

# Inhibition of G protein-activated inwardly rectifying K<sup>+</sup> channels by fluoxetine (Prozac)

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**1** The effects of fluoxetine, a commonly used antidepressant drug, on G protein-activated inwardly rectifying K<sup>+</sup> channels (GIRK, Kir3) were investigated using *Xenopus* oocyte expression assays.

**2** In oocytes injected with mRNAs for GIRK1/GIRK2, GIRK2 or GIRK1/GIRK4 subunits, fluoxetine reversibly reduced inward currents through the basal GIRK activity. The inhibition by fluoxetine showed a concentration-dependence, a weak voltage-dependence and a slight time-dependence with a predominant effect on the instantaneous current elicited by voltage pulses and followed by slight further inhibition. Furthermore, in oocytes expressing GIRK1/2 channels and the cloned *Xenopus* A<sub>1</sub> adenosine receptor, GIRK current responses activated by the receptor were inhibited by fluoxetine. In contrast, ROMK1 and IRK1 channels in other Kir channel subfamilies were insensitive to fluoxetine.

**3** The inhibitory effect on GIRK channels was not obtained by intracellularly applied fluoxetine, and not affected by extracellular pH, which changed the proportion of the uncharged to protonated fluoxetine, suggesting that fluoxetine inhibits GIRK channels from the extracellular side.

**4** The GIRK currents induced by ethanol were also attenuated in the presence of fluoxetine.

**5** We demonstrate that fluoxetine, at low micromolar concentrations, inhibits GIRK channels that play an important role in the inhibitory regulation of neuronal excitability in most brain regions and the heart rate through activation of various G-protein-coupled receptors. The present results suggest that inhibition of GIRK channels by fluoxetine may contribute to some of its therapeutic effects and adverse side effects, particularly seizures in overdose, observed in clinical practice.

*British Journal of Pharmacology* (2003) **138**, 1119–1128. doi:10.1038/sj.bjp.705172

**Keywords:** Fluoxetine; antidepressant; GIRK; Kir channel; ethanol; *Xenopus* oocyte

**Abbreviations:** Ado, adenosine; DMSO, dimethyl sulfoxide;  $E_K$ , K<sup>+</sup> equilibrium potential; EtOH, ethanol; GIRK, G protein-activated inwardly rectifying K<sup>+</sup> channel; hK, high potassium; 5-HT, serotonin; Kir channel, inward-rectifier K<sup>+</sup> channel; ND98, K<sup>+</sup>-free high sodium;  $n_H$ , Hill coefficient; NMDG, *N*-methyl-D-glucamine; QX-314, lidocaine *N*-ethyl bromide; SSRI, selective serotonin reuptake inhibitor; *wv*, *weaver*; XA1 receptor, *Xenopus* A<sub>1</sub> adenosine receptor

## Introduction

Fluoxetine, known by the trade name of Prozac, has been used widely in the treatment of depression and other psychiatric disorders, such as obsessive-compulsive disorder, bulimia nervosa, post-traumatic stress disorder and alcoholism (Wong *et al.*, 1995; Baldessarini, 2001). Inhibition of serotonin (5-hydroxytryptamine; 5-HT) transporters in the brain is generally thought to have important implications in its therapeutic effects, and fluoxetine is classified as a selective serotonin reuptake inhibitor (SSRI) (Wong *et al.*, 1995). Recent studies have also shown that fluoxetine inhibits the functions of several receptors and ion channels, such as 5-HT<sub>2C</sub> (Ni & Miledi, 1997) and 5-HT<sub>3</sub> receptors (Fan, 1994), nicotinic acetylcholine receptors (García-Colunga *et al.*, 1997; Maggi *et al.*, 1998), voltage-gated Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> channels (Pancrazio *et al.*, 1998; Choi *et al.*, 1999; 2001; Yeung *et al.*, 1999; Deák *et al.*, 2000; Perchenet *et al.*, 2001; Thomas *et al.*,

2002) and Cl<sup>−</sup> channels (Maertens *et al.*, 1999). In addition to inhibition of serotonin transporters, these effects might be involved in the molecular and cellular mechanisms underlying the multiple therapeutic effects and side effects of fluoxetine.

G protein-activated inwardly rectifying K<sup>+</sup> (GIRK) channels (also known as Kir3 channels) are members of a family of inward-rectifier K<sup>+</sup> (Kir) channels that includes seven subfamilies (Doupnik *et al.*, 1995; Reimann & Ashcroft, 1999). Four GIRK channel subunits have been identified in mammals (Kubo *et al.*, 1993b; Lesage *et al.*, 1995; Wickman *et al.*, 1997). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions (Kobayashi *et al.*, 1995; Lesage *et al.*, 1995; Karschin *et al.*, 1996; Liao *et al.*, 1996) or homomultimers composed of GIRK2 subunits in the substantia nigra (Inanobe *et al.*, 1999), and atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky *et al.*, 1995). Various G-protein-coupled receptors, such as M<sub>2</sub> muscarinic, α<sub>2</sub> adrenergic, D<sub>2</sub> dopamine, 5-HT<sub>1A</sub>, opioid,

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nociceptin/orphanin FQ receptors and A<sub>1</sub> adenosine (Ado) receptor, activate GIRK channels (North, 1989; Ikeda *et al.*, 1995, 1996, 1997) through direct action of G protein  $\beta\gamma$ -subunits released from pertussis toxin-sensitive G proteins (Reuveny *et al.*, 1994). In addition, ethanol (EtOH) activates GIRK channels independent of G-protein-coupled signalling pathways (Kobayashi *et al.*, 1999; Lewohl *et al.*, 1999). Activation of GIRK channels causes membrane hyperpolarization, and thus the channels play an important role in the inhibitory regulation of the neuronal excitability and the heart rate (North, 1989; Signorini *et al.*, 1997; Wickman *et al.*, 1998). Therefore, modulators of GIRK channel activity may affect many brain and cardiac functions. Here we show that fluoxetine inhibits brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes, whereas two different SSRIs: fluvoxamine and zimelidine have no significant effect on the channels. In contrast, ROMK1 (Kir1.1) and IRK1 (Kir2.1) channels in other Kir channel subfamilies were insensitive to fluoxetine. Moreover, fluoxetine inhibited EtOH-induced GIRK currents. We propose that some effects of fluoxetine in clinical practice may involve inhibition of GIRK channels in the brain and heart.

## Methods

### *Preparation of specific mRNAs*

Plasmids containing the entire coding sequences for the mouse GIRK1, GIRK2, *weaver* (*wv*) GIRK2 and GIRK4 channel subunits and the *Xenopus* A<sub>1</sub> (XA1) Ado receptor were obtained using the polymerase chain reaction method as described previously (Kobayashi *et al.*, 1995, 1999, 2000, 2002). In addition, cDNAs for rat ROMK1 in pSPORT and mouse IRK1 in pcDNA1 were provided by Dr Steven C. Hebert and Dr Lily Y. Jan, respectively. These plasmids were linearized by digestion with an appropriate enzyme as described previously (Ho *et al.*, 1993; Kubo *et al.*, 1993a; Kobayashi *et al.*, 2000), and the specific mRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE™ *In Vitro* Transcription Kit (Ambion, Austin, TX, U.S.A.).

### *Electrophysiological analyses*

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until use. Frogs were anesthetized by immersion in water including 0.15% tricaine (Sigma Chemical Co., St Louis, MO, U.S.A.). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely killed after the final collection. Oocytes (Stages V and VI) were isolated manually from the ovary and maintained in Barth's solution (Kobayashi *et al.*, 2002). *Xenopus laevis* oocytes were injected with mRNA(s) for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each ~0.4 ng), GIRK2 (~5 ng), *wv*GIRK2 (~12.5 ng), ROMK1 (~5 ng) or IRK1 (~0.5 ng) and/or XA1 (~10 ng). The oocytes were incubated at 19°C in Barth's solution, and defolliculated following treatment with 0.8 mg ml<sup>-1</sup> collagenase as described previously (Kobayashi *et al.*, 2002). Whole-cell currents of the oocytes were recorded from 2 to 10 days after the injection with a conventional two-electrode voltage clamp (Kobayashi *et al.*,

1999). The membrane potential was held at -70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-potassium (hK) solution (composition in mM: KCl 96, NaCl 2, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.5 and HEPES 5), a K<sup>+</sup>-free high-sodium (ND98) solution (composition in mM: NaCl 98, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.5 and HEPES 5) or a large organic cation *N*-methyl-D-glucamine (NMDG) solution (composition in mM: NMDG 98, MgCl<sub>2</sub> 1 and HEPES 5) at a flow rate of 2.5 ml min<sup>-1</sup>. The acidic or basic solutions were made by adding HCl or NaOH, respectively. For examining the effect of intracellular fluoxetine, 23 nl of 10 mM fluoxetine or 30 mM lidocaine *N*-ethyl bromide (QX-314) dissolved in distilled water was injected into an oocyte using a Nanoliter injector (World Precision Instruments, Sarasota, FL, U.S.A.) (Kobayashi *et al.*, 1999) and the oocyte currents were continuously recorded for 30–40 min. Owing to a volume of ~1  $\mu$ l in the oocyte, the intracellular concentrations of fluoxetine or QX-314 were presumed as ~225 or ~674  $\mu$ M, respectively. In the hK solution, the K<sup>+</sup> equilibrium potential ( $E_K$ ) was close to 0 mV and inward K<sup>+</sup> current flow through Kir channels was observed at negative holding potentials. Data were fitted to a standard logistic equation using SigmaPlot (SPSS Science, Chicago, IL, U.S.A.) in the analysis of concentration–response relations. The EC<sub>50</sub> value, which is the concentration of a drug that produces 50% of the maximal current response for that drug, IC<sub>50</sub> value which is the concentration of a drug that reduces control current responses by 50% and the Hill coefficient ( $n_H$ ) were obtained from the concentration–response relations.

### *Statistical analysis of results*

The values obtained are expressed as mean  $\pm$  s.e.m., and  $n$  is the number of oocytes tested. Statistical analysis of differences between groups was carried out using paired *t*-test, Student's *t*-test or one-way ANOVA followed by Tukey–Kramer *post hoc* test. A probability of 0.05 was taken as the level of statistical significance.

### *Compounds*

Fluoxetine hydrochloride and fluvoxamine maleate were purchased from Tocris Cookson Limited (Bristol, U.K.). Zimelidine dihydrochloride and Ado were purchased from Research Biochemical International (Natick, MA, U.S.A.). QX-314 was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Fluoxetine and fluvoxamine were dissolved in dimethyl sulfoxide (DMSO) or distilled water. Other drugs were dissolved in distilled water. The stock solutions of these compounds were stored at -30°C until use. EtOH was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

## Results

### *Inhibition of GIRK channels by fluoxetine*

To investigate whether fluoxetine interacts with brain-type GIRK1/2 and cardiac-type GIRK1/4 channels, we used

*Xenopus* oocyte expression assays. In oocytes coinjected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which are known to depend on free G protein  $\beta\gamma$ -subunits present in the oocytes because of the inherent activity of G proteins (Dascal, 1997), were observed under the conditions of a hK solution containing 96 mM  $K^+$  and negative membrane potentials (Kobayashi *et al.*, 2000; Figure 1a). Application of fluoxetine, at 10  $\mu$ M, immediately and reversibly caused a reduction of the inward currents through basally active channels in the hK solution (Figure 1a). The current responses were abolished in

the presence of 3 mM  $Ba^{2+}$ , which blocks the Kir channel family including GIRK channels ( $n = 4$ ; Figure 1a). Fluoxetine produced no significant response in a  $K^+$ -free ND98 solution containing 98 mM  $Na^+$  instead of the hK solution ( $5 \pm 5$  nA,  $n = 3$ ), suggesting that the fluoxetine-sensitive current components show  $K^+$  selectivity. In uninjected oocytes, fluoxetine and  $Ba^{2+}$  caused no significant response (0 nA at 200  $\mu$ M and  $5.5 \pm 0.7$  nA at 3 mM at a membrane potential of  $-70$  mV,  $n = 4$  and 29, respectively; Figure 1a), suggesting no effect of fluoxetine on intrinsic oocyte channels. In addition, application of DMSO, the solvent vehicle, at the highest concentration (0.3%) used had no significant effect on the current responses in oocytes coinjected with GIRK1 and GIRK2 mRNAs ( $n = 4$ ; data not shown). These results suggest that fluoxetine inhibits GIRK1/2 channels. However, the channels were insensitive to two different SSRIs: fluvoxamine and zimelidine ( $6.1 \pm 2.3\%$  inhibition and  $0.7 \pm 1.2\%$  inhibition of the 3 mM  $Ba^{2+}$ -sensitive current component at 100  $\mu$ M,  $n = 11$  and 10, respectively). Furthermore, similar results were obtained in oocytes co-injected with GIRK1 and GIRK4 mRNAs (Figure 1a), suggesting that fluoxetine also inhibits GIRK1/4 channels.

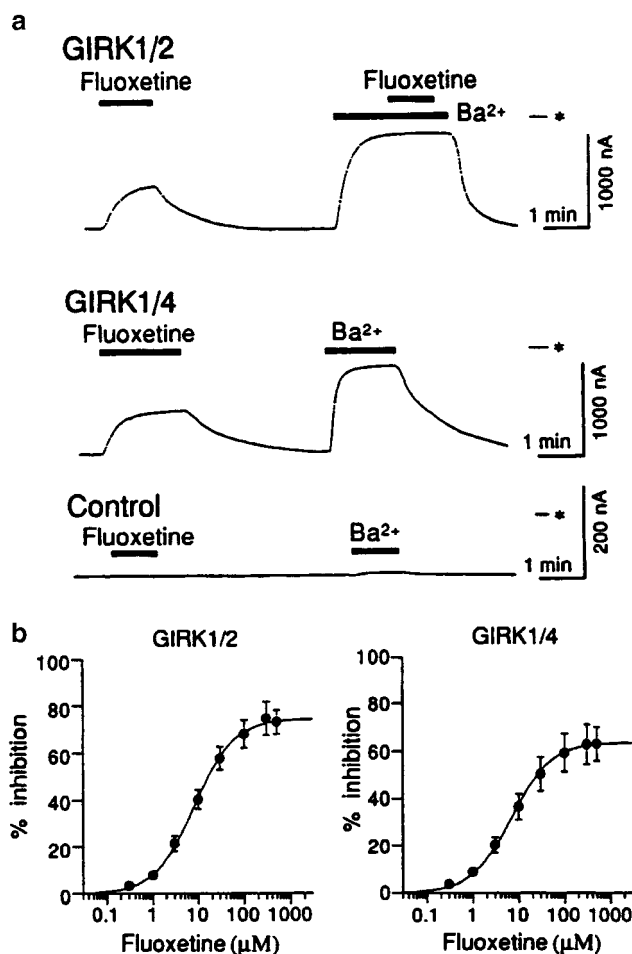
#### Characteristics in fluoxetine inhibition of GIRK channels

We investigated the concentration–response relations of the inhibitory effects of fluoxetine on GIRK channels expressed in *Xenopus* oocytes, compared with the current components sensitive to 3 mM  $Ba^{2+}$  which blocked basal GIRK currents (Kobayashi *et al.*, 2002). Inhibition of GIRK1/2 and GIRK1/4 channels by fluoxetine was concentration-dependent with similar potency and effectiveness at micromolar concentrations (Figure 1b). Fluoxetine reduced the GIRK currents to a limited extent even at high concentrations. Table 1 shows the  $EC_{50}$  and  $n_H$  values obtained from the concentration–response relations for fluoxetine and the percentage inhibition of the GIRK currents by the drug at the highest concentrations tested. In addition, to further compare the effects of fluoxetine on GIRK channels, the drug concentrations required to inhibit the GIRK currents by 50% are shown in Table 1.

The instantaneous GIRK1/2 currents elicited by the voltage step to  $-100$  mV from a holding potential of 0 mV diminished in the presence of 10  $\mu$ M fluoxetine (Figure 2a). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by fluoxetine was slightly higher than that of the instantaneous current ( $4.6 \pm 0.2\%$ ,  $4.6 \pm 0.1\%$  and  $5.2 \pm 0.1\%$  at  $-80$ ,  $-100$  and  $-120$  mV; paired *t*-test,  $P < 0.05$ , 0.005 and 0.005;  $n = 11$ , 10 and 12, respectively). These results suggest that the channels were primarily inhibited at the holding potential of 0 mV and in only a slight time-dependent manner by external fluoxetine.

Like 3 mM  $Ba^{2+}$ -sensitive currents corresponding to basal GIRK currents, fluoxetine-sensitive currents in oocytes expressing GIRK channels increased with negative membrane potentials and the current–voltage relations showed strong inward rectification (Figure 2b), indicating a characteristic of GIRK currents.

For GIRK1/2 channels, the percentage inhibition of GIRK currents by 10  $\mu$ M fluoxetine tended to decrease with membrane potentials from  $-80$  to  $-20$  mV, although there was no significant difference in the percentage inhibition at membrane potentials between  $-120$  and  $-40$  mV (Figure 2c). For

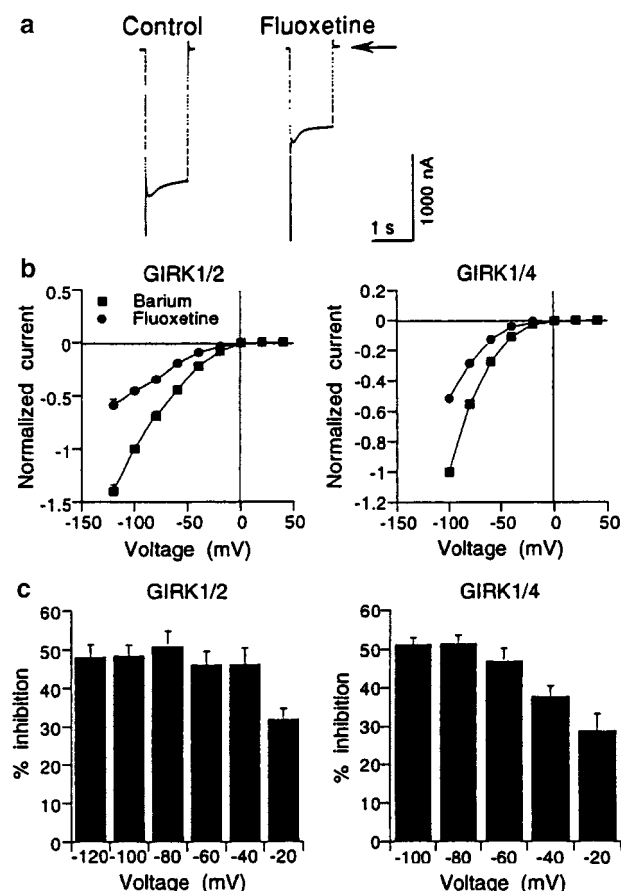


**Figure 1** Inhibition by fluoxetine of brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. (a) *Top*: In an oocyte coinjected with GIRK1 and GIRK2 mRNAs, current responses to 10  $\mu$ M fluoxetine and 10  $\mu$ M fluoxetine in the presence of 3 mM  $Ba^{2+}$ . *Middle*: In an oocyte coinjected with GIRK1 and GIRK4 mRNAs, current responses to 10  $\mu$ M fluoxetine and 3 mM  $Ba^{2+}$ . *Bottom*: In an uninjected oocyte, no significant current responses to 200  $\mu$ M fluoxetine and 3 mM  $Ba^{2+}$ . Asterisks show the zero current level. Bars show the duration of application. (b) Concentration–response relations for fluoxetine in regard to the effects on GIRK1/2 channels (left) and GIRK1/4 channels (right). The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM  $Ba^{2+}$ -sensitive current components, which were  $741.6 \pm 118.0$  nA ( $n = 5$ ) in oocytes expressing GIRK1/2 channels and  $600.8 \pm 268.0$  nA ( $n = 5$ ) in oocytes expressing GIRK1/4 channels, respectively. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution containing 96 mM  $K^+$  (pH 7.4). Each point and error bar represents the mean and s.e.m. of the percentage responses obtained from five oocytes. Data points were fitted using a logistic equation.

**Table 1** Inhibitory effects of fluoxetine on GIRK channels

Channel	EC <sub>50</sub>	IC <sub>50</sub>	% inhibition ( $\mu$ M)	n <sub>H</sub>	(n)
GIRK1/2	7.89 $\pm$ 0.60	16.9 $\pm$ 4.0	73.6 $\pm$ 6.1 (300)	0.83 $\pm$ 0.06	(5)
GIRK1/4	7.05 $\pm$ 0.65	18.4 $\pm$ 4.3	63.3 $\pm$ 8.5 (300)	0.85 $\pm$ 0.03	(5)
GIRK2	13.03 $\pm$ 1.89	89.5 $\pm$ 24.3	57.5 $\pm$ 7.5 (300)	0.89 $\pm$ 0.02	(7)
wtGIRK2	7.85 $\pm$ 2.24	9.37 $\pm$ 2.81	92.0 $\pm$ 3.4 (200)	0.82 $\pm$ 0.05	(4)

The mean  $\pm$  s.e.m. of the EC<sub>50</sub> values and the drug concentrations required to reduce basal GIRK currents by 50% (IC<sub>50</sub>) are shown in  $\mu$ M. The values of % inhibition indicate the mean  $\pm$  s.e.m. % inhibition of basal GIRK currents by fluoxetine at the highest concentrations tested. The highest concentrations tested ( $\mu$ M) and the number of oocytes tested (n) are indicated in parentheses. The n<sub>H</sub> values indicate the mean  $\pm$  s.e.m. of the Hill coefficients.



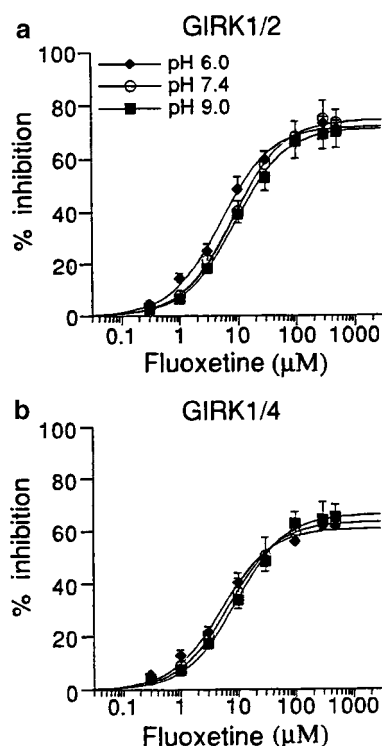
**Figure 2** Characteristics in the effects of fluoxetine on GIRK currents. (a) Representative GIRK1/2 currents elicited by voltage step to  $-100$  mV for 1 s from a holding potential of 0 mV in the absence and presence of  $10 \mu$ M fluoxetine. Current responses were recorded in a high-potassium solution containing  $96$  mM  $K^+$ . Arrow indicates the zero current level. (b) Current-voltage relations of  $3$  mM  $Ba^{2+}$ -sensitive inward currents and  $10 \mu$ M fluoxetine-sensitive inward currents in oocytes expressing GIRK1/2 channels or GIRK1/4 channels. Current responses were normalized to the  $3$  mM  $Ba^{2+}$ -sensitive current component measured at a membrane potential of  $-100$  mV. The  $Ba^{2+}$ -sensitive current components were  $2426.9 \pm 661.3$  nA ( $n = 7$ ) in oocytes expressing GIRK1/2 channels and  $1203.3 \pm 307.0$  nA ( $n = 4$ ) in oocytes expressing GIRK1/4 channels. (c) The percentage inhibition of GIRK channels by fluoxetine over the voltage range of  $-120$  to  $-20$  mV. There was a significant interaction between the fluoxetine effect and the membrane potential effect ( $P < 0.05$  for GIRK1/2,  $n = 5-16$  for the groups, and  $P < 0.001$  for GIRK1/4,  $n = 4$  for each group; one-way ANOVA), and then there were significant differences between the effects at  $-80$  or  $-100$  mV and that at  $-20$  mV for GIRK1/2 channels, and between the effects at  $-60$ ,  $-80$  or  $-100$  mV and that at  $-20$  mV and between the effects at  $-80$  or  $-100$  mV and that at  $-40$  mV for GIRK1/4 channels ( $P < 0.05$ ; Tukey-Kramer *post hoc* test). All values are mean and s.e.m.

GIRK1/4 channels, the percentage inhibition of GIRK currents by  $10 \mu$ M fluoxetine decreased with membrane potentials from  $-80$  to  $-20$  mV (Figure 2c). When membrane potentials were more negative than  $-100$  mV, the percentage inhibitions by  $10 \mu$ M fluoxetine were almost the same both for GIRK1/2 and GIRK1/4 channels, suggesting that some changes in the interaction between fluoxetine and GIRK channels may occur at very negative membrane potentials.

At physiological pH or below, fluoxetine exists mainly in a protonated form and the proportion of the uncharged form increases by increasing pH, because of a  $pK_a$  value of 9.5 (Maertens *et al.*, 1999). We examined whether changes in pH affect fluoxetine inhibition of GIRK channels. The effects of pH on the fluoxetine inhibition were not observed in the concentration-response relations for fluoxetine (Figure 3), suggesting that the inhibition is mediated by both forms of fluoxetine with almost the same effectiveness. It also appears unlikely that the inhibition by fluoxetine is caused by hydrophobic interactions with GIRK channels within the membrane bilayer.

Moreover, we examined the effect of intracellular fluoxetine on GIRK channels. Injection of fluoxetine into oocytes expressing GIRK1/2 channels had no or little effect on the basal currents ( $3.8 \pm 1.8\%$  inhibition of control at the presumed intracellular concentration of  $\sim 225 \mu$ M,  $n = 9$ ). Therefore, the result suggests that fluoxetine directly inhibits GIRK channels from the extracellular side of the cell membrane.

We further investigated the effects of fluoxetine on GIRK channels activated by G-protein-coupled receptors. In oocytes coexpressing GIRK1/2 channels and XA1 receptor (Kobayashi *et al.*, 2002), application of  $10$  nM Ado induced inward GIRK currents (Figure 4). Effects of fluoxetine were evaluated by measuring the amplitude of the Ado-induced current response during application of fluoxetine at different concentrations. The current responses to  $10$  nM Ado were reversibly inhibited by fluoxetine with an IC<sub>50</sub> value of  $9.26 \pm 2.00 \mu$ M and a  $n_H$  value of  $0.88 \pm 0.08$  ( $n = 5$ , Figure 4). The extent of inhibition by fluoxetine was similar to that of basally active GIRK1/2 channels ( $P > 0.05$  at each concentration, Student's *t*-test), suggesting interaction of fluoxetine with GIRK channels. In addition, the Ado-induced GIRK currents were not significantly affected by intracellularly applied fluoxetine ( $112.2 \pm 14.4\%$  of pretreated control current, paired *t*-test,  $P > 0.1$ ,  $n = 5$ ), whereas the GIRK currents were significantly inhibited by intracellularly applied QX-314 ( $55.2 \pm 11.9\%$  of pretreated control current, paired *t*-test,  $P < 0.01$ ,  $n = 8$ ) as reported previously (Zhou *et al.*, 2001). The results, therefore, suggest that extracellular fluoxetine can inhibit the effect of GIRK channels activated by G-protein-coupled receptors.

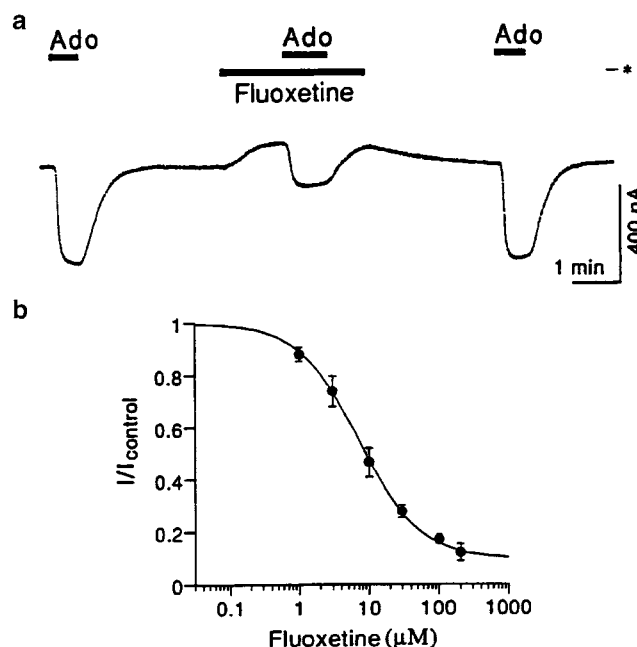


**Figure 3** Concentration–response relations for inhibition of GIRK1/2 channels and GIRK1/4 channels by fluoxetine at three different pH values. The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM Ba<sup>2+</sup>-sensitive current components, which were  $1033.3 \pm 184.8$  nA ( $n=6$ , pH 6.0),  $741.6 \pm 118.0$  nA ( $n=5$ , pH 7.4) and  $822.0 \pm 166.3$  nA ( $n=4$ , pH 9.0) in oocytes expressing GIRK1/2 channels (a), and  $625.5 \pm 117.4$  nA ( $n=6$ , pH 6.0),  $600.8 \pm 268.0$  nA ( $n=5$ , pH 7.4) and  $328.8 \pm 114.0$  nA ( $n=4$ , pH 9.0) in oocytes expressing GIRK1/4 channels (b), respectively. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution. Each point and error bar represents the mean and s.e.m. of the percentage responses obtained. Data points were fitted using a logistic equation.

#### Comparison of the fluoxetine effects on members of Kir channels

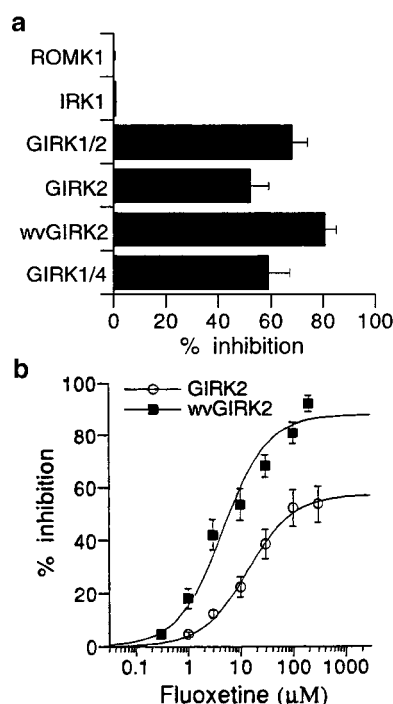
We examined whether fluoxetine interacts with ROMK1, an ATP-regulated inwardly rectifying K<sup>+</sup> channel, and IRK1, a constitutively active Kir channel, among G-protein-insensitive Kir channels. In oocytes expressing ROMK1 or IRK1 channels, application of 100 μM fluoxetine had no significant effect on the inward currents through the channels in the hK solution ( $0.3 \pm 0.3$  inhibition and  $0.8 \pm 0.3\%$  inhibition of the 3 mM Ba<sup>2+</sup>-sensitive current components that were  $946.0 \pm 126.0$  and  $967.7 \pm 418.1$  nA at a membrane potential of  $-70$  mV,  $n=3$  for ROMK1 and  $n=6$  for IRK1, respectively; Figure 5a).

GIRK2 channels form homomultimers (Lesage *et al.*, 1995; Inanobe *et al.*, 1999). The GIRK2 channels of *wv* mutant mice, which have a missense point mutation in the pore-forming region (Patil *et al.*, 1995), show constitutive activation in a G-protein- and EtOH-independent manner and permeability to sodium as well as potassium ions because of the lack of K<sup>+</sup> selectivity (Navarro *et al.*, 1996; Slesinger *et al.*, 1997; Kobayashi *et al.*, 1999). To further address the relation



**Figure 4** Inhibitory effect of fluoxetine on GIRK channels activated by a G-protein-coupled receptor. (a) In an oocyte coexpressed with mRNAs for GIRK1 and GIRK2 channels and XA1 receptor, current responses to adenosine (Ado), Ado in the presence of 10 μM fluoxetine and Ado are shown. The concentration of Ado used was 10 nM. Bars show the duration of application. Asterisk indicates the zero current level. (b) Concentration-dependent inhibition of fluoxetine on Ado-induced GIRK currents.  $I_{\text{control}}$  is the amplitude of GIRK currents induced by 10 nM Ado ( $407.2 \pm 109.0$  nA,  $n=5$ ) and  $I$  is the current amplitude in the presence of fluoxetine. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution. Each point and error bar represents the mean and s.e.m. of the relative responses. Data points were fitted using a logistic equation.

between fluoxetine and GIRK channels, we investigated the effects of fluoxetine on these homomeric channels. In oocytes expressing GIRK2 channels, fluoxetine inhibited the channels to the similar extent when compared with GIRK heteromeric channels ( $P > 0.05$  at 300 μM, Student's *t*-test), although the IC<sub>50</sub> value for GIRK2 channels was  $\sim 5$  times higher than those for the heteromeric channels (Table 1). On the other hand, in oocytes expressing *wv*GIRK2 channels, large inward currents were observed when the oocytes were perfused with either ND98 or hK solution instead of NMDG solution, which does not contain Na<sup>+</sup> or K<sup>+</sup> ( $3118.6 \pm 190.1$  and  $3094.6 \pm 364.4$  nA,  $n=7$  and 25, respectively). The addition of 100 μM fluoxetine to ND98 or hK solutions immediately reduced the inward currents ( $89.9 \pm 1.9\%$  of the ND98 current component and  $80.9 \pm 4.1\%$  of the hK current component,  $n=4$  and 7, respectively; Figure 5a). As shown in Figure 5 and Table 1, the inhibitory effect of fluoxetine on *wv*GIRK2 channels was more efficacious than that of GIRK2 channels ( $P < 0.01$ , Student's *t*-test). Moreover, injection of fluoxetine inhibited only  $8.4 \pm 2.3\%$  of the hK current component, which was  $1329.8 \pm 232.9$  nA, by the presumed intracellular concentration of  $\sim 225$  μM ( $n=5$ ). The results suggest the possibilities that extracellular fluoxetine may easily approach its action sites on *wv*GIRK2 channels because of a mutation in the pore-

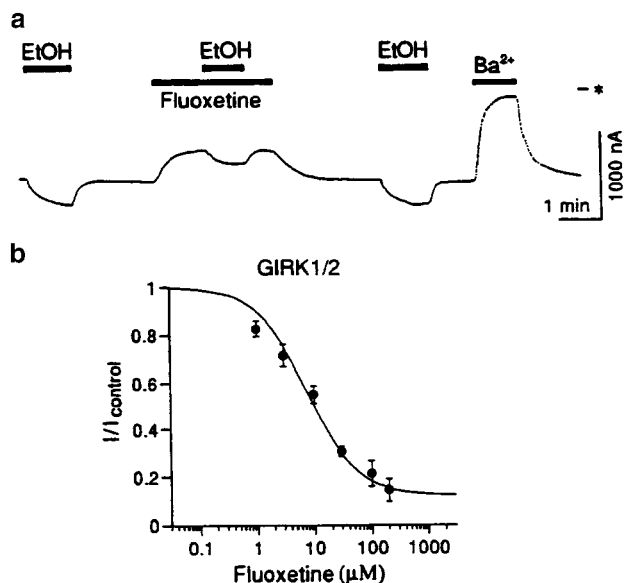


**Figure 5** Comparison of the fluoxetine effects on members of inwardly rectifying potassium channels. (a) Action of fluoxetine on GIRK, ROMK1 and IRK1 channels expressed as homomeric or heteromeric channels in *Xenopus* oocytes. The concentration of fluoxetine used was 100  $\mu$ M. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution. (b) Distinct concentration-dependent responses to fluoxetine for GIRK2 channels and *weaver* (wv) GIRK2 channels. The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM  $\text{Ba}^{2+}$ -sensitive current components ( $685.8 \pm 183.6$  nA,  $n = 5$ ) in oocytes expressing GIRK2 channels, and with the hK current components ( $2857.8 \pm 321.0$  nA,  $n = 4$ ) in oocytes expressing wvGIRK2 channels, respectively. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution or a NMDG solution. Each point and error bar represents the mean and s.e.m. of the percentage responses obtained. Data points were fitted using a logistic equation.

forming region and/or that it may easily cause a distinct conformational change in the channels with the different channel properties.

#### Fluoxetine inhibits EtOH-induced GIRK currents

GIRK channels are also activated by EtOH independent of G-protein signalling pathways (Kobayashi *et al.*, 1999). Several studies have shown that fluoxetine reduces EtOH consumption (Rockman *et al.*, 1982; Naranjo & Knoke, 2001), suggesting a relation between the fluoxetine and EtOH effects. We next examined the effect of fluoxetine on EtOH-induced GIRK currents. In oocytes expressing GIRK1/2 channels, the EtOH-induced GIRK currents were attenuated in the presence of fluoxetine, with an  $\text{IC}_{50}$  value of  $11.1 \pm 2.5$   $\mu$ M and a  $n_H$  value of  $0.78 \pm 0.02$ , in a reversible manner ( $n = 5$ ; Figure 6). Furthermore, we compared the EtOH-induced GIRK currents before and after injecting



**Figure 6** Inhibitory effect of fluoxetine on the ethanol-induced GIRK currents in *Xenopus* oocytes expressing GIRK1/2 channels. (a) In an oocyte coinjected with GIRK1 and GIRK2 mRNAs, current responses to ethanol (EtOH), EtOH in the presence of 10  $\mu$ M fluoxetine, EtOH and 3 mM  $\text{Ba}^{2+}$  are shown. The concentration of EtOH used was 100 mM. Asterisk indicates the zero current level. Bars show the duration of application. (b) Concentration-dependent inhibition of fluoxetine on EtOH-induced GIRK currents.  $I_{\text{control}}$  is the amplitude of GIRK currents induced by 100 mM EtOH ( $340.8 \pm 18.1$  nA,  $n = 5$ ) and  $I$  is the current amplitude in the presence of fluoxetine. Current responses were measured at a membrane potential of  $-70$  mV in a high-potassium solution containing 96 mM  $\text{K}^+$ . Each point and error bar represents the mean and s.e.m. of the relative responses. Data points were fitted using a logistic equation.

fluoxetine into the oocytes. The EtOH-induced GIRK currents were not significantly affected by intracellularly applied fluoxetine ( $105.9 \pm 4.6\%$  of pretreated control current, paired  $t$ -test,  $P > 0.1$ ,  $n = 5$ ). The results, therefore, suggest that extracellular fluoxetine can inhibit the effect of GIRK channels induced by EtOH.

## Discussion

We have demonstrated that extracellular fluoxetine inhibits both brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels to a limited extent at high concentrations. However, two different SSRIs fluvoxamine and zimelidine have no significant effect on the channels. The inhibition by fluoxetine showed a concentration-dependence, a weak voltage-dependence and a slight time-dependence with a predominant effect on the instantaneous current elicited by voltage pulses and followed by slight further inhibition. On the other hand, blockade by extracellular  $\text{Ba}^{2+}$  and  $\text{Cs}^+$ , typical of Kir channel blockers that occlude the pore of the open channel, shows a concentration-dependence, a strong voltage-dependence and a time-dependence with a comparatively small effect on the instantaneous current, but a marked inhibition on the steady-state current at the end of voltage pulses (Lesage *et al.*, 1995). These observations suggest that

fluoxetine probably causes a conformational change in the channels, but does not act as typical open channel blockers of Kir channels like  $\text{Ba}^{2+}$  and  $\text{Cs}^{+}$ , although all these molecules act at the channels from the extracellular side. The different mechanism from  $\text{Ba}^{2+}$  for GIRK channels may be involved in the incomplete blockade and slower time course of inhibition, compared with the inhibition by  $\text{Ba}^{2+}$  as shown in Figure 1. Since *ww*GIRK2 channels were highly sensitive to fluoxetine, but ROMK1 and IRK1 channels among members of the Kir channel family were insensitive to fluoxetine, further studies using chimeric GIRK channels replaced with the homologous regions from ROMK1 and IRK1 channels and mutant GIRK channels may clarify the critical sites mediating the effects of fluoxetine on GIRK channels. In addition, single channel analyses of wild-type GIRK channels and *ww*GIRK2 channels in the presence of fluoxetine may be useful for understanding the mechanisms of the action of fluoxetine on GIRK channels and the greater inhibition by fluoxetine of the mutant channels with different channel properties.

The therapeutic plasma concentrations of fluoxetine range approximately from 0.3 to 1.5  $\mu\text{M}$  (Baldessarini, 2001). The brain concentrations of fluoxetine are approximately 10 or 20 times higher than the corresponding blood levels (Karson *et al.*, 1993; Bolo *et al.*, 2000). Therefore, the present findings suggest that GIRK channels in the brain may be inhibited by fluoxetine at clinically relevant concentrations.

Neuronal GIRK channels are widely present in various brain regions, such as the frontal cortex, hippocampus, amygdala, thalamus, substantia nigra, ventral tegmental area, brain stem and cerebellum (Kobayashi *et al.*, 1995; Karschin *et al.*, 1996; Liao *et al.*, 1996). GIRK channels are thought to play an important role in inhibiting the neuronal excitability through activation of various G-protein-coupled receptors. Therefore, inhibition of GIRK channels by fluoxetine may affect many brain functions. Recent studies have demonstrated that there are regional abnormalities in the prefrontal cortex, hippocampus, amygdala, thalamus and ventral striatum in depressed individuals (Manji *et al.*, 2001). It is generally thought that the therapeutic effects of fluoxetine and fluvoxamine are primarily because of the inhibition of the reuptake of serotonin in the brain. Although a comparative study of fluoxetine and fluvoxamine showed that both drugs were equally effective in the treatment of patients with major depression (Rapaport *et al.*, 1996), cohort studies showed that fluvoxamine was significantly less effective in the treatment of depression, anxiety and other disorders than fluoxetine (Mackay *et al.*, 1997). In addition, fluvoxamine is approved for the treatment of obsessive-compulsive disorder in the US, but not for the treatment of depression (Barbey & Roose, 1998). The present study demonstrates that in contrast to fluoxetine, fluvoxamine has no significant effect on GIRK channels. GIRK2-deficient mice show an increase in motor activity and less anxiety (Blednov *et al.*, 2001). Taken together, inhibition of neuronal GIRK channels by fluoxetine may contribute to additive therapeutic effects for depression and other related psychiatric disorders.

On the other hand, the incidence of seizures during treatment with SSRIs including fluoxetine and fluvoxamine is a serious side effect in contrast with various benign side effects (Barbey & Roose, 1998; Neely, 1998; Baldessarini, 2001). The molecular mechanisms underlying seizures during treatment with SSRIs remains unclear. The serotonin syn-

drome, which is a toxic hyperserotonergic state, typically includes not only changes in mental state, myoclonus, tremor and various autonomic responses, but also seizures in severe cases (Sternbach, 1991; Baldessarini, 2001). Seizures associated with SSRIs may be related to a consequence of aberrant responses in serotonergic system. Moreover, overdoses of fluoxetine have been reported to be associated with seizures (Barbey & Roose, 1998; Neely, 1998). The plasma concentrations of fluoxetine in several patients who experienced seizures were reported to be approximately 3.3–7.1  $\mu\text{M}$  (Barbey & Roose, 1998; Neely, 1998). Therefore, fluoxetine at the corresponding brain levels may potentially inhibit neuronal GIRK channels. Bupivacaine, a local anesthetic, also inhibits GIRK channels but does not affect other Kir channels (Zhou *et al.*, 2001). Overdoses of bupivacaine are associated with seizures, and the blood levels are close to the  $\text{IC}_{50}$  values for GIRK channels. In addition, fluoxetine inhibits two types of neuronal voltage-gated  $\text{K}^{+}$  channels, Kv1.1 and Kv3.1 channels (Yeung *et al.*, 1999; Choi *et al.*, 2001). GIRK2-deficient mice show spontaneous seizures (Signorini *et al.*, 1997), and the inhibition of these  $\text{K}^{+}$  channels could lead to an increase in neuronal excitability. Therefore, potent inhibition of neuronal GIRK channels by fluoxetine together with the Kv channels may also contribute to the cause of seizures and some of other neuropsychiatric toxicity.

Antidepressant drugs including fluoxetine, fluvoxamine and tricyclic antidepressants show analgesic activity (Messing *et al.*, 1975; Lin *et al.*, 1980; Schreiber *et al.*, 1996; Korzeniewska-Rybicka & Plaznik, 2000; Galeotti *et al.*, 2001). Studies using *ww* mutant mice or GIRK2-deficient mice have shown that activation of GIRK channels by opioids or EtOH may be involved in analgesia induced by the drugs, suggesting that the GIRK channel is one of the key molecules in analgesia (Kobayashi *et al.*, 1999; Ikeda *et al.*, 2000, 2002; Mitrovic *et al.*, 2000). However, the present study demonstrates that fluoxetine inhibits GIRK channels, while fluvoxamine has no significant effect on GIRK channels. Therefore, the analgesic effect of fluoxetine and fluvoxamine may be caused by interaction with some of other several targets. Previous studies have shown that the actions of 5-HT may contribute to the analgesic effect (Messing *et al.*, 1975; Lin *et al.*, 1980; Tura & Tura, 1990). Various antidepressant drugs including fluoxetine and tricyclic antidepressants inhibit voltage-gated  $\text{Na}^{+}$  channels like local anesthetics (Pancrazio *et al.*, 1998). Therefore, the analgesic effect of the antidepressant drugs may share these common mechanisms. In addition, Galeotti *et al.* (2001) suggest that the analgesic effects of tricyclic antidepressants may involve the opening of voltage-gated, ATP-sensitive and  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels. Further studies are required to clarify whether the analgesic effects of SSRIs are also mediated by these channels.

In the heart, acetylcholine released from the stimulated vagus nerve opens atrial GIRK channels via activation of  $\text{M}_2$  muscarinic acetylcholine receptors, and ultimately causes slowing of the heart rate (Brown & Birnbaumer, 1990). The binding affinity of fluoxetine for the receptor exhibits low micromolar concentrations (Stanton *et al.*, 1993). In the present study, micromolar concentrations of fluoxetine inhibited cardiac-type GIRK1/4 channels. In clinical practice, fluoxetine overdoses are associated with the incidence of sinus tachycardia (Barbey & Roose, 1998; Neely, 1998). The clinically relevant plasma concentrations become significantly

higher than the micromolar concentrations in the therapeutic use, although the corresponding heart concentrations have not been determined in humans. Sinus tachycardia during fluoxetine treatment may be related to not only antagonism of  $M_2$  muscarinic acetylcholine receptors, but also inhibition of atrial GIRK channels. In addition, 5-HT can elicit tachycardia in humans, although it is known that 5-HT elicits complex changes in the cardiovascular system comprising bradycardia or tachycardia, hypotension or hypertension and vasodilatation or vasoconstriction (Saxena & Villalon, 1990). The 5-HT effect caused by inhibition of 5-HT uptake might also be related to the incidence of tachycardia during treatment with SSRIs. Sinus tachycardia is also observed in fluvoxamine overdose (Barbey & Roose, 1998). Although fluvoxamine has no significant effect on GIRK channels, it has less affinity for muscarinic acetylcholine receptors than fluoxetine (Baldessarini, 2001). Therefore, sinus tachycardia observed in fluvoxamine overdose might be related to the effects of 5-HT and antagonism of muscarinic acetylcholine receptors.

Interestingly, we also demonstrate that extracellular fluoxetine at low micromolar concentrations can inhibit the effect of brain-type GIRK channels induced by ethanol. Fluoxetine reduces EtOH consumption (Rockman *et al.*, 1982; Naranjo & Knoke, 2001). However, GIRK2-deficient mice display no significant change in EtOH consumption (Blednov *et al.*, 2001). So far, there is no evidence to help clarify the *in vivo*

effects of fluoxetine on GIRK channel activation by EtOH. Further studies using mice treated with antisense oligodeoxynucleotides to the GIRK mRNAs, mutant mice with GIRK channels insensitive to EtOH alone and a selective unidentified GIRK channel activator or inhibitor may be useful for the understanding of the antagonism of EtOH-induced GIRK effects by fluoxetine *in vivo*. In addition, mutagenesis studies of GIRK channels may clarify the molecular mechanism underlying the inhibitory action of fluoxetine on EtOH activation of GIRK channels.

In conclusion, we demonstrate that fluoxetine, at clinically relevant brain concentrations, acts as an inhibitor at GIRK channels, which play an important role in the inhibitory regulation of neuronal excitability in most brain regions. Moreover, fluoxetine can inhibit the effect of GIRK channels induced by EtOH. Our results suggest that inhibition of GIRK channels by fluoxetine may contribute to some of the therapeutic effects and the adverse side effects, particularly seizures in overdose, observed in clinical practice.

We thank Drs Toshiro Kumanishi and Kansaku Baba for their cooperation, and Tomio Ichikawa and Kazuo Kobayashi for their assistance. We also thank Dr Steven C. Hebert for providing the ROMK1 cDNA, and Dr Lily Y. Jan for providing the IRK1 cDNA. This work was supported by research grants from the Ministry of Education, Science, Sports and Culture of Japan and the RIKEN Brain Science Institute.

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(Received August 26, 2002

Revised November 4, 2002

Accepted January 3, 2003)