Research Report

Fluoxetine inhibition of glycine receptor activity in rat hippocampal neurons

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\textbf{ABSTRACT}

Fluoxetine is a selective serotonin reuptake inhibitor widely used for treating depression. However, fluoxetine treatment may lead to seizures at higher doses, which underlying mechanism remains largely unknown. In this study, we examined the effects of fluoxetine on glycine receptor (GlyR) activity. Using the whole-cell patch-clamp recording method, we found that fluoxetine and its metabolite norfluoxetine inhibited glycine-induced currents in cultured rat hippocampal neurons. This inhibition was dose-dependent, and voltage-independent. Fluoxetine shifted the glycine concentration-response curve to the right without altering the maximal current. Both Lineweaver-Burk and Schild plots suggest competitive inhibition. The amount of fluoxetine inhibition significantly increased when homomeric GlyRs were selectively inhibited with picrotoxin. Moreover, fluoxetine inhibited the current mediated by heteromeric \(\alpha_2\beta\)- but not homomeric \(\alpha_2\)-GlyRs transiently expressed in HEK293T cells. These results suggest that fluoxetine is a competitive and subtype-selective GlyR inhibitor, which may explain its capacity to induce seizures.

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1. Introduction

Fluoxetine (Prozac) is widely prescribed to treat depression. However, fluoxetine treatment can occasionally lead to seizures (Rosenstein et al., 1993). Seizures associated with fluoxetine therapy generally occur at higher doses (Braitberg and Curry, 1995; Oke et al., 2001; Pisani et al., 1999). Less commonly, seizures can also occur at therapeutic doses (Ware and Stewart, 1989; Weber, 1989). Unfortunately, the underlying mechanism remains largely unknown.

Fluoxetine has been shown to interact with many neuronal receptors. It has been reported to inhibit the NMDA receptor (Szasz et al., 2007) and enhance GABA type-A receptor (GABA\textsubscript{A}R) activity (Derry et al., 2007; Robinson et al., 2003). However, both of these interactions fail to explain the fluoxetine-associated seizures. The glycine receptor (GlyR), one of the major inhibitory receptors in the adult mammalian central nervous system (CNS), has never been investigated as a potential target during fluoxetine therapy.
Recent studies indicate that the GlyR plays an important inhibitory role in the CNS (Ye, 2008). The GlyR can be activated by endogenous amino acids including glycine, taurine, and β-alanine to mediate neural inhibition in the mature brain (Chattipakorn and McMahon, 2002; Mori et al., 2002). In the mature rat hippocampus, which expresses functional extrasynaptic GlyRs, there is increasing evidence that these extrasynaptic receptors play important roles in maintaining the normal excitatory balance for proper CNS function under physiological and/or pathological conditions (Chattipakorn and McMahon, 2003; Mitchell and Silver, 2003; Song et al., 2006; Zhang et al., 2008, 2006). Activation of GlyRs suppresses neuronal excitation and seizure-like events in the rat entorhinal cortex and hippocampus (Chattipakorn and McMahon, 2003; Kirchner et al., 2003; Song et al., 2006; Zhang et al., 2008, 2006). Meanwhile, the potent convulsant agent strychnine (STN) selectively antagonizes the GlyR, demonstrating the importance of GlyRs in CNS seizure activity. Therefore, we investigated whether fluoxetine has any effects on the GlyR using whole-cell patch-clamp recording in cultured rat hippocampal neurons and transfected HEK293T cells.

## Results

### 2.1. Fluoxetine inhibited the GlyR activity

Under our experimental conditions, we found that fluoxetine (100 μM) alone could directly induce an inward membrane current in cultured rat hippocampal neurons (Fig. 1A), which was almost completely abolished by 10 μM bicuculline (BIC), a selective antagonist of GABA<sub>A</sub>R (for 5 neurons, P<0.001). In addition, the selective GlyR antagonist strychnine (STN, 1 μM), NMDA receptor antagonist APV (50 μM) and non-NMDA receptor antagonist CNQX (10 μM) failed to block the fluoxetine-induced current (I<sub>FLX</sub>). Fig. S1 shows that this I<sub>FLX</sub> and the GABA-induced current are linearly correlated (n=8). Moreover, the I–V curve of I<sub>FLX</sub> is linear (R<sup>2</sup> =0.986) with a reversal potential close to the calculated Nernst potential for Cl<sup>−</sup> (E<sub>Cl</sub>= −1.28 mV in our experimental conditions). These results suggest that the I<sub>FLX</sub> may be mediated by GABA<sub>A</sub>Rs.

At a holding potential of −60 mV under our experimental conditions, we have found that glycine (100 μM) can induce a...
STN-sensitive GlyR-mediated current in cultured rat hippocampal neurons (Sun et al., 2007). As illustrated in Figs. 1B and D, fluoxetine significantly inhibited the peak amplitude of glycine-induced current ($I_{Gly}$) when co-applied with 100 μM glycine immediately after 20 s pre-application (protocol Pre-Co, $P<0.001$, $n=6$). The amount of fluoxetine inhibition in protocol Pre-Co showed no significant difference from that seen in the pre-application protocol (protocol Pre) (to 61.5±0.7% vs. 62.7±0.6%, $n=6$). The inhibited $I_{Gly}$ could be recovered after 2 min of washing with standard external solution. However, fluoxetine did not significantly inhibit the peak amplitude of $I_{Gly}$ in the co-application protocol (protocol Co, $n=6$).

In rat hippocampus, our previous studies have demonstrated that GABA can dose-dependently inhibit the $I_{Gly}$ induced by 100 μM glycine through activating GABAARs, and that this inhibition can be blocked with 10 μM BIC (Li and Xu, 2002). In this study, our data show that the amount of fluoxetine inhibition with protocol Pre did not change when blocking $I_{FLX}$ with BIC (Figs. 1C and D, $n=5$), suggesting that the fluoxetine inhibition is independent of $I_{FLX}$. Based on these findings, all following experiments were performed in the absence of BIC with protocol Pre unless otherwise specified.

2.2. Dose–response of fluoxetine and its active metabolite norfluoxetine on $I_{Gly}$

We further examined the effects of fluoxetine in the concentration range of 1 to 100 μM. Figs. 2A and C show that fluoxetine inhibited $I_{Gly}$ in a dose-dependent manner. We found that hippocampal neuron does not tolerate high doses (>100 μM) of fluoxetine, so we failed to obtain an exact half-maximal inhibition concentration ($IC_{50}$) value of fluoxetine. Nonetheless, our data show that the $IC_{50}$ value appears to be larger than 30 μM.

We found that 100 μM norfluoxetine, an active metabolite of fluoxetine, could also evoke a BIC-sensitive current. However, the amplitude showed no significant difference from that of $I_{FLX}$ (100 μM) recorded in the same neurons (data not shown). Figs. 2B and C recorded that norfluoxetine inhibited the $I_{Gly}$ in a dose-dependent manner in the protocol Pre-Co, with an $IC_{50}$ value of 21.1±2.4 μM ($n=5$). Meanwhile, norfluoxetine appeared to be more potent than fluoxetine (Fig. 2C).

2.3. Fluoxetine inhibition of $I_{Gly}$ was independent of voltage

To explore the voltage dependence of fluoxetine inhibition, we examined the I–V relationship of $I_{Gly}$ with a voltage ramp protocol ranging from −60 to+60 mV in the absence or presence of 100 μM fluoxetine. As illustrated in Fig. 3, the I–V curves are linear. Fluoxetine inhibited $I_{Gly}$ to a similar extent at all voltages tested, suggesting that the fluoxetine inhibition of $I_{Gly}$ is not voltage-dependent. Furthermore, the glycine-activated channel remained selectively permeable to Cl− because the reversal potential of $I_{Gly}$ was approximately −1 mV in the absence or presence of 100 μM fluoxetine ($n=5$).

2.4. Effects of fluoxetine on the activation of $I_{Gly}$

We then examined the concentration–response relationship of $I_{Gly}$ in the absence or presence of fluoxetine. Fig. 4A shows that fluoxetine shifted the glycine concentration–response curve to the right without affecting the maximal response ($n=4$–6). Fluoxetine at 10, 30 and 100 μM increased the average glycine EC50 from 77.9±5.9 to 84.0±6.8 (P>0.05), 101.8±12.0 (P>0.05) and 161.7±21.3 μM (P<0.01), respectively. The Line–weaver–Burk plot of Fig. 4B shows that fluoxetine did not
change the Y-intercept (Fig. 4B). The Schild plot analysis yielded a linear regression of a high reliability ($R^2 = 0.997$), with a slope of $-1.14$ and a fluoxetine $pA_2$ value of 4.04 that corresponds to a $K_i$ value (antilog of $-pA_2$) of 90.74 μM (Fig. 4C).

Fig. 4 – Effects of fluoxetine on the activation of $I_{\text{Gly}}$. (A) The dose–response curves of $I_{\text{Gly}}$ in the absence and presence of fluoxetine. Each data point is the mean (±S.E.M.) for 4–6 neurons in the absence (○) or the presence of fluoxetine at 10 (▲), 30 (▼) or 100 μM (●). The dashed line represents the current used for normalization. (B) Double reciprocal plot of fluoxetine inhibition on the $I_{\text{Gly}}$. (C) Schild plot analysis of fluoxetine inhibition on the $I_{\text{Gly}}$.

2.5. Dependence of subunit composition

Previous studies indicated that picrotoxin (PTX) can distinguish between homomeric and heteromeric GlyRs, and that...
100 μM PTX selectively inhibits approximately 80% of currents mediated by homomeric GlyR (Pribilla et al., 1992). Therefore, to test whether fluoxetine inhibition depends on the subunit composition of GlyRs, we examined fluoxetine inhibition in the absence and presence of 100 μM PTX in the cultured rat hippocampal neurons. Fig. 5A shows that the amount of fluoxetine inhibition in the presence of PTX (to 48.9±3.5%, n=5) was significantly larger than that seen in the absence of PTX (to 62.7±0.6 %, n=6) (P<0.01). Since the GlyR is mainly composed of α2 and β subunits in mature rat hippocampus (Betz and Laube, 2006; Malosio et al., 1991), these results suggest that heteromeric α2β-GlyRs are more sensitive to fluoxetine inhibition.

To further examine the GlyR subtype-sensitivity of fluoxetine, we transiently expressed homomeric α2- and heteromeric α2β-GlyRs in HEK293T cells. Since cells co-transfected with GlyR α2 and β subunit cDNAs do not always express a pure population of heteromeric GlyRs, we added 100 μM PTX throughout to prevent contamination by homomeric α2-GlyRs when recording the \( I_{\text{Gly}} \) mediated by recombinant heteromeric α2β-GlyRs. Fig. 5B shows that fluoxetine (100 μM) significantly inhibited the \( I_{\text{Gly}} \) mediated by recombinant heteromeric α2β- (n=4, P<0.001) but not homomeric α2-GlyRs. These results suggest that fluoxetine is a subtype-selective GlyR inhibitor in mature rat hippocampus.

### 3. Discussion

In this study, we have demonstrated that the antidepressant fluoxetine can inhibit \( I_{\text{Gly}} \) in a dose-dependent manner in cultured rat hippocampal neurons. The fluoxetine metabolite norfluoxetine is even more potent of an inhibitor. In addition, our results suggest that fluoxetine may be a competitive and subtype-selective inhibitor of GlyRs in mature rat hippocampus.

Previous studies have indicated that fluoxetine is an allosteric antagonist of 5-HT type-3 receptors (Eisensamer et al., 2003) and a competitive antagonist of 5-HT type-2C receptors (Ni and Miledi, 1997). In this study, our data show that fluoxetine inhibited \( I_{\text{Gly}} \) in a voltage-independent manner. Fluoxetine shifted the glycine concentration–response curve to the right without affecting the maximal response to glycine. Lineweaver–Burk plot showed that fluoxetine did not change the Y-intercept. Schild plot analysis showed that fluoxetine increased glycine EC\(_{50}\) with a slope of linear regression of –1.14. Although Schild plot analysis for competitive antagonism is based on EC\(_{50}\) measurements in the Hill equation, which does not have a proper physical basis (because the most competitive antagonist does not behave ideally), the scope of the linear regression is considered to be optimal within the limits of 0.8–1.2 (Tallarida and Murray,

![Fig. 5 – Subtype-sensitivity of fluoxetine inhibition. (A) Representative current traces recorded from the same neuron showing the fluoxetine inhibition in the absence and presence of PTX in rat hippocampal neurons. The bar graph compares the fluoxetine inhibition of \( I_{\text{Gly}} \) in the absence and presence of PTX. (n=5, ***P<0.001, compared with the response to 100 μM glycine alone in the absence (control) or presence of PTX, respectively; **P<0.01, indicates the significant difference between the fluoxetine inhibition in the absence (control) and presence of PTX, Student’s t-test). (B) Representative current traces show the effects of 100 μM fluoxetine on recombinant α2- and α2β-GlyRs transiently expressed in HEK293T cells. The bar graph shows the average effect of fluoxetine on currents mediated by α2- or α2β-GlyRs (n=4, ***P<0.001, compared with the response to glycine alone, Student’s t-test).](image-url)
fluoxetine selectively inhibits the activity of heteromeric GlyRs was shown to be weak because fluoxetine inhibited the activity of action than glycine. The binding affinity of fluoxetine for glycine has a molecular weight of 309.33, much greater than glycine. If fluoxetine and glycine have different but overlapping binding sites. In that case, the fluoxetine binding site would become inaccessible when the GlyR is in the glycine-bound state. Further study is warranted to confirm these possibilities.

The GlyR subtype-selectivity of fluoxetine in mature rat hippocampus was also investigated in this study. In rat hippocampal neurons, the amount of inhibition by fluoxetine significantly increased after selectively inhibiting homomeric GlyRs with 100 μM picrotoxin. In transiently-transfected HEK293T cells, fluoxetine inhibited I_{Gly} mediated by heteromeric α2β2- but not homomeric α2-β2-GlyRs. These results suggest that fluoxetine selectively inhibits the activity of homomeric α2β2-GlyRs in mature rat hippocampus.

The clinical significance of fluoxetine inhibition on I_{Gly} may be of interest with respect to its association with seizure activity at higher doses. In this study, we found that fluoxetine inhibited GlyR activities in a dose-dependent manner. The metabolite norfluoxetine is even more potent than fluoxetine. In addition to the direct effect shown here, fluoxetine might also inhibit the GlyR activity indirectly by enhancing the synthesis of neuroactive steroids (Pinna et al., 2004; Uzunova et al., 1998) that negatively modulate GlyR activities (Jiang et al., 2006).

One might doubt whether the concentrations of fluoxetine or norfluoxetine used in this study are comparable with those expected to arise from therapeutic doses or overdoses. It has been reported that the cumulative brain concentration of fluoxetine/norfluoxetine in chronically treated patients with therapeutic doses could reach 30 μM (Karson et al., 1993), which is nearly 20-fold higher than plasma levels and comparable to the concentration used in the present study. In view of the critical importance of GlyRs in the CNS, the inhibitory action of fluoxetine/norfluoxetine on the GlyR activity may lead to CNS disinhibition and result in seizure activity after long-term, high dose fluoxetine treatment. Since norfluoxetine is more potent and has a significantly longer elimination half-life than fluoxetine (Sanchez and Hyttel, 1999), it might be that many of the fluoxetine-associated seizures observed in vivo are mediated by norfluoxetine. The mechanism underlying the action of fluoxetine in vivo remains far from clear, however, and requires further study, including the interplay between GlyRs, GABA<sub>A</sub>Rs, and 5-HT<sub>3</sub> receptors with respect to the effects of fluoxetine.

Taken together, these data suggest that the inhibition of I_{Gly} by fluoxetine/norfluoxetine might play a significant role in fluoxetine-associated seizures, and underscore the need for development of novel, more highly selective antidepressants.

### 4. Experimental procedures

The use and care of animals in the present study followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Science and Technology of China. All efforts were made to minimize the number of animals used.

#### 4.1. Cell culture

Hippocampal neurons were isolated from Wistar rats (postnatal day 0) by a standard enzyme treatment protocol. Briefly, hippocampal tissues were dissociated with trypsin-EDTA solution (0.25% trypsin and 1 mM EDTA, Gibco, Grand Island, NY, USA) and the neurons were plated (1–5×10<sup>5</sup> cell/ml) on poly-lysine (Sigma, St. Louis, MO, USA) coated cover glasses in DMEM (Gibco) with L-glutamine plus 10% fetal bovine serum (Gibco), 10% F-12 nutrient mixture (Gibco), and 100 U/ml penicillin/streptomycin (Gibco). After the attachment of neurons to the glasses within 24 h, the culture medium was replaced with neuron-basal medium (1 ml) with 2% B27 (Gibco) every 3–4 days. To stabilize the cell population, the culture was treated with 5-fluoro-2′-deoxyuridine (20 μg/ml, Sigma) on the fourth day after plating to block cell division of non-neuronal cells. The cultures were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. Cells for electrophysiological recordings were used at 7–14 days after plating.

#### 4.2. Transfection

All constructs were expressed in HEK293T cells. HEK293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were maintained in DMEM (Gibco) supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (Gibco). HEK293T cells were transfected by lipofection using 1 μg of cDNA and 2 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per 3.5×10<sup>5</sup> cells plated on 35 mm culture dishes. The GlyR subunits were co-transfected with pEGFP, a green fluorescent protein expression vector that was used to identify the transfected cells for patch clamping by monitoring its fluorescence. The α2- and β-subunits were co-transfected at the ratio of 1:4. Electrophysiological measurements were performed 24–48 h after transfection.

The human α2 subunit cDNA was kindly provided by Dr. Heinrich Betz (Department of Neurochemistry, Max-Planck-Institute for Brain Research, Germany), the human β subunit cDNA by Dr. Yu-tian Wang (University of British Columbia, Canada) and the pEGFP by Dr. Jian-hong Luo (Faculty of Medicine, Zhejiang University, China).

#### 4.3. Solutions and drugs

The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, and 10 Hepes. The pH was adjusted to 7.4 with Tris base. The osmolarity of the solutions

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1987; Tallarida et al., 1979). Thus, these results indicate a competitive inhibition and exclude an allosteric effect of fluoxetine on GlyRs. If fluoxetine indeed has any allosteric effects on GlyRs, this effect would be secondary and minimal.
was adjusted with sucrose to 310–320 mOsm/l detected with a micro-osmometer (Model 3300, Norwood, MA, USA). 0.3 μM tetrodotoxin (TTX) was added all the time to prevent contamination by spontaneous synaptic activity. The pipette solution for whole-cell patch-clamp recording contained (in mM): 150 CsCl, 1 MgCl₂, 0.5 CaCl₂, 5 EGTA, 2 Mg-ATP, and 10 Hepes. The pH was also adjusted to 7.4 with Tris base. When examining the relationship between the effect of fluoxetine on the Iₘ, and the membrane potential, voltage-activated Ca²⁺ channel was blocked by adding 0.2 mM CdCl₂ to the standard external solution. The drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use. All drugs for electrophysiological experiments were purchased from Sigma and applied using a rapid application technique termed as ‘Y-tube’ method throughout the experiments (Murase et al., 1989). This application system allows a complete exchange of external solution surrounding a neuron within 20 min.

4.4. Electrophysiological recordings

The electrophysiological recordings were performed in conventional whole-cell patch-clamp recording configuration under voltage-clamp mode. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 3–5 MΩ. Membrane currents were measured using a patch-clamp amplifier (Axon 200B, Molecular Device, CA, USA), sampled using a Digidata 1320A interface connected to a personal computer and analyzed with Clampex and Clampfit software (Version 9.0.1, Axon Instruments). In this study, 70–90% series resistance was compensated. Unless otherwise specified, the membrane potential was held at −60 mV on hippocampal neurons and −50 mV on HEK293T cells throughout the experiment. All the experiments were carried out at room temperature (22–25 °C).

4.5. Data analysis

Clampfit software was used for data analysis. Data were statistically compared using Student’s t-test for comparison between two groups and One-way ANOVA for comparison between multiple groups. For all experiments, average values are expressed as mean ± S.E.M. Levels of statistical significance were assumed as P<0.05. P and n represent the value of significance and the number of neurons, respectively.

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Appendix A. Supplementary data


References


