxetine was co-applied for 5 sec. with 10 μM GABA to cells expressing the α1β3γ2L isoform (Figure 1). Fluoxetine increased the response to GABA in a concentration dependent manner, with an average EC₅₀ of 134.3 ± 35.1 μM (N=4). At lower fluoxetine concentrations the current often continued to increase throughout the 5 sec 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 μM fluoxetine in the absence of GABA produced an average peak current of 4.0 ± 0.3 pA at -50 mV (N=3), comparable to the variation produced by baseline noise. The average response to 10 μM 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 (α1-α6). 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 (β3) and γ (γ2L) subunits (Figure 2). The GABA concentration was sub-maximal (EC_{20-30}) for each isoform. With the exception of the α5-containing receptors, enhancement of the GABA-activated current by 100 μM fluoxetine was similar for all these
combinations (p > 0.05 compared among isoforms). Only the α5β3γ2L receptor isoform was not significantly potentiated by 100 μM fluoxetine (p<0.001 compared to the α1β3γ2L receptor).

**Fluoxetine potentiation does not depend upon the β or γ subunit subtype**

Three different GABAR β (β1-β3) and γ (γ1-γ3) subtypes have been described in mammalian species. We examined the sensitivity to fluoxetine of receptors containing each of the β subtypes in combination with α1 and γ2L (Figure 2). The responses of the α1β1γ2L, α1β2γ2L and α1β3γ2L isoforms to 100 μM 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 α1 and β3. We also examined the α1β3 isoform, to determine whether the presence of a γ or δ subunit was important for the response (Figure 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 μM 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 co-applied 100 μM fluoxetine with 100 μM or 1 mM GABA (Figure 3). The amount of potentiation decreased with increasing GABA concentrations 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 sec 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 α1β3γ2L receptor exhibits outward rectification in its response to GABA, due to a voltage-dependent shift in sensitivity to GABA (Fisher, 2002a). While significant potentiation by 100 μM fluoxetine was still observed at +50 mV, the amount of potentiation was decreased compared to -50 mV (Figure 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 (~EC50) at +50 mV than at -50 mV (~EC20-30). 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 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 α1β3γ2L receptor to GABA in a concentration-dependent manner, with an average EC50 of 0.6 ± 0.3 μM (N = 3) (Figure 5). However, the maximum potentiation was significantly smaller than that seen with fluoxetine,
with an average peak enhancement of 227.5 ± 15.8% compared to 358.1 ± 51.5% (N=4) for fluoxetine.

**Structural determinants of fluoxetine sensitivity**

The α5 subunit appears to confer a unique response in its lower sensitivity to fluoxetine. To begin to elucidate the structures within the α5 subunit responsible for this property, we examined amino acid residues previously shown to be important in determining its pharmacological properties. The α5 subunit is also distinctive in its higher sensitivity to positive modulation by several benzodiazepine derivatives (Quirk et al., 1996; Liu 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 α5 subunit (Fisher, 2002b). Since these residues have been shown to contribute to properties associated with the α5 subunit, we examined whether they are also important in fluoxetine sensitivity.

The wild-type α5 residues were exchanged for the residues found in the homologous sites of the α1 subunit with histidine195 changed to aspartate and isoleucine215 changed to valine. The mutated α5 subunits were co-expressed with wild-type β3 and γ2L subunits and their sensitivity to 100 μM fluoxetine determined (Figure 6). Both the α5(H195D)β3γ2L and the α5(I215V)β3γ2L receptors were similar to wild-type in their lack of sensitivity to 100 μM 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 GABARs. The response to submaximal GABA concentrations was increased but the maximal current response was unaffected. Fluoxetine showed an interesting and unique dependence on the GABAR subunit composition. Only receptors containing an α5 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, as αβδ and αβ receptors were also potentiated. Fluoxetine did not directly activate the GABAR, and its effect was not altered by membrane voltage. Norfluoxetine, a metabolic product of fluoxetine, was even more potent in its enhancement of GABAR activity. Mutations of amino acid residues in the α5 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 GABAR and the affinity or efficacy of most of these depend upon the subunit composition of the receptor (for review see Korpi et al., 2002). While several benzodiazepine derivatives selectively enhance α5-containing receptors, fluoxetine is the first positive modulator reported for which the α5 subunit is selectively insensitive. In addition, the activity of the benzodiazepines also depends upon the nature of the γ subunit, while the activity of fluoxetine does not. Other modulators which 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 α5 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 α5 subunit is expressed primarily in the CA1 and CA3 regions.
of the hippocampus, and its production decreases with development (Wisden et al., 1992; Laurie et al., 1992). The physiological importance of these subunits in hippocampal function is unknown. However, changes in expression of the α5 subunit 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 (Uzonova 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, as 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-HT3 serotonin receptors (Fan, 1994; Breitinger et al., 2001) as well as several Cl⁻ channels (Maertens et al., 1999) and voltage-gated Ca²⁺ 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 1-10 μM. In contrast to the GABAR however, fluoxetine was found to inhibit channel activity in all these cases. At both the nAChR and 5-HT3 receptors fluoxetine reduced the response in a non-competitive manner. For the nAChR, the inhibition was voltage-dependent and consistent with an open channel block mechanism.

Plasma fluoxetine and norfluoxetine concentrations in patients are typically reported near 1 μM (Orsulak et al., 1988; Pato et al., 1991). While this level of fluoxetine would have little
effect on the GABAR isoforms studied here, accumulation of fluoxetine in the brains of chronically treated patients has been reported to increase the concentration nearly 20 fold compared to plasma levels (Karson et al., 1993). Therefore, in the central nervous system, levels can be reached that might enhance the activity of most GABARs. In addition, the more potent metabolite norfluoxetine would effectively modulate GABARs at these concentrations. It may be that much of the anti-convulsant effect of fluoxetine treatment is mediated through this metabolic product.
References


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Footnotes

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Figure Legends

Figure 1 - Fluoxetine enhances the response to GABA

A. 10 μM GABA or 10 μM GABA + fluoxetine was applied for 5 sec (bar) to cells expressing the α1β3γ2L 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 co-application of fluoxetine as a percentage of the response to 10 μM GABA alone. Symbols indicate mean ± SEM from 4 cells and were fit with a four-parameter logistic equation (solid line). The EC50 from the fit of the averaged data is 128.1 μM with a hill number of 1.4.

Figure 2 - Only the α5 subunit confers a reduced sensitivity to fluoxetine

A. Representative whole-cell current traces from cells transfected with different combinations of GABAR subunits in response to GABA and GABA plus 100 μM fluoxetine. Drugs were applied for 5 sec (solid line). The current in response to GABA alone is the smaller in all cases. Cells were voltage clamped at -50mV.

B. Average potentiation of the response to GABA by 100 μM fluoxetine. α subunits were co-expressed with β3 and γ2L. The current in response to GABA + fluoxetine was normalized to the response to GABA alone. Bars show the mean ± SEM 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) indicate a significant difference from the response to GABA alone for each isoform (paired t-test).
Figure 3 - Effect of GABA concentration on potentiation by fluoxetine.

A. Representative traces from cells expressing the α1β3γ2L isoform in response to varying GABA concentrations alone or with 100 μM fluoxetine. Drugs were applied for 5 sec 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 ± SEM 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).

Figure 4 - Effect of membrane voltage on potentiation by fluoxetine.

A. Representative whole cell traces from cells expressing the α1β3γ2L isoform at membrane potentials of -50mV or +50 mV. 10 μM GABA or GABA + 100 μM fluoxetine was applied for 5 sec 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 ± SEM 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) indicate a significant difference from the response to GABA alone at each potential (paired t-test).

Figure 5 - Norfluoxetine is a more potent modulator of GABAR activity

A. 10 μM GABA or 10 μM GABA + norfluoxetine was applied for 5 sec (bar) to cells expressing the α1β3γ2L 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 co-application of norfluoxetine as a percentage of the response to 10 μM GABA alone. Symbols indicate mean ± SEM from 3 cells and were fit with a four-parameter logistic equation (solid line). The EC$_{50}$ from the fit of the averaged data is 0.7 μM with a hill number of 0.5.

**Figure 6 - Histidine195 and isoleucine215 of the α5 subunit do not influence fluoxetine sensitivity**

Cells were transfected with mutated α5$_{(H195D)}$ or α5$_{(I215V)}$ subunits along with wild-type β3 and γ2L and the peak current response to GABA and GABA + 100 μM fluoxetine was measured. Bars indicate mean ± SEM 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).
A

10 μM GABA + 10 μM fluoxetine + 100 μM fluoxetine + 1 mM fluoxetine

B

% of response to GABA alone

[Fluoxetine], M

100 200 300 400

-6 -5 -4 -3
A

10 µM GABA + 1 µM norfluoxetine + 100 µM norfluoxetine

B

% of response to GABA alone

[norfluoxetine], M