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Inhibition of G Protein-Activated Inwardly Rectifying K^+ Channels by Different Classes of Antidepressants

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Abstract

Various antidepressants are commonly used for the treatment of depression and several other neuropsychiatric disorders. In addition to their primary effects on serotonergic or noradrenergic neurotransmitter systems, antidepressants have been shown to interact with several receptors and ion channels. However, the molecular mechanisms that underlie the effects of antidepressants have not yet been sufficiently clarified. G protein-activated inwardly rectifying K^+ (GIRK, Kir3) channels play an important role in regulating neuronal excitability and heart rate, and GIRK channel modulation has been suggested to have therapeutic potential for several neuropsychiatric disorders and cardiac arrhythmias. In the present study, we investigated the effects of various classes of antidepressants on GIRK channels using the *Xenopus* oocyte expression assay. In oocytes injected with mRNA for GIRK1/GIRK2 or GIRK1/GIRK4 subunits, extracellular application of sertraline, duloxetine, and amoxapine effectively reduced GIRK currents, whereas nefazodone, venlafaxine, mianserin, and mirtazapine weakly inhibited GIRK currents even at toxic levels. The inhibitory effects were concentration-dependent, with various degrees of potency and effectiveness. Furthermore, the effects of sertraline were voltage-independent and time-independent during each voltage pulse, whereas the effects of duloxetine were voltage-dependent with weaker inhibition with negative membrane potentials and time-dependent with a gradual decrease in each voltage pulse. However, Kir2.1 channels were insensitive to all of the drugs. Moreover, the GIRK currents induced by ethanol were inhibited by sertraline but not by intracellularly applied sertraline. The present results suggest that GIRK channel inhibition may reveal a novel characteristic of the commonly used antidepressants, particularly sertraline, and contributes to some of the therapeutic effects and adverse effects.

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Introduction

Depression is one of the most common illnesses in the world [1,2]. After the efficacy of tricyclic antidepressants (TCAs), including imipramine, amitriptyline and amoxapine, was well established, various classes of antidepressants were introduced, including selective serotonin reuptake inhibitors (SSRIs; fluoxetine, paroxetine and sertraline), serotonin-norepinephrine reuptake inhibitors (SNRIs; venlafaxine and duloxetine), selective norepinephrine reuptake inhibitors (NRIs; reboxetine), noradrenergic and specific serotonergic antidepressants (NaSSAs; mirtazapine and mianserin), and 5-hydroxytryptamine type 2 (5-HT_2) receptor antagonists (nefazodone) [1–3]. Antidepressants are commonly used for the treatment of depression and several neuropsychiatric disorders, such as anxiety disorders, eating disorders, obsessive-compulsive disorders, and chronic pain disorders [1–3]. Their clinical efficacy is hypothesized to be linked mainly with facilitation of noradrenergic or serotonergic function in the brain [2]. In contrast, the interaction between antidepressants and muscarinic, α_1 adrenergic, and H_1 histamine receptors is involved in some of their adverse side effects, such as dry mouse, orthostatic hypotension, and sedation [2]. Antidepressants have also been shown to modulate the function of several other receptors and ion channels, including 5-HT_{2C} and 5-HT_3 receptors, nicotinic acetylcholine receptors, *N*-methyl-D-aspartate (NMDA) receptor channels, $P2X_2$ receptors, voltage-gated Ca^{2+}, Na^+, and K^+ channels, Ca^{2+}-activated K^+ channels, two-pore-domain K^+ channels, and volume regulated anion channels [4–23]. The modulation of these receptors and channels might also be relevant to the pharmacological effects of antidepressants. However, the molecular mechanisms that underlie the effects of various antidepressants have not yet been sufficiently clarified.

G protein-activated inwardly rectifying K^+ (GIRK) channels (also known as Kir3 channels) are members of a major subfamily of inwardly rectifying K^+ (Kir) channels that includes seven subfamilies [24]. Four GIRK channel subunits have been identified in mammals [25–27]. Neuronal GIRK channels are predominantly heterotetramers composed of GIRK1 and GIRK2 subunits in most brain regions or homotetramers composed of GIRK2 subunits in the substantia nigra [27–30], whereas atrial GIRK channels are heterotetramers composed of GIRK1 and GIRK4 subunits [26]. The channels are activated by various $G_{i/o}$-protein-coupled receptors, such as M_2 muscarinic, α_2 adrenergic, D_2 dopaminergic, opioid, nociceptin/orphanin FQ, CB_1 cannabinoid, and A_1 adenosine receptors, through the direct action of G-protein $\beta\gamma$ subunits [31–33]. Additionally, ethanol activates GIRK

channels independently of G-protein-coupled signaling pathways [34,35]. GIRK channels play an important role in regulating neuronal excitability, synaptic transmission, and heart rate [31,36–39]. Furthermore, recent studies have suggested that GIRK channel modulation has the potential for treating several neuropsychiatric disorders and cardiac arrhythmias [33,40,41]. Therefore, GIRK channel modulators may affect various brain and cardiac functions. We have demonstrated the distinctive effects of several antidepressants on GIRK channels, even among the same class, particularly SSRIs [42,43]. To further clarify the interaction between various classes of commonly used antidepressants and GIRK channels may be useful for advancing our understanding of the pharmacological effects of antidepressants. In the present study, we examined the effects of various antidepressants on GIRK channels using the *Xenopus* oocyte expression assay.

Materials and Methods

Preparation of specific mRNAs

Plasmids that contain the entire coding sequences for the mouse GIRK1, GIRK2, and GIRK4 channel subunits were obtained previously [34,44,45]. cDNAs for mouse Kir2.1 in pcDNA1 [46] were generously provided by Dr. Lily Y. Jan (University of California, San Francisco). These plasmids were linearized by digestion with the appropriate enzymes as described previously [45,46]. The specific mRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE™ *In Vitro* Transcription Kit (Ambion, Austin, TX, USA).

Electrophysiological analysis

Adult female *Xenopus laevis* frogs (Copacetic, Soma, Aomori, Japan) were anesthetized by immersion in water that contained 0.15% tricaine (Sigma-Aldrich, St. Louis, MO, USA). A small incision was made on the abdomen to remove several ovarian lobes from the frogs, which were humanely killed after the final collection. All procedures for the care and treatment of animals were performed in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Niigata University (Permit Number: 172-2). *Xenopus* oocytes (Stages V and VI) were manually isolated from the ovary and maintained in Barth's solution [47]. Oocytes were injected with mRNA for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (0.15 ng each) or Kir2.1 (0.3 ng). The oocytes were incubated at 19°C in Barth's solution and manually defolliculated after treatment with 0.8 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 1 h. The whole-cell currents of the oocytes were recorded from 3 to 9 days after injection with a conventional two-electrode voltage clamp [34,48]. 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 continuously superfused with a high-potassium (hK) solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 with KOH) or a K^+-free high-sodium (ND98) solution (98 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 with NaOH) at a flow rate of 2.5 ml/min. In the hK solution, the K^+ equilibrium potential was close to 0 mV, and the inward K^+ current flow through the Kir channels was observed at negative holding potentials as previously shown [25,27,43]. Additionally, to examine the effects of antidepressants on outward K^+ currents, a perfusion solution that contained 4 mM K^+ (K4 solution) was made by substituting NaCl with KCl in the ND98 solution. To examine the effects of an antidepressant on GIRK channels activated by G-protein activation, 13.8 nl of 100 mM Li_4-guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS; Sigma-Aldrich), a nonhydrolyzable G-protein activator, dissolved in distilled water was injected into an oocyte using a nanoliter injector (World Precision Instruments, Sarasota, FL, USA) as described previously [49]. Furthermore, to examine the effects of intracellular sertraline, 23 nl of 10 mM sertraline dissolved in distilled water was injected into an oocyte using a Nanoliter injector as described previously [50], and the oocyte currents were then continuously recorded for approximately 30–40 min. Because the volume of the *Xenopus* oocytes used was approximately 1 μl, the intracellular concentration of sertraline was presumed to be approximately 225 μM. For the analysis of concentration-response relationships, the data were fitted to a standard logistic equation [51] using KaleidaGraph (Synergy Software, Reading, PA, USA). The concentration of a drug that produces 50% of the maximal current response for that drug (EC_{50}), the concentrations required to reduce control currents by 25% and 50% (IC_{25} and IC_{50}, respectively), and the Hill coefficient (n_H) were obtained from the concentration-response relationships.

Data analyses

The data are expressed as mean ± SEM, and n is the number of oocytes tested. The statistical analysis of differences between groups was performed using paired t-test, one-way analysis of variance (ANOVA), or two-way ANOVA followed by the Tukey-Kramer *post hoc* test. Values of $P<0.05$ were considered statistically significant.

Compounds

All of the antidepressants tested were commercially purchased. Amoxapine and nefazodone hydrochloride were obtained from Sigma-Aldrich. Mirtazapine and mianserin hydrochloride were obtained from Tocris Bioscience (Bristol, UK). Sertraline hydrochloride and duloxetine hydrochloride were obtained from Tronto Research Chemicals (North York, Canada). Venlafaxine hydrochloride was obtained from LKT Laboratories (St. Paul, MN, USA). Sertraline was dissolved in dimethyl sulfoxide (DMSO) or distilled water, and venlafaxine was dissolved in distilled water. The other antidepressants were dissolved in DMSO. The stock solution of each compound was stored at −30°C until use. Ethanol was purchased from Wako Pure Chemical Industries. Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

Results

Inhibition of GIRK channels by antidepressants

In *Xenopus* oocytes injected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which depend on free G-protein βγ subunits present in the oocytes because of the inherent activity of G-proteins [32], were observed at a holding potential of −70 mV in an hK solution that contained 96 mM K^+ (Fig. 1A). The 3 mM Ba^{2+}-sensitive current components (1042.8±90.1 nA, $n=30$) correspond to the magnitude of GIRK currents in oocytes that express GIRK channels [34]. Extracellular application of 30 μM sertraline, an SSRI, reversibly reduced the inward currents through the expressed GIRK channels (Fig. 1A). The current responses to an additional 100 μM sertraline during the application of 3 mM Ba^{2+}, which blocks Kir channels, were not significant (reduction of inward currents by 4.5±3.5 nA; less than 1% inhibition of the Ba^{2+}-sensitive current components, $n=4$). Sertraline at 100 μM produced no significant response in a K^+-free ND98 perfusion solution that contained 98 mM Na^+ instead of the hK solution (3.0±1.8 nA, $n=4$), suggesting that the SSRI-sensitive current components show K^+ selectivity. Additionally, the application of DMSO or distilled water, the solvent vehicles, at the

highest concentration (0.3%) induced no significant current response in the hK or ND98 solutions ($n=5$; data not shown). In contrast, in oocytes injected with mRNA for Kir2.1, a constitutively active Kir channel [46], extracellular application of 300 μM sertraline had no significant effect on the inward currents through the channels in the hK solution (less than 2% change of the Ba^{2+}-sensitive current components; 848.3±322.0 nA, $n=4$; Fig. 1B). In uninjected oocytes, 300 μM sertraline and 3 mM Ba^{2+} caused no significant response (2.0±2.0 nA, $n=4$, and 3.1±1.7 nA, $n=4$, respectively; Fig. 1C) compared with oocytes injected with GIRK mRNA, suggesting no significant effect of sertraline or Ba^{2+} on intrinsic oocyte channels. Furthermore, in oocytes injected with GIRK1 and GIRK4 mRNAs, 30 μM sertraline similarly inhibited basal GIRK currents under the same conditions (51.6±4.3% inhibition of 3 mM Ba^{2+}-sensitive current components, 561.7±58.2 nA, $n=11$). Additionally, the Ba^{2+}-sensitive current components in oocytes injected with mRNA for GIRK1/GIRK2 or GIRK1/GIRK4 combinations were very significantly larger than those in oocytes injected with the same small amount of a single GIRK mRNA (less than 20 nA, $n=7$, respectively). The results indicate that sertraline predominantly inhibited GIRK1/2 and GIRK1/4 heteromultimeric channels, but not Kir2.1 channels. Moreover, the effects of different classes of antidepressants on GIRK channels were examined using the same expression assay. Amoxapine, a second generation TCA, and duloxetine, an SNRI, significantly inhibited basal GIRK currents at 100 μM (45.6±4.4 and 65.6±1.0% inhibition for GIRK1/2, $n=5$ and 7, respectively; 27.6±4.0 and 49.7±2.2% inhibition for GIRK1/4, $n=4$ and 6, respectively). However, the 5-HT_2 receptor antagonist nefazodone, NaSSAs mianserin and mirtazapine, and SNRI venlafaxine weakly inhibited the currents at 100 μM (35.9±3.5, 24.1±5.5, 17.6±3.5, and 19.4±4.6% inhibition for GIRK1/2, $n=11$, 4, 4, and 4, respectively; 30.3±3.4, 18.8±1.3, 12.1±2.4, and 22.8±3.2% inhibition for GIRK1/4, $n=13$, 5, 5, and 5, respectively). Additionally, the inhibitions were reversible with washout, similar to sertraline (data not shown). In contrast, Kir2.1 channels were insensitive to these drugs at 100 μM (less than 4% change of the Ba^{2+}-sensitive current components; 912.5±182.8 nA, $n=4$). In uninjected oocytes, 300 μM of the drugs caused no significant response (less than 6 nA; $n=4$ for each of the drugs). Altogether, the results suggest significant inhibition of GIRK channels by sertraline, duloxetine, and amoxapine, weak inhibition of the channels by nefazodone, mianserin, mirtazapine, and venlafaxine, and no significant effects of the drugs on Kir2.1 channels.

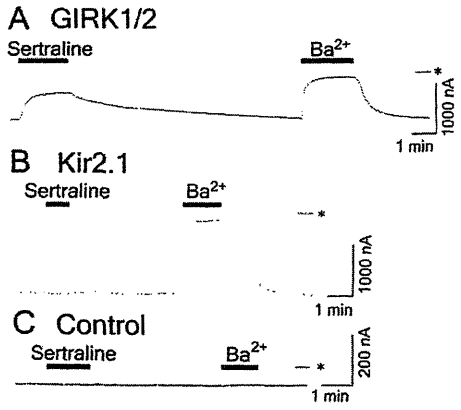

Figure 1. Inhibitory effects of sertraline on GIRK channels expressed in Xenopus oocytes. (A) In an oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 30 μM sertraline and 3 mM Ba^{2+} are shown. (B) In an oocyte injected with Kir2.1 mRNA, current responses to 100 μM sertraline and 3 mM Ba^{2+} are shown. (C) In an uninjected oocyte, no significant current responses to 300 μM sertraline or 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of −70 mV in an hK solution that contained 96 mM K^+. Asterisks show the zero current level. Horizontal bars indicate the duration of application.
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Concentration-dependent inhibition of GIRK channels by various antidepressants

The concentration-response relationships for the inhibitory effects of different classes of antidepressants on GIRK1/2 and GIRK1/4 channels were investigated. Figure 2 shows that the inhibitions of both types of GIRK channels by various antidepressants were concentration-dependent with distinctive potency and effectiveness at micromolar concentrations. The rank order of the inhibition of GIRK channels by 100 μM of these drugs was the following: duloxetine≥sertraline>amoxapine>nefazodone>mianserin≥venlafaxine≈mirtazapine for GIRK1/2 channels and sertraline>duloxetine≫nefazodone, amoxapine>venlafaxine, mianserin>mirtazapine for GIRK1/4 channels. Table 1 shows the EC_{50} and n_H values obtained from the concentration-response relationships for sertraline, duloxetine and amoxapine, and the percentage inhibition of the GIRK currents by the drugs at the highest concentrations tested. Additionally, because the drugs could not completely block these types of GIRK channels even at the highest concentrations tested, the IC_{25} and IC_{50} values were also calculated to further compare the effects of the drugs (Table 1). The inhibition of GIRK1/2 channels by sertraline was similar to that by duloxetine (Fig. 2). Furthermore, the inhibition of GIRK1/2 channels by sertraline was statistically similar to the inhibition of GIRK1/4 channels ($P>0.05$ at each concentration, Tukey-Kramer *post hoc* test; Fig. 2, Table 1). In contrast, the inhibition of GIRK1/2 channels by duloxetine and amoxapine was more effective than the inhibition of GIRK1/4 channels ($P<0.05$ at 30, 100, and 300 μM for duloxetine and $P<0.05$ at 300, 500, and 1000 μM for amoxapine, Tukey-Kramer *post hoc* test; Fig. 2, Table 1).

Characteristics of inhibition of GIRK channels by the SSRI sertraline and SNRI duloxetine

Sertraline and duloxetine, which belong to commonly used classes of antidepressants, effectively inhibited GIRK channels, and we further investigated the effects of these drugs in more detail. Instantaneous GIRK1/2 currents elicited by the voltage step to −100 mV from a holding potential of 0 mV were diminished in the presence of 30 μM sertraline applied for 3 min (Fig. 3A). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by sertraline was not significantly different from that of the instantaneous current ($P>0.05$, paired *t*-test; $n=9$ at −40, −60, −80, −100, and −120 mV, respectively). For duloxetine, the instantaneous currents were primarily diminished in the presence of 30 μM duloxetine, and the currents gradually increased in the voltage step (Fig. 3A). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by duloxetine significantly decreased compared with that of the instantaneous current ($P<0.05$ at −80, −100 and −120 mV, paired *t*-test, $n=6$). Figure 3B shows that 30 μM sertraline- and duloxetine-sensitive currents in oocytes that expressed GIRK1/2 channels increased with negative membrane potentials, and the current-voltage relationships showed strong inward rectification ($n=9$ and 6,

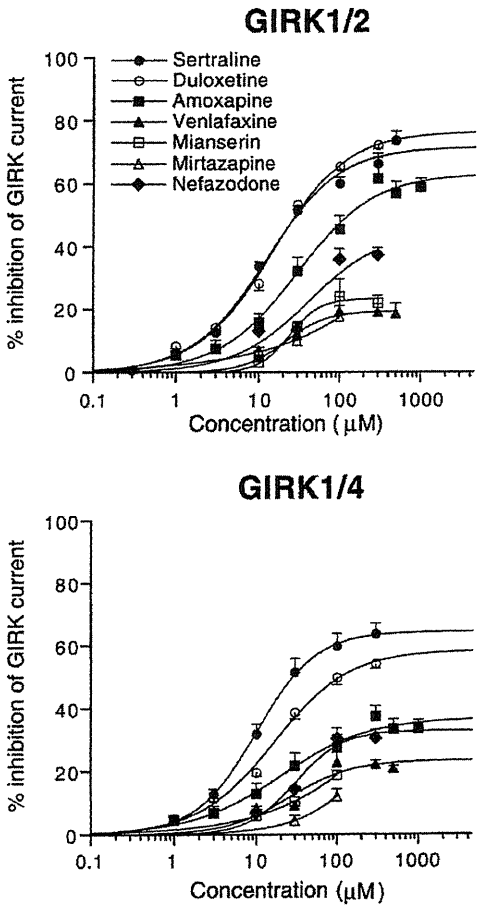

Figure 2. Concentration-response relationships for the effects of various antidepressants on GIRK1/2 and GIRK1/4 channels. The magnitudes of inhibition of GIRK currents by the drugs were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes that expressed GIRK1/2 channels or GIRK1/4 channels (762.8±36.0 nA, $n=50$, and 585.0±44.0 nA, $n=40$, respectively). Each point and error bar represent the mean ± SEM of the percentage responses.
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respectively), similar to 3 mM Ba^{2+}-sensitive currents that corresponded to basal GIRK currents, indicating a characteristic of GIRK currents. The percentage inhibition of GIRK1/2 currents by 30 μM sertraline at the end of the voltage pulses showed no significant difference across voltages between −120 and −40 mV (no significant sertraline effect×membrane potential effect interaction, $P>0.1$, one-way ANOVA; $P>0.1$ across voltages, Tukey-Kramer *post hoc* test; Fig. 3C), suggesting voltage-independent inhibition of GIRK channels by sertraline. In contrast, the GIRK current inhibition by duloxetine at the end of the voltage pulses was voltage-dependent, with weaker inhibition at more negative membrane potentials (significant duloxetine effect×membrane potential effect interaction, $P<0.05$, one-way ANOVA; significant differences between −120 and −60 mV, between −120 and −40 mV, between −100 and −60 mV, and between −100 and −40 mV, $P<0.05$, Tukey-Kramer *post hoc* test, $n=6$, Fig. 3C). The voltage-dependency was associated with a time-dependent decrease in the inhibition by duloxetine in the voltage pulses at more negative membrane potentials. Furthermore, similar results were obtained in oocytes that expressed GIRK1/4 channels ($n=4$ for each of the drugs; data not shown). Altogether, sertraline and duloxetine primarily inhibited GIRK channels at the holding potential of 0 mV before the voltage pulses. The inhibitory effects of sertraline were voltage-independent and time-independent during each voltage pulse, whereas those of duloxetine decreased voltage-dependently with negative membrane potentials and time-dependently up to a steady state current level in each voltage pulse.

Furthermore, the effects of the two antidepressants on GIRK channels under a physiological K^+ condition were examined. In oocytes injected with GIRK1 and GIRK2 mRNAs, outward currents observed at a holding potential of −10 mV in a K4 solution that contained 4 mM K^+ were reversibly reduced by 30 μM sertraline ($n=4$), 30 μM duloxetine ($n=4$), and 3 mM Ba^{2+} (the Ba^{2+}-sensitive current components, 49.0±2.8 nA, $n=8$; Fig. S1), whereas in uninjected oocytes, the drugs at 100 μM and 3 mM Ba^{2+} caused no significant response (3.0±0.9 nA for sertraline, 0±0 nA for duloxetine, and 7.6±1.3 nA for Ba^{2+}; $n=4$, 4, and 8, respectively). The results suggest that the antidepressants also inhibited outward GIRK currents at a physiologically extracellular K^+ concentration.

Sertraline and duloxetine possess a secondary amine group with pK_a values of 8.9 and 9.34, respectively (Data Sheets of Pfizer and

Table 1. Inhibitory effects of sertraline, duloxetine and amoxapine on GIRK channels.

	Sertraline		Duloxetine		Amoxapine	
	GIRK1/2	GIRK1/4	GIRK1/2	GIRK1/4	GIRK1/2	GIRK1/4
EC_{50} (μM)	11.7±1.0	12.6±2.5	14.9±0.4	17.0±1.3	38.7±6.2	17.7±4.4
IC_{25} (μM)	6.9±0.6	7.0±1.0	6.6±0.6	12.6±1.2	21.5±8.3	39.7±15.8
IC_{50} (μM)	29.1±3.4	36.7±7.8	28.3±2.5	124.2±34.3	181.1±48.3	ND
% max	73.7±2.9	63.7±3.5	72.2±1.1	54.1±1.5	58.9±2.9	36.0±1.6
(μM; *n*)	(500; 16)	(300; 11)	(300; 7)	(300; 6)	(1000; 5)	(1000; 4)
n_H	1.02±0.05	0.89±0.09	0.94±0.06	0.97±0.07	0.87±0.03	0.87±0.07

Mean ± SEM concentrations of antidepressants (μM) that produce 50% of the maximal effect (EC_{50}) and are required to reduce basal GIRK currents by 25% and 50% (IC_{25} and IC_{50}, respectively) are shown. The % max values indicate the mean ± SEM percentage inhibition of basal GIRK currents by a drug at the highest concentrations tested. The highest concentrations tested (μM) and the number of oocytes tested (*n*) are shown in parentheses. The n_H values indicate the mean ± SEM of Hill coefficients. ND indicates that the value was not determined because of a low effectiveness of the drug.
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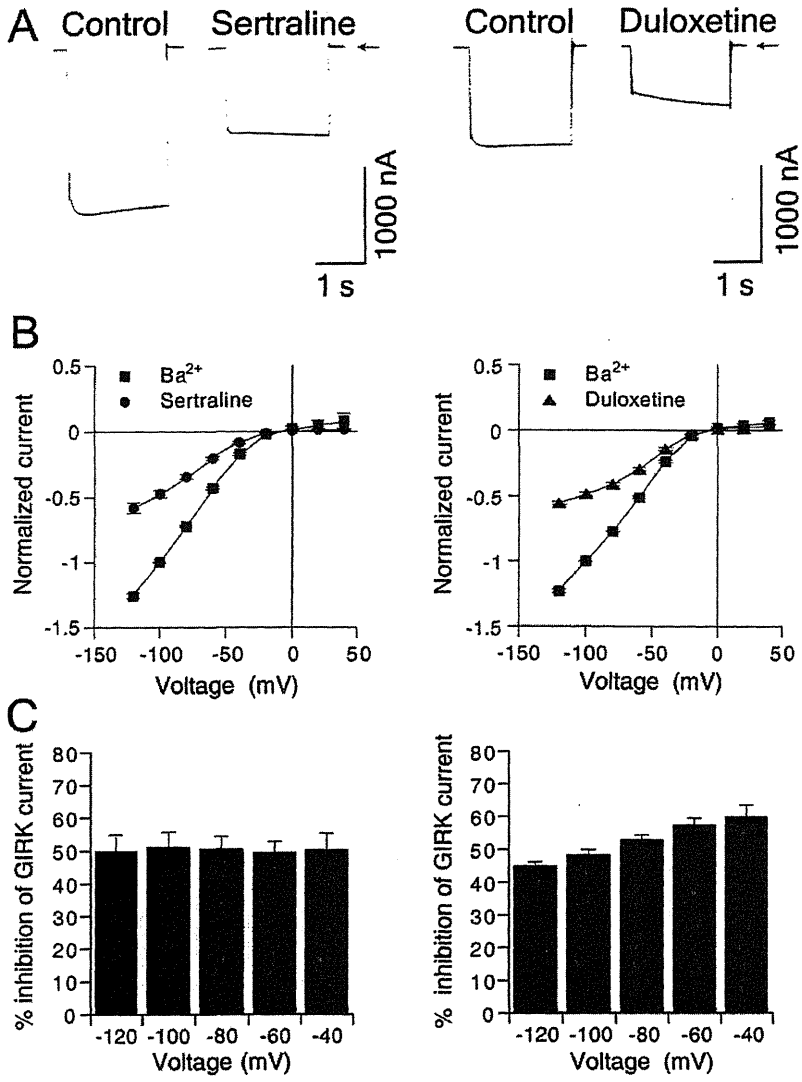

Figure 3. Characteristics of the inhibitory effects of sertraline and duloxetine on GIRK currents. (A) Representative GIRK1/2 currents elicited by a voltage step to −100 mV for 2 s from a holding potential of 0 mV in the presence or absence of 30 μM sertraline (left) or 30 μM duloxetine (right) applied for 3 min. Current responses were recorded in an hK solution that contained 96 mM K^+. Arrows indicate the zero current level. (B) Current-voltage relationships of the magnitudes of 3 mM Ba^{2+}-sensitive currents and the magnitudes of currents reduced by 30 μM sertraline (left, $n=9$) or 30 μM duloxetine (right, $n=6$) in oocytes that expressed GIRK1/2 channels. Current responses were normalized to the 3 mM Ba^{2+}-sensitive current component measured at a membrane potential of −100 mV (1851.0±220.4 nA, $n=15$). (C) Percentage inhibition of GIRK1/2 channels by 30 μM sertraline or 30 μM duloxetine over the voltage range of −120 to −40 mV. The magnitudes of inhibition of GIRK currents by 30 μM sertraline (left, $n=9$) and duloxetine (right, $n=6$) at the end of the voltage pulses were compared with the 3 mM Ba^{2+}-sensitive current components. All values are expressed as mean ± SEM.
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Eli Lilly and Company). At physiological pH or below, sertraline and duloxetine exist mainly in a protonated form, approximately 96.9% and 98.9% at pH 7.4, respectively, and the proportion of the uncharged form increases with an increase in pH. We examined whether changes in extracellular pH would affect GIRK channel inhibition by sertraline or duloxetine. However, in oocytes that expressed GIRK1/2 channels, the percentage inhibition of GIRK channels by sertraline or duloxetine at the same concentrations was not significantly affected by extracellular pH 7.4 and 9.0 (no significant pH×drug interaction, $P>0.05$, two-way ANOVA; $P>0.05$ at each concentration, Tukey-Kramer *post hoc* test; Fig. 4). The results indicate that a marked increase in the proportion of the uncharged form of sertraline and duloxetine may not significantly affect all of the inhibitory effects on GIRK channels, suggesting that GIRK channel inhibition may be mediated by both forms of the drugs with similar effectiveness. Additionally, the inhibition by the antidepressants was unlikely mediated by nonspecific membrane perturbation induced by the uncharged form.

Effects of sertraline on GIRK channels activated by GTPγS, a nonhydrolyzable GTP

GIRK channels are activated by various $G_{i/o}$-protein-coupled receptors through the direct action of G-protein βγ subunits

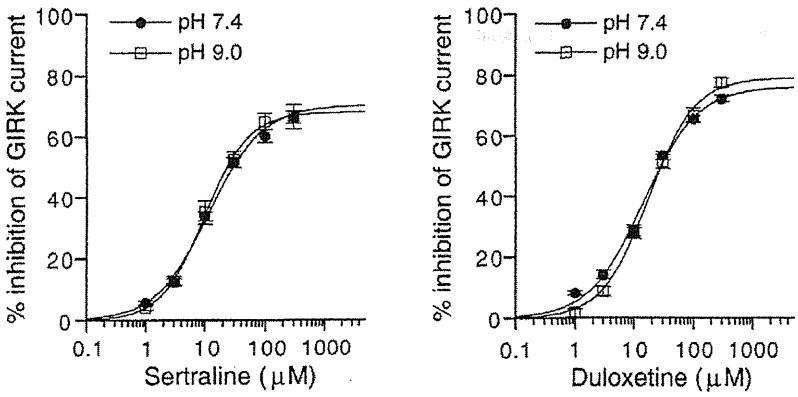

Figure 4. Concentration-dependent inhibition of GIRK channels by sertraline or duloxetine at different pH values. The magnitudes of inhibition of GIRK currents by the antidepressants were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes that expressed GIRK1/2 channels (1020.8±96.2 nA at pH 7.4, $n=16$ for sertraline and $n=7$ for duloxetine; 1079.5±173.8 nA at pH 9.0, $n=7$ for sertraline and $n=6$ for duloxetine, respectively). Current responses were measured at a membrane potential of −70 mV in an hK solution that contained 96 mM K^+. Each point and error bar represent the mean ± SEM of the percentage responses.
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released from the heterotrimeric G-protein complex [32,33]. The effects of sertraline on GIRK channels activated by G-protein-coupled signaling mechanisms were further examined using GTPγS, a nonhydrolyzable GTP analog that maintains G-proteins in an activated state. Injection of GTPγS into *Xenopus* oocytes injected with GIRK1 and GIRK2 mRNAs increased inward currents with time and reached a steady-state level (516.0±123.7 nA, $n=5$) as reported previously [49,51]. The increased inward currents were completely blocked by 3 mM Ba^{2+}, whereas GTPγS injection into uninjected oocytes had no significant effect on current responses to 3 mM Ba^{2+} (3.9±2.1 nA, $n=5$). Increased GIRK currents composed of basal GIRK currents and GTPγS-induced GIRK currents were inhibited by sertraline ($IC_{25}=5.5\pm0.7$ μM; $IC_{50}=18.1\pm3.0$ μM; $n_H=1.24\pm0.09$; $n=5$; Fig. 5). The concentration response curve for the inhibition of total GIRK currents by sertraline was partially different from that for the inhibition of basal GIRK currents in GTPγS-untreated oocytes injected with GIRK1 and GIRK2 mRNAs ($P<0.05$ at 30 μM, Tukey-Kramer *post hoc* test, Fig. 5). The results suggest that the potency of the inhibition of GIRK channels activated by GTPγS-induced G-protein activation may be slightly higher than that of basally active GIRK channels, although the maximal efficacy was similar.

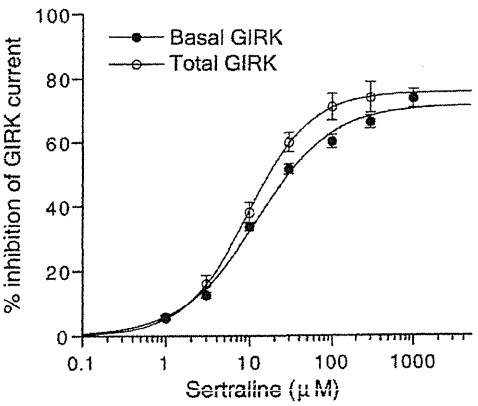

Figure 5. Effects of sertraline on total GIRK currents composed of GTPγS-induced and basal GIRK currents. For comparison, the effects on GTPγS-untreated basal GIRK currents shown in Figure 2 are also shown. The magnitudes of inhibition of GIRK currents by sertraline were compared with the 3 mM Ba^{2+}-sensitive current components. Each point and error bar represent the mean ± SEM of the percentage responses ($n=5$ for GTPγS-injected oocytes and $n=16$ for GTPγS-untreated oocytes). Current responses were measured at a membrane potential of −70 mV in an hK solution that contained 96 mM K^+.
doi:10.1371/journal.pone.0028208.g005

Sertraline inhibits ethanol-induced GIRK currents. GIRK channels are also activated by ethanol independent of G-protein signaling pathways [34]. Sertraline was shown to reduce ethanol consumption in mice [52] and was effective in alcoholics [53]. Therefore, we also examined the effects of sertraline on GIRK channel activation induced by ethanol. The effects of sertraline were evaluated by measuring the amplitude of the ethanol-induced current response during the extracellular application of sertraline at different concentrations. In oocytes injected with GIRK1 and GIRK2 mRNAs, the GIRK currents induced by 100 mM ethanol (344.2±40.3 nA, $n=6$) were reversibly attenuated in the presence of sertraline ($IC_{25}=6.2\pm1.4$ μM; $IC_{50}=29.6\pm5.5$ μM; $n_H=0.87\pm0.17$; $n=6$; Fig. 6A, 6B). However, the 100 mM ethanol-induced GIRK currents were not significantly affected by intracellularly applied sertraline (104.9±9.1% of untreated control current, paired *t*-test, $P>0.1$, $n=6$; Fig. 6C). Moreover, in oocytes that expressed GIRK channels, the basal currents were not substantially affected by intracellularly applied sertraline (92.5±1.6% of untreated control current, $n=6$). The results indicate that intracellular sertraline could not inhibit GIRK channels. In contrast, GIRK channel inhibition induced by extracellularly applied sertraline, which is mainly protonated at pH 7.4, was reversible with washout (Figs. 1A and 6A). Because the protonated form may not readily permeate the cell membrane, extracellularly applied sertraline may exist mainly on the extracellular side. Altogether, extracellular sertraline may inhibit GIRK channels activated by ethanol. Additionally, the extent of inhibition by sertraline of GIRK1/2 channels activated by ethanol was higher at 100 and 300 μM than that of basally active GIRK1/2 channels by G-proteins ($P<0.05$, Tukey-Kramer *post hoc* test), indicating a significant difference in the maximal efficacy of sertraline between ethanol activation of GIRK channels and G-protein activation of the channels.

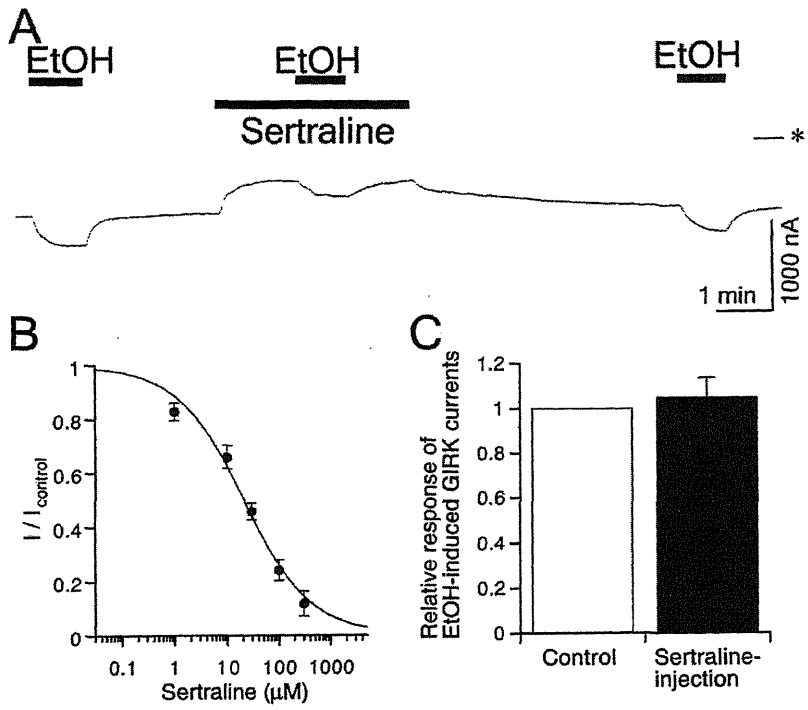

Figure 6. Effect of sertraline on ethanol-induced GIRK currents. (A) Current responses to 100 mM ethanol (EtOH), 100 mM EtOH in the presence of 30 μM sertraline, and 100 mM EtOH in an oocyte injected with GIRK1 and GIRK2 mRNAs. Asterisk indicates the zero current level. Bars show the duration of application. (B) Concentration-dependent inhibition of EtOH-induced GIRK currents by sertraline. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM EtOH (344.2±40.3 nA, $n=6$), and I is the current amplitude in the presence of sertraline. (C) Lack of effect of intracellular sertraline on 100 mM EtOH-induced GIRK currents. The amplitude of EtOH-induced GIRK currents after sertraline injection (black bar) was compared with EtOH-induced GIRK currents before the injection (control, white bar) in the same oocyte that expressed GIRK channels ($n=5$). Current responses were measured at a membrane potential of −70 mV in an hK solution that contained 96 mM K^+. All values are expressed as mean ± SEM.
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Discussion

The present study demonstrated that the SSRI sertraline, SNRI duloxetine, and second-generation TCA amoxapine effectively inhibited brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. However, the 5-HT_2 receptor antagonist nefazodone, SNRI venlafaxine, and NaSSAs mianserin and mirtazapine weakly inhibited both types of GIRK channels even at high concentrations. The inhibitions by different classes of antidepressants were concentration-dependent with various degrees of potency and effectiveness. In contrast, Kir2.1 channels in other Kir channel subfamilies were insensitive to all of the drugs. Furthermore, the present results suggest that sertraline and duloxetine primarily inhibited GIRK channels at the holding potential of 0 mV before the voltage pulses. The effects of sertraline on GIRK channels were voltage-independent and time-independent during each voltage pulse, similar to the effects of various TCAs [42]. The effects of duloxetine decreased voltage-dependently with negative membrane potentials and time-dependently up to a steady current level in each voltage pulse, and the voltage-dependency was associated with a time-dependent decrease in the inhibition by duloxetine at more negative membrane potentials. The present results also suggest that the site of action on the channels may be extracellular. In contrast, blockade of GIRK channels by extracellular Ba^{2+} and Cs^+, which occlude the pore of the open channel, increases concentration-dependently, voltage-dependently with negative membrane potentials, and time-dependently with a comparatively small effect on the instantaneous current but marked inhibition on the steady-state current at the end of the voltage pulses [27]. These observations suggest that sertraline and duloxetine may cause an allosteric conformational change in GIRK channels, rather than simple occlusion of the open channel. Additionally, sertraline may stably bind to the channels during the voltage pulses, whereas duloxetine may partially dissociate from the channels in the voltage pulses. The n_H values obtained from the concentration-response relationships for sertraline and duloxetine were almost 1 (Table 1), suggesting an one-to-one interaction between the drug and the binding site. Interestingly, GIRK channels were significantly inhibited by the SSRI sertraline and SNRI duloxetine, despite a great difference in the pharmacological profiles for monoamine transporters. The chemical structure of sertraline is distinct from that of duloxetine [2,54]. These antidepressants may act at different binding sites on the channels, and agents with similar structures may interact with GIRK channels. However, the SNRIs venlafaxine and milnacipran [43] had weak or little effects on GIRK channels, respectively. The distinctive effects of the SNRIs on GIRK channels may be attribute to their diverse chemical structures [54]. The *Xenopus* oocyte expression system is useful to determine drug actions on membrane proteins, such as voltage-gated Na^+ and Ca^{2+} channels, glutamate receptor channels, $5HT_{1C}$ receptor [55]. Since neuronal and cardiac GIRK channels are considered to consist predominantly of GIRK1/2 channels and GIRK1/4 channels, respectively [26,29,36], the effects of antidepressants on GIRK1/2 and

GIRK1/4 channels expressed in *Xenopus* oocytes were investigated in the present study. However, GIRK subunits have been suggested to form functional GIRK channels composed of several types of tetrameric stoichimetries in various cell populations, particularly neurons [56]. GIRK1 subunits are posttranslationally modified by glycosylation [26,56,57]. Furthermore, GIRK channels are regulated by not only G proteins but also phosphatidylinositol 4,5-bisphosphate in the cell membrane, polyamines and protein kinases [33]. The effects of antidepressants on GIRK channels might be influenced by differences in composition of the channel subunits, levels of glycosylation of GIRK1 subunits, and interaction with membrane and intracellular factors between the *Xenopus* oocyte expression system and neurons. Further studies using neurons and cardiac myocytes may be useful for advancing our understanding of the effects of antidepressants on GIRK channels.

The therapeutic serum concentrations range from approximately 0.16 to 0.82 μM for sertraline, 0.07 to 0.27 μM for duloxetine, 0.57 to 1.9 μM for amoxapine, 0.02 to 0.64 μM for nefazodone, 0.06 to 0.26 μM for mianserin, 0.08 to 0.37 μM for mirtazapine, and 0.72 to 1.44 μM for venlafaxine [2,58–60]. Additionally, increases in antidepressant doses are associated with increases in blood concentrations [59]. The concentrations in cases of overdose were reported to reach up to 13.7 μM for sertraline [61], 8.4 μM for duloxetine [62], 57.4 μM for amoxapine [63], 11.7 μM for nefazodone, 18.9 μM for mianserin [59], 8.7 μM for mirtazapine [64], and 302.8 μM for venlafaxine [65]. Most of the doses of antidepressants are distributed in various tissues from the blood, and antidepressants generally accumulate in the brain [2,58,66]. Indeed, brain levels of antidepressants were 40-fold higher for sertraline [67], 15-fold higher for duloxetine [68], 8.7- to 35.5-fold higher for amoxapine [69], 1.3- to 1.8-fold higher for nefazodone [70], 12.1-fold higher for mianserin [71], 3.2-fold higher for mirtazapine, and 4.9-fold higher for venlafaxine [66] compared with blood levels. Altogether, due to the high brain-to-blood partition ratios, presumed brain concentrations during treatment with therapeutic doses would range from approximately 6.4 to 32.8 μM for sertraline and 5.0 to 67.5 μM for amoxapine, and those after overdose would reach up to 548 μM for sertraline, 126 μM for duloxetine, 499 or 2038 μM for amoxapine, 229 μM for mianserin, and 1484 μM for venlafaxine. In addition, it has been shown that the therapeutic concentrations of some SSRIs in the brain were much higher than binding affinities of the antidepressants to monoamine transportors [72–75]. Brain concentrations at therapeutic doses of sertraline and amoxapine and after overdose of sertraline, duloxetine, amoxapine, mianserin and venlafaxine overlap with their effective concentrations in inhibiting predominant brain-type GIRK1/2 channels (Fig. 2). Therefore, the present results suggest that some inhibition of GIRK channels in the brain might occur with the antidepressant medication, particularly sertraline. However, mirtazapine and nefazodone may have small or little effects on GIRK channels even at toxic levels. Inhibition of GIRK channels causes a depolarization of membrane potential, resulting in an increase in cell excitability [38]. GIRK channels play an important role in regulating neuronal excitability and synaptic transmission [36,41]. Therefore, even partial inhibition of GIRK channels by the antidepressants may affect various brain functions.

Interestingly, GIRK2 knockout mice exhibit reduced anxiety-related behavior [76]. Animal studies have shown that sertraline has anxiolytic properties [77,78]. Indeed sertraline is clinically effective in the treatment of panic disorder and posttraumatic stress disorder [79]. Although the therapeutic effects are generally thought to be primarily attributable to inhibition of serotonin reuptake in the brain [2], some inhibition of GIRK channels might also contribute to improvement of anxiety symptoms.

Although the risk of seizures with antidepressants is generally very low, the association with overdose is well established [80]. However, the molecular mechanisms by which antidepressants cause seizures have not been clarified. GIRK2 knockout mice exhibit spontaneous seizures and are more susceptible to seizures induced by pentylenetetrazol than wild-type mice [37]. The risk of seizures in overdoses with sertraline, duloxetine, mianserin, and venlafaxine significantly increases [80–82], and amoxapine overdose is more likely to cause seizures [83]. Brain levels of the drugs in overdose cases may be considerably higher than levels during treatment at therapeutic doses, suggesting significant inhibition of neuronal GIRK channels by the drugs. Additionally, other types of K^+ channels are inhibited by antidepressants at micromolar concentrations, that is, the two-pore-domain K^+ channel, TREK-1 for sertraline and voltage-gated K^+ channels for amoxapine and mianserin [16,17,21]. Therefore, the inhibition of GIRK channels by the drugs after overdose together with the different types of K^+ channels may contribute to increased seizure activity and the occurrence of other neurological side effects by increasing neuronal excitability.

In the heart, GIRK channels cause a slowing of heart rate in response to activation of M_2 muscarinic receptors through acetylcholine release from the stimulated vagus nerve [25,26]. GIRK1 and GIRK4 knockout mice exhibit slightly elevated resting heart rates [39]. The present results indicate that sertraline, duloxetine, amoxapine, and venlafaxine can partially inhibit cardiac-type GIRK1/4 channels at blood levels after overdose, although the corresponding heart concentrations were not determined. These antidepressants are associated with sinus tachycardia in cases of toxicity after overdose [81,82,84,85]. In addition, the drugs exhibit low micromolar binding affinities for the muscarinic receptor, with the exception of venlafaxine [2,86], and nanomolar to low micromolar binding affinities for norepinephrine transporters [2,68]. Altogether, sinus tachycardia associated with drug overdose may be related to partial inhibition of atrial GIRK channels as well as antagonism of the muscarinic receptor and enhancement of sympathetic nerve activity.

Sertraline was shown to be effective in the treatment of alcoholics [53]. Interestingly, GIRK2 knockout mice show reduced ethanol-induced conditioned taste aversion and conditioned place preference and are less sensitive than wild-types to some of the acute effects of ethanol, including anxiolysis, habituated locomotor stimulation, and acute handling-induced convulsions [76,87]. In the present study, sertraline inhibited ethanol-induced GIRK1/2 currents. Sertraline may suppress some of the GIRK-related effects of ethanol. Furthermore, GIRK knockout mice show an attenuation of the morphine withdrawal syndrome [88]. Sertraline reduced the severity of the naloxone-precipitated opioid withdrawal syndrome in rats [89]. GIRK knockout mice also show reduced cocaine self-administration [90]. Inhibition of GIRK channels by sertraline may play a role in the treatment of addiction to these drugs.

Supporting Information

Figure S1 Effect of sertraline on outward GIRK currents. In a *Xenopus* oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 30 μM sertraline and 3 mM Ba^{2+} at a membrane potential of −10 mV in a K4 solution that contained 4 mM K^+ are shown. Asterisk indicates the zero current level.
(DOC)

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Author Contributions

Conceived and designed the experiments: TK. Performed the experiments: TK. Analyzed the data: TK KW KI. Contributed reagents/materials/analysis tools: TK KW KI. Wrote the paper: TK KI.

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Article

Family Dysfunction Differentially Affects Alcohol and Methamphetamine Dependence: A View from the Addiction Severity Index in Japan

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Abstract: We investigated the differential influence of family dysfunction on alcohol and methamphetamine dependence in Japan using the Addiction Severity Index (ASI), a useful instrument that multilaterally measures the severity of substance dependence. The participants in this study were 321 male patients with alcohol dependence and 68 male patients with methamphetamine dependence. We conducted semi-structured interviews with each patient using the ASI, which is designed to assess problem severity in seven functional domains: Medical, Employment/Support, Alcohol use, Drug use, Legal, Family/Social relationships, and Psychiatric. In patients with alcohol dependence, bad relationships with parents, brothers and sisters, and friends in their lives were related to current severe psychiatric problems. Bad relationships with brothers and sisters and partners in their lives were related to current severe employment/support problems, and

bad relationships with partners in their lives were related to current severe family/social problems. The current severity of psychiatric problems was related to the current severity of drug use and family/social problems in patients with alcohol dependence. Patients with methamphetamine dependence had difficulty developing good relationships with their father. Furthermore, the current severity of psychiatric problems was related to the current severity of medical, employment/support, and family/social problems in patients with methamphetamine dependence. The results of this study suggest that family dysfunction differentially affects alcohol and methamphetamine dependence. Additionally, family relationships may be particularly related to psychiatric problems in these patients, although the ASI was developed to independently evaluate each of seven problem areas.

Keywords: alcohol dependence; methamphetamine dependence; Addiction Severity Index; family relationship

1. Introduction

In 2003, approximately 800,000 adults of the Japanese general population of 120 million could be classified with alcohol dependence, making this group one of the largest among the various mental disorders [1]. Additionally, stimulant dependence is a serious problem not only for patients, but also for Japanese society [2]. For example, approximately 25% of convicted prisoners committed offenses under the Stimulant Control Law [3].

Previous studies have suggested that social support is an important factor for improving the symptoms of substance dependence. Coping and social support are related to substance use behavior and treatment outcomes in adolescents [4,5]. Social support also plays an important role in relapse avoidance efforts for individuals who undergo substance use treatment. Social support is a "social fund" from which individuals draw assistance when confronting stressors [6].

On the other hand, bad relationships may be an aggravating factor. Previous studies have reported an association between familial relationships and substance dependence. Multidimensional Family Therapy is uniquely suited to address adolescent substance abuse and related disorders, given its comprehensive interventions that systematically target the multiple interacting risk factors that underlie many of the developmental disruptions of adolescence [7]. A previous study of alcohol dependence suggested that among the many biological, morphological, and social markers of increased maturation, visible signs of maturity are important triggers of alcohol use and alcohol use disorders, especially when they occur early and in young people with conduct problems, deviant peers, problem families, and inadequate parental supervision [8]. Another study of drug dependence reported that drug use prevention should not simply focus on reducing drug availability, but also help young people develop good family/peer relationships and find healthy ways to enjoy themselves [9].

The Addiction Severity Index (ASI) is a semi-structured clinical research interview widely used in substance abuse treatment settings in the United States and many other countries. This instrument was designed to assess problem severity in seven functional domains: Medical, Employment/Support,

Alcohol use, Drug use, Legal, Family/Social relationships, and Psychiatric [10]. Therefore, family relationships are an important factor in assessing the severity of substance dependence using the ASI.

A comparison of the characteristics of family relationships and the association between family relationships and various problems related to substance dependence in patients with alcohol and drug dependence using the ASI may be useful for establishing personalized programs for individuals with substance dependence. However, no study of which we are aware has compared the differences in the association between family dysfunction and problems related to substance dependence between alcohol and drug dependence. Moreover, the ratio of individuals who use methamphetamine is the highest in individuals with drug dependence in Japanese hospitals, suggesting that it may be meaningful to focus on the characteristics of individuals with methamphetamine dependence. Therefore, we investigated the differences in the influence of family dysfunction on alcohol dependence and methamphetamine dependence in Japanese patients using the ASI as an exploratory survey. We hypothesized that family dysfunction in patients with alcohol and patients with methamphetamine dependence may be related to different aspects of problems related to substance dependence. The present exploratory study may provide future direction for more detailed investigations that lead to the development of more effective methods for finding appropriate psychological interventions for each patient.

2. Methods

2.1. Participants

We surveyed 370 patients with alcohol dependence and 83 patients with drug dependence. Valid data were obtained from 321 male patients with alcohol dependence (86.76%; mean age, 49.7 ± 11.0 years) and 80 male patients with drug dependence (96.39%; mean age, 32.9 ± 9.4 years). The participants with alcohol dependence were recruited from nine nationwide hospitals or recovery facilities for addiction treatment located in Japan: National Hospital Organization Kurihama Alcoholism Center, Kanagawa (n = 91), Wakamiya Hospital, Yamagata (n = 55), Komakino Hospital, Tokyo (n = 50), Mie Prefectural Mental Medical Center, Mie (n = 42), Asahiyama Hospital, Hokkaido (n = 26), Ishikawa Prefectural Takamatsu Hospital, Ishikawa (n = 17), National Hospital Organization Hizen Psychiatric Center, Saga (n = 14), Akagi-Kohgen Hospital, Gunma (n = 13), and Tohokukai Mental Hospital, Miyagi (n = 12). The participants with drug dependence were recruited from five nationwide hospitals or recovery facilities for addiction treatment in Japan: Tokyo Metropolitan Matsuzawa Hospital, Tokyo (n = 37), Self-Support Services (*i.e.*, a recovery facility run by a non-profit organization for addiction recovery), Tokyo (n = 16), National Center of Neurology and Psychiatry Musashi Hospital, Tokyo (n = 17), GAIA (i.e., a recovery facility run by a non-profit organization for addiction recovery), Okinawa (n = 8), and Fukko-kai Tarumi Hospital, Hyogo (n = 2).

2.2. Methods

The Japanese version of the ASI [11,12] was used in the present study. The ASI is a semi-structured clinical research interview designed to assess problem severity in seven functional domains: Medical status, Employment/Support status, Alcohol use, Drug use, Legal status, Family/Social relationships,

and Psychiatric status [10]. The Medical status domain gathers basic information about medical history. It addresses information about lifetime hospitalizations, long-term medical problems, and recent physical ailments. The Employment/Support status domain gathers basic information about work experience and current sources of income. The Drug/Alcohol use domain gathers basic information about the patient's substance abuse history. It addresses information about current and lifetime substance abuse, the consequences of abuse, periods of abstinence, treatment episodes, and the financial burden of substance abuse. The Legal status domain gathers basic information about the patient's legal history. It addresses information about probation or parole, legal charges, convictions, incarcerations or detainments, and illegal activities. The Family/Social relationship domain assesses relationship problems with family members or friends. The Psychiatric status domain is used not to diagnose psychiatric disorders but to assess the experience of various psychiatric symptoms other than those associated with the effects of alcohol or drugs.

Acceptable reliability and validity of the ASI were confirmed in patients with drug [11] and alcohol dependence [12]. The ASI provides a composite score (CS). The CS in each problem area is a mathematically calculated score mainly based on patient responses to sets of items that ask the patient to report behaviors during the 30 days prior to the interview. The CS is calculated using a weighted formula designed to provide an equal contribution from each item and varies from 0 to 1, with a higher score indicating greater problem severity. Additionally, we analyzed the items of the ASI related to education years, employment status, marital status, cohabitation, years of current cohabitation, experience of abuse, family history of substance dependence or psychiatric disorders, and family relationships in their life.

2.3. Procedure

The recruitment criteria were the following: at least 18 years of age, a history of substance addiction problems diagnosed as alcohol dependence or drug dependence based on the criteria of the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV), and the ability to understand Japanese.

The inpatients with alcohol dependence were provided an average 80-day treatment program (e.g., group meetings, alcohol education, family treatment programs, psychotherapy, and so on) after detoxification. After recovery from serious physical and mental instability (nearly 1 month after hospitalization), informed consent was obtained from the subjects, excluding the patients who had serious cognitive impairment and psychiatric problems.

The participants with drug dependence were inpatients or outpatients at a Japanese mental hospital or recovery facility or non-patients who were recovering from stimulant abuse in a recovery facility. Considering the time required for an interview and the reliability of the responses, we excluded patients in a state of acute drug-induced psychosis.

The ASI was administered by psychiatrists and clinical psychologists who were experts in alcohol or drug dependence, carefully read the ASI manual [13], and learned the interview methods themselves. The average time required for administration of the questionnaire was 60 min. Inpatient subjects were requested to answer the questions during the 30 days prior to the start of inpatient treatment. The

Institutional Review Board of each institution approved the study, and all of the participants provided written informed consent.

2.4. Statistical Analysis

Comparisons between groups with regard to age, number of convictions, and ASI CS were conducted using the *t*-test. Comparisons between groups with regard to the characteristics of education, employment, marital status, cohabitation, experience of abuse, and psychiatric symptoms were performed using the x^2 test and Fisher exact test (multiple comparisons were performed using residual analysis). The relationships between ASI CSs were analyzed using partial correlation analysis. The significance level was set at less than 0.05 or 0.01. Statistical analyses were performed with SPSS 18 (SPSS Inc., Chicago, IL).

3. Results

3.1. Participant Characteristics

Table 1 shows the substances that the participants with drug dependence in this study mainly used. Most of the patients with drug dependence (85.00%) used methamphetamine, and others used cannabis (6.25%), inhalants (3.75%), analgesics/hypnotics/tranquilizers (2.50%), antitussive drugs (1.25%), or hallucinogens (1.25%). We performed the subsequent statistical analysis only in individuals who mainly used methamphetamine as patients with methamphetamine dependence ($n = 68$).

Table 1. Substances that participants with drug dependence in this study mainly used.

Drug	%
Methamphetamine	85.00
Cannabis	6.25
Inhalants	3.75
Analgesics/hypnotics/tranquilizers	2.50
Antitussive drugs	1.25
Hallucinogens (e.g., lysergic acid diethylamide)	1.25

Table 2 shows the characteristics of the participants. The mean age of the patients with alcohol dependence was significantly higher than that of patients with methamphetamine dependence ($t = 12.31$, $p < 0.0001$). Significant differences were found in educational background ($z = 17.72$, $p = 0.003$). Patients with alcohol dependence had a higher ratio of being a junior high school graduate ($p < 0.05$), and patients with drug dependence had a higher ratio of being a high school dropout ($p < 0.05$). Significant differences were found in employment status ($z = 36.26$, $p < 0.0001$). Patients with alcohol dependence had higher ratios of full-time employment ($p < 0.05$) and retirement ($p < 0.05$). Patients with methamphetamine dependence had higher ratios of part-time employment ($p < 0.05$) and unemployment ($p < 0.05$). A significant difference was found in marital status ($z = 64.08$, $p < 0.0001$). Patients with alcohol dependence had a higher ratio of being

married ($p < 0.05$). Patients with methamphetamine dependence had a higher ratio of never being married ($p < 0.05$). Significant differences were found in cohabitation ($z = 62.71$, $p < 0.0001$). More patients with alcohol dependence lived with their family ($p < 0.05$). More patients with methamphetamine dependence lived with their parents ($p < 0.05$). Significant differences were found in years at their current residence ($z = 12.24$, $p = 0.002$). More patients with alcohol dependence had lived in their current residence for more than 10 years ($p < 0.05$). More patients with methamphetamine dependence had lived in their current residence for less than 10 years ($p < 0.05$). With regard to abuse, patients with methamphetamine dependence had a higher ratio of physical abuse experience ($z = 8.48$, $p = 0.0007$). With regard to psychiatric symptoms in the past month, patients with methamphetamine dependence had higher ratios of "hallucinations" ($z = 17.11$, $p = 0.0003$) and "trouble understanding, concentrating, or remembering" ($z = 16.57$, $p = 0.0002$). Patients with methamphetamine dependence had more convictions ($t = 5.35$, $p < 0.0001$).

Table 2. Participant characteristics.

Characteristic	**Alcohol dependence (*n* = 321)**	**Methamphetamine dependence (*n* = 68)**	***p***	
Mean age (years [SD])	49.46 (10.34)	34.82 (8.51)	< 0.0001	*
Education				
Education (mean years [SD])	11.75 (2.69)	11.87 (2.32)	n.s.	
% Junior high school graduate	29.91	14.71	< 0.05	*
% Some high school	9.66	25.00	< 0.05	*
% High school graduate	31.15	30.88	n.s.	
% Some college	7.48	11.76	n.s.	
% College graduate	18.38	13.24	n.s.	
% Unclear	3.43	4.41	n.s.	
Employment (past 3 years)				
% Full-time	69.47	41.18	< 0.05	*
% Part-time	10.59	25.00	< 0.05	*
% Retired	6.85	0.00	< 0.05	*
% Unemployed	10.90	25.00	< 0.05	*
% Other	2.18	8.82	< 0.05	*
% Public assistance recipient (past 30 days)	8.41	16.18	n.s.	
Marital status				
% Married	54.21	8.82	< 0.05	*
% Never married	21.18	66.18	< 0.05	*
% Separated/Widowed/Divorced	24.61	25.00	n.s.	
Cohabitation				
% With family	46.11	10.29	< 0.05	*
% With spouse	14.64	10.29	n.s.	
% With parents	13.40	39.71	< 0.05	*
% Alone	21.81	19.12	n.s.	
% Other	4.05	20.59	< 0.05	*

Table 2. *Cont.*

Characteristic	*Alcohol dependence (n = 321)*	*Methamphetamine dependence(n = 68)*	*p*	
Years of current cohabitation				
% < 10 years	41.32	65.57	< 0.05	*
% 10-20 years	22.08	11.48	< 0.05	*
% > 20 years	36.59	22.95	< 0.05	*
Abuse				
% Emotional abuse	22.19	14.93	0.25	
% Physical abuse	6.85	17.91	0.007	*
% Sexual abuse	0.00	0.00	-	
Psychiatric symptoms				
% Serious depression	15.26	14.71	1.00	
% Serious anxiety or tension	24.61	32.35	0.22	
% Hallucinations	2.80	14.71	0.0003	*
% Trouble understanding, concentrating, or remembering	12.46	32.35	0.0002	*
% Trouble controlling violent behavior	4.05	10.29	0.06	
% Serious thoughts of suicide	18.07	16.18	0.86	
% Attempted suicide	3.12	4.40	0.71	
Number of convictions (mean years [SD])	0.14 (0.74)	1.32 (1.79)	< 0.0001	*

SD, standard deviation; * significant difference.

3.2. Relationship between ASI Composite Scores

Table 3 shows the correlations between ASI CSs after controlling for age. For patients with alcohol dependence, the CS of psychiatric problems was significantly correlated with the CSs of drug use (r = 0.27, p < 0.0001) and family/social problems (r = 0.28, p < 0.0001). For patients with methamphetamine dependence, the CS of psychiatric problems was significantly correlated with the CSs of medical problems (r = 0.33, p = 0.008), employment/support problems (r = 0.40, p = 0.001), drug use (r = 0.40, p = 0.0009), and family/social problems (r = 0.43, p = 0.0004). The CS of family/social problems was significantly correlated with the CS of medical problems (r = 0.33, p = 0.007) and legal problems (r = 0.35, p = 0.004).

Table 3. Correlations between ASI composite scores after controlling for age.

Alcohol dependence		**Employment**	**Alcohol use**	**Drug use**	**Legal**	**Family/Social**	**Psychiatric**
Medical	*r*	0.08	−0.13	0.08	0.05	0.03	0
	p	0.2	0.03	0.16	0.38	0.62	0.97
Employment	*r*		0.01	0.07	0.13	0.12	−0.02
	p		0.85	0.26	0.02	0.04	0.69
Alcohol use	*r*			−0.04	0.01	0.14	0.09
	p			0.51	0.85	0.02	0.14
Drug use	*r*				0	0.14	0.26
	p				0.96	0.02	< 0.0001 *
Legal	*r*					0.02	−0.07
	p					0.69	0.23
Family/Social	*r*						0.28
	p						< 0.0001 *

Table 3. *Cont.*

Methamphetamine dependence		**Employment**	**Alcohol use**	**Drug use**	**Legal**	**Family/Social**	**Psychiatric**
Medical	*r*	0.17	0.04	−0.04	0.19	0.33	0.33
	p	0.17	0.76	0.78	0.12	0.007 *	0.008 *
Employment	*r*		−0.04	0.22	−0.01	0.05	0.40
	p		0.76	0.07	0.92	0.71	0.001 *
Alcohol use	*r*			0.12	−0.06	0.20	0.06
	p			0.34	0.61	0.11	0.65
Drug use	*r*				−0.05	0.07	0.40
	p				0.68	0.60	0.0009 *
Legal	*r*					0.35	0.09
	p					0.004 *	0.48
Family/Social	*r*						0.43
	p						0.0004 *

* Significant correlation ($p < 0.01$).

Table 4 shows the comparison of ratios of each psychiatric symptom in the past month between groups of high and low CSs of Family/Social relationship problems. These groups were divided on the basis of median CSs of Family/Social relationships. In patients with alcohol dependence, high CSs of Family/Social relationship problems was associated with higher ratios of serious depression ($z = 10.98$, $p = 0.001$), serious anxiety or tension ($z = 6.17$, $p = 0.02$), and serious thoughts of suicide ($z = 6.81$, $p = 0.01$) than the low CS group. In patients with methamphetamine dependence, no significant difference was found between groups of high and low CSs of Family/Social relationship problems in ratio of each psychiatric symptom.

Table 4. Comparison of ratios of each psychiatric symptom between groups of high and low CSs of Family/Social relationship problems.

Alcohol dependence	Family/Social			
	High	Low	*p*	
Serious depression (%)	22.62	9.41	0.001	*
Serious anxiety or tension (%)	32.56	19.08	0.02	*
Hallucinations (%)	2.33	3.82	0.72	
Trouble understanding, concentrating, or remembering (%)	14.73	12.21	0.59	
Trouble controlling violent behavior (%)	5.43	3.88	0.77	
Serious thoughts of suicide (%)	25.78	12.98	0.01	*
Attempted suicide (%)	3.10	3.08	1.00	

Table 4. *Cont.*

Methamphetamine dependence	Family/Social		
	High	Low	*p*
Serious depression (%)	21.88	6.06	0.08
Serious anxiety or tension (%)	39.39	24.24	0.29
Hallucinations (%)	18.18	12.12	0.73
Trouble understanding, concentrating, or remembering (%)	42.42	24.24	0.19
Trouble controlling violent behavior (%)	18.18	3.03	0.10
Serious thoughts of suicide (%)	18.18	12.12	0.73
Attempted suicide (%)	3.03	3.03	1.00

* Significant difference.

3.3. Family History of Alcohol Dependence, Methamphetamine Dependence, and Psychiatric Disorders

Table 5 shows the family histories of alcohol dependence, methamphetamine dependence, and psychiatric disorders in the participants of the present study. Of the patients with alcohol dependence, 36.33% had fathers with alcohol-related problems, 20.87% had uncles (paternal) with alcohol-related problems, and 25.94% had brothers with alcohol-related problems, and these ratios were significantly higher compared with patients with methamphetamine dependence (father, $z = 7.97$, $p = 0.005$; uncle, $z = 6.31$, $p = 0.009$; brother, $z = 8.81$, $p = 0.002$). Of the patients with methamphetamine dependence, 9.52% had brothers with drug-related problems, and the ratio was significantly higher than that of patients with alcohol dependence ($z = 22.90$, $p = 0.005$).

Table 5. Family history of alcohol dependence, methamphetamine dependence, and psychiatric disorders.

Relation	**Alcohol dependence**	**Methamphetamine dependence**	***p***
Grandmother (maternal) (%)			
Alcohol	1.44	2.04	0.57
Drug	0.00	0.00	—
Psychiatric disorder	0.00	0.00	—
Grandfather (maternal) (%)			
Alcohol	15.42	6.12	0.11
Drug	0.00	0.00	—
Psychiatric disorder	0.00	0.00	—
Mother (%)			
Alcohol	1.30	1.92	0.54
Drug	0.33	0.00	1.00
Psychiatric disorder	2.64	7.41	0.09