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Inhibition of G-Protein-Activated Inwardly Rectifying K^+ Channels by the Selective Norepinephrine Reuptake Inhibitors Atomoxetine and Reboxetine

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Atomoxetine and reboxetine are commonly used as selective norepinephrine reuptake inhibitors (NRIs) for the treatment of attention-deficit/hyperactivity disorder and depression, respectively. Furthermore, recent studies have suggested that NRIs may be useful for the treatment of several other psychiatric disorders. However, the molecular mechanisms underlying the various effects of NRIs have not yet been sufficiently clarified. G-protein-activated inwardly rectifying K^+ (GIRK or Kir3) channels have an important function in regulating neuronal excitability and heart rate, and GIRK channel modulation has been suggested to be a potential treatment for several neuropsychiatric disorders and cardiac arrhythmias. In this study, we investigated the effects of atomoxetine and reboxetine on GIRK channels using the *Xenopus* oocyte expression assay. In oocytes injected with mRNA for GIRK1/GIRK2, GIRK2, or GIRK1/GIRK4 subunits, extracellular application of atomoxetine or reboxetine reversibly reduced GIRK currents. The inhibitory effects were concentration-dependent, but voltage-independent, and time-independent during each voltage pulse. However, Kir1.1 and Kir2.1 channels were insensitive to atomoxetine and reboxetine. Atomoxetine and reboxetine also inhibited GIRK currents induced by activation of cloned A_1 adenosine receptors or by intracellularly applied GTPγS, a nonhydrolyzable GTP analogue. Furthermore, the GIRK currents induced by ethanol were concentration-dependently inhibited by extracellularly applied atomoxetine but not by intracellularly applied atomoxetine. The present results suggest that atomoxetine and reboxetine inhibit brain- and cardiac-type GIRK channels, revealing a novel characteristic of clinically used NRIs. GIRK channel inhibition may contribute to some of the therapeutic effects of NRIs and adverse side effects related to nervous system and heart function.
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Keywords: atomoxetine; reboxetine; selective norepinephrine reuptake inhibitor; GIRK channel; ethanol; *Xenopus* oocyte

INTRODUCTION

Atomoxetine (originally named tomoxetine) and reboxetine are commonly used as selective norepinephrine reuptake inhibitors (NRIs) for the treatment of attention-deficit/hyperactivity disorder and depression, respectively (Hajós *et al*, 2004; Garland and Kirkpatrick 2004; Simpson and Plosker, 2004; Supplementary Figure S1). Their clinical efficacy is hypothesized to be linked mainly with potent inhibition of presynaptic norepinephrine transporters (Wong *et al*, 2000; Hajós *et al*, 2004; Simpson and Plosker, 2004). Furthermore, recent studies have suggested that the drugs are potentially useful for the treatment of several other psychiatric conditions, including anxiety disorders, eating disorders, substance use disorders, and narcolepsy (Kadhe *et al*, 2003; Hajós *et al*, 2004; Szerman *et al*, 2005; McElroy *et al*, 2007; Geller *et al*, 2007; Wilens *et al*, 2008). However, the molecular mechanisms underlying the various effects of NRIs 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 include seven subfamilies (Reimann and Ashcroft, 1999). Four GIRK channel subunits have been identified in mammals (Kubo *et al*, 1993b; Krapivinsky *et al*, 1995; Lesage *et al*, 1995). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions or homomultimers composed of GIRK2 subunits in the substantia nigra (Lesage *et al*, 1995; Karschin *et al*, 1996; Liao *et al*, 1996; Inanobe *et al*, 1999), whereas atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky *et al*, 1995). The channels are activated by various

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G_i-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 (North, 1989; Dascal, 1997; Kobayashi and Ikeda, 2006). Additionally, ethanol activates GIRK channels independently of G-protein-coupled signaling pathways (Kobayashi *et al*, 1999; Lewohl *et al*, 1999). GIRK channels have an important function in regulating neuronal excitability, synaptic transmission, and heart rate (North, 1989; Lüscher *et al*, 1997; Signorini *et al*, 1997; Kuzhikandathil and Oxford, 2002; Kovoor *et al*, 2001). Furthermore, recent studies have suggested that GIRK channel modulation has the potential for treating several neuropsychiatric disorders and cardiac arrhythmias (Hashimoto *et al*, 2006; Kobayashi and Ikeda 2006; Cruz *et al*, 2008). Therefore, GIRK channel modulators may affect various brain and cardiac functions. In this study, the effects of atomoxetine and reboxetine on GIRK channels were examined using the *Xenopus* oocyte expression assay.

MATERIALS AND METHODS

Preparation of Specific mRNAs

Plasmids containing the entire coding sequences for the mouse GIRK1, GIRK2, and GIRK4 channel subunits and the *Xenopus* A_1 adenosine receptor (A_1R) were obtained previously (Kobayashi *et al*, 1995, 1999, 2000, 2002). cDNAs for rat Kir1.1 in pSPORT (Ho *et al*, 1993) and mouse Kir2.1 in pcDNA1 (Kubo *et al*, 1993a) were generously provided by Dr Steven C Hebert (Yale University) and Dr Lily Y Jan (University of California, San Francisco), respectively. These plasmids were linearized by digestion with the appropriate enzymes as described previously (Ho *et al*, 1993; Kubo *et al*, 1993a; Kobayashi *et al*, 2000). 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 containing 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 carried out in accordance with National Institutes of Health guidelines and were approved by our Institutional Animal Care and Use Committee. *Xenopus* oocytes (Stages V and VI) were manually isolated from the ovary and maintained in Barth's solution (Kobayashi *et al*, 2002). Oocytes were injected with mRNA for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each 0.15 ng), GIRK2 (1 ng), Kir1.1 (2 ng), Kir2.1 (0.3 ng), or A_1R (5 ng). The oocytes were incubated at 19°C in Barth's solution and manually defolliculated after treatment with 0.8 mg ml^{-1} collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 1 h. Whole-cell currents of the oocytes were recorded 3–8 days after injection with a conventional two-electrode voltage clamp (Kobayashi *et al*, 1999; Ikeda *et al*, 2003). All recordings were carried out at room temperature (19°C) to avoid damage to *Xenopus* oocytes and the effects of temperature (Fraser and Djamgoz, 1992; Weber, 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 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 shown earlier (Ho *et al*, 1993; Kubo *et al*, 1993a; Lesage *et al*, 1995; Kobayashi *et al*, 2006). Additionally, to examine the effects of the NRIs on outward K^+ currents, a perfusion solution containing 4 mM K^+ (K4 solution) was made by substituting NaCl with KCl in the ND98 solution. To examine the effects of the drugs 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 earlier (Kovoor *et al*, 1995). Furthermore, to examine the effects of intracellular atomoxetine, 23 nl of 10 mM atomoxetine dissolved in distilled water was injected into an oocyte using a nanoliter injector (Kobayashi *et al*, 2003), and the oocyte currents were then continuously recorded for ~30–40 min. As the volume of the *Xenopus* oocytes used was ~1 μl, the intracellular concentration of atomoxetine was presumed to be ~225 μM. For analysis of concentration–response relationships, data were fitted to the following logistic equation: drug inhibition = max/1 + $(EC_{50}/[drug])^{nH}$, with max being the maximal inhibition attainable, EC_{50} being the concentration of a drug that produces 50% of the maximal current response for that drug, [drug] being the concentration of an NRI and n_H being the Hill coefficient, using KaleidaGraph (Synergy Software, Reading, PA, USA). The concentrations required to reduce control currents, by 25 and 50% (IC_{25} and IC_{50}, respectively), were calculated from the concentration–response relationships.

Statistical Analysis

Data are expressed as mean ± SEM, and n is the number of oocytes tested. Statistical analysis of the differences between groups was performed using Student's t-test, paired t-test, one-way analysis of variance (ANOVA), or two-way ANOVA followed by Tukey–Kramer *post hoc* test. Values of $P < 0.05$ were considered statistically significant.

Compounds

Tomoxetine hydrochloride (recently renamed atomoxetine hydrochloride) and reboxetine mesylate were purchased from Tocris Cookson (Bristol, UK) and dissolved in dimethyl sulfoxide (DMSO) or distilled water. 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 Atomoxetine and Reboxetine

In *Xenopus* oocytes injected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which depend on free G-protein $\beta\gamma$ subunits present in the oocytes because of the inherent activity of G-proteins (Dascal, 1997), were observed at a holding potential of −70 mV in an hK solution containing 96 mM K^+ (Figure 1a). Extracellular application of 30 μM atomoxetine or reboxetine reversibly reduced the inward currents through the expressed GIRK channels (Figure 1a). The current responses to an additional 100 μM atomoxetine during the application of 3 mM Ba^{2+}, which blocks Kir channels, were not significant (reduction of inward currents by 5.5 ± 5.0 nA; <1% inhibition of the Ba^{2+}-sensitive current components; $n=4$). The 3 mM Ba^{2+}-sensitive current components (910.5 ± 65.7 nA, $n=14$) are considered to correspond to the magnitude of GIRK currents in oocytes expressing GIRK channels (Kobayashi *et al*, 1999). Atomoxetine and reboxetine produced no significant response in a K^+-free ND98 perfusion solution containing 98 mM Na^+ instead of the hK solution ($n=4$; data not shown), suggesting that the NRI-sensitive current components show K^+ selectivity. Additionally, application of DMSO or distilled water, the solvent vehicle, at the highest concentration (0.3%) induced no significant current response in the hK or ND98 solutions ($n=5$; data not shown). However, in oocytes injected with mRNA for Kir1.1, an ATP-regulated Kir channel (Ho *et al*, 1993), or Kir2.1, a constitutively active Kir channel (Kubo *et al*, 1993a), extracellular application of 300 μM atomoxetine or reboxetine had no significant effects on the inward currents through the channels in the hK solution (<3% change of the Ba^{2+}-sensitive current components; 583.3 ± 59.7 nA for Kir1.1, $n=4$; 1306.7 ± 179.8 nA for Kir2.1, $n=4$; Figure 1b). In uninjected oocytes, 300 μM atomoxetine and reboxetine as well as 3 mM Ba^{2+} caused no significant response (3.8 ± 2.9, 0 ± 0, and 6.8 ± 0.7 nA, respectively; $n=4$, 4, and 7, respectively; Figure 1c) compared with oocytes injected with GIRK mRNA, suggesting no significant effects of atomoxetine, reboxetine, or Ba^{2+} on intrinsic oocyte channels. Furthermore, in oocytes injected with GIRK1 and GIRK2 mRNAs, outward currents observed at a holding potential of −30 mV in a K4 solution containing 4 mM K^+ were reversibly reduced by 30 μM atomoxetine ($n=4$), 30 μM reboxetine ($n=4$), or 3 mM Ba^{2+} (the Ba^{2+}-sensitive current components, 85.2 ± 32.8 nA, $n=8$; Supplementary Figure S2), whereas in uninjected oocytes, the NRIs at 100 μM and 3 mM Ba^{2+} caused no significant response (3.0 ± 0.9 nA for atomoxetine, 0 ± 0 nA for reboxetine, and 7.6 ± 1.3 nA for Ba^{2+}; $n=4$, 4, and 8, respectively). The results suggest that the NRIs also inhibited outward GIRK currents. Similarly, in oocytes injected with either GIRK1 and GIRK4 mRNAs or GIRK2 mRNA, atomoxetine and reboxetine inhibited basal GIRK currents under the same conditions (3 mM Ba^{2+}-sensitive current components for GIRK1/4, 1027.5 ± 112.6 nA, $n=10$; 3 mM Ba^{2+}-sensitive current components for GIRK2, 757.0 ± 51.5 nA, $n=12$; Figure 2). The results suggest that atomoxetine and reboxetine inhibited GIRK channels, but not Kir1.1 and Kir2.1 channels.

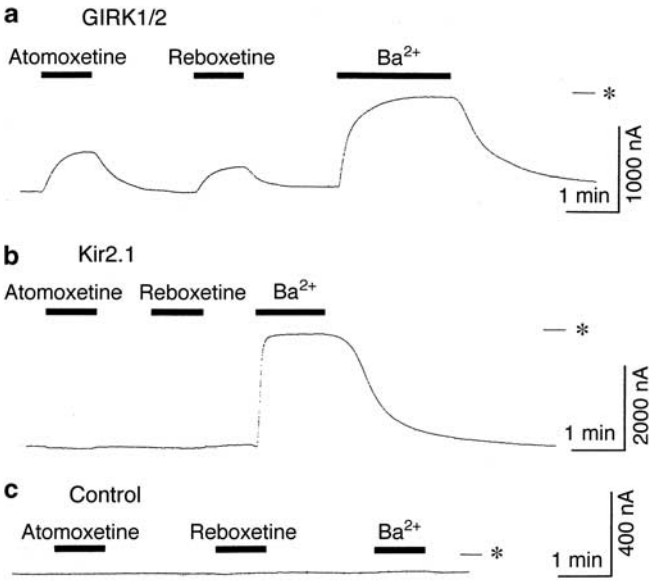

Figure 1 Inhibitory effects of atomoxetine and reboxetine on GIRK channels expressed in *Xenopus* oocytes. (a) In an oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 10 μM atomoxetine, 10 μM reboxetine, and 3 mM Ba^{2+} are shown. (b) In an oocyte injected with Kir2.1 mRNA, current responses to 100 μM atomoxetine, 100 μM reboxetine, and 3 mM Ba^{2+} are shown. (c) In an uninjected oocyte, no significant current responses to 300 μM atomoxetine, 300 μM reboxetine, or 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+. Asterisks show the zero current level. Horizontal bars indicate the duration of application.

Characteristics of Inhibition of GIRK Channels by Atomoxetine and Reboxetine

The concentration–response relationships of the inhibitory effects of atomoxetine and reboxetine on GIRK1/2, GIRK2, and GIRK1/4 channels were investigated. Figure 2 shows that inhibition of these types of GIRK channels by atomoxetine and reboxetine was concentration-dependent. Table 1 shows the EC_{50} and n_H values obtained from the concentration–response relationships and the percentage inhibition of the GIRK currents by the NRIs 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 GIRK1/4 channels by atomoxetine was more effective at 10 and 30 μM than inhibition of GIRK2 channels ($P<0.05$, Tukey–Kramer *post hoc* test), although the effects of atomoxetine at the highest concentration on three types of channels were similar ($P>0.05$, Tukey–Kramer *post hoc* test; Figure 2a; Table 1). In contrast, the inhibitory effects of reboxetine on these types of channels were statistically similar ($P>0.05$ at each concentration, Tukey–Kramer *post hoc* test), although the inhibition of GIRK2 channels by 100 and 300 μM reboxetine was slightly less effective than inhibition of the other

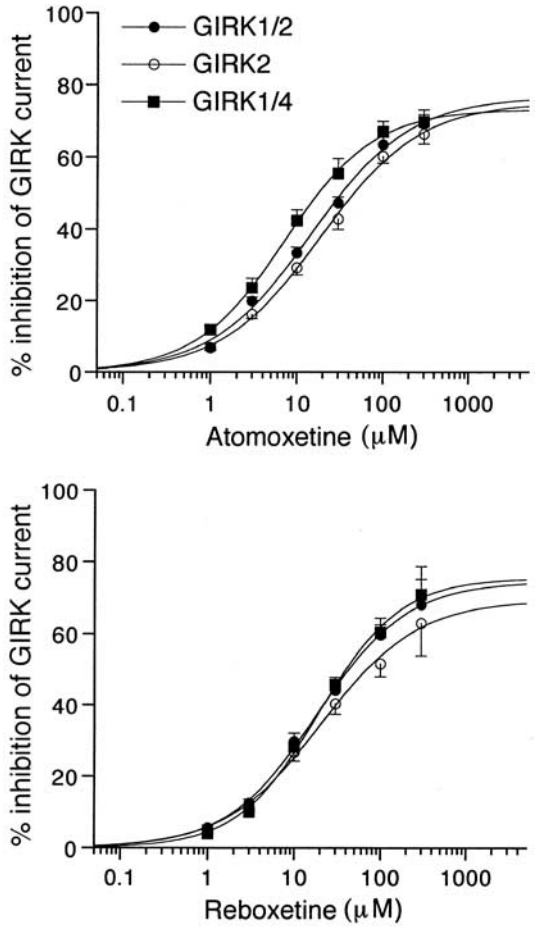

Figure 2 Concentration–response relationships for the inhibitory effects of atomoxetine and reboxetine on GIRK1/2, GIRK2, and GIRK1/4 channels. The magnitudes of inhibition of GIRK currents by the drugs were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing GIRK1/2, GIRK2, and GIRK1/4 channels (910.5 ± 65.7 nA, $n = 14$; 757.0 ± 51.5 nA, $n = 12$; and 1027.5 ± 112.6 nA, $n = 10$, respectively). Each point and error bar represents the mean ± SEM of the percentage responses.

Table 1 Inhibitory Effects of Atomoxetine and Reboxetine on GIRK Channels

	Atomoxetine			Reboxetine		
	GIRK1/2	**GIRK2**	**GIRK1/4**	**GIRK1/2**	**GIRK2**	**GIRK1/4**
EC_{50} (μM)	10.9 ± 1.3	12.4 ± 1.5	6.5 ± 0.4	13.7 ± 1.3	15.5 ± 2.1	19.4 ± 1.7
IC_{25} (μM)	5.4 ± 0.4	6.1 ± 0.5	2.9 ± 0.2	7.8 ± 1.1	8.8 ± 1.4	9.0 ± 0.4
IC_{50} (μM)	33.3 ± 4.9	52.2 ± 10.2	14.3 ± 1.6	48.2 ± 11.1	64.0 ± 18.3	41.0 ± 4.9
% Max	69.3 ± 2.5	67.0 ± 1.3	73.1 ± 0.8	68.3 ± 10.6	63.1 ± 9.1	71.1 ± 4.2
(*n*)	(8)	(6)	(5)	(6)	(6)	(5)
n_H	0.96 ± 0.09	0.89 ± 0.09	0.88 ± 0.07	0.94 ± 0.03	0.91 ± 0.04	0.93 ± 0.04

Mean ± SEM of the concentration of a drug that produces 50% of the maximal effect (EC_{50}) and the concentrations required to reduce basal GIRK currents by 25 and 50% (IC_{25} and IC_{50}, respectively) are shown in μM. The values of % max indicate the mean ± SEM percentage inhibition of basal GIRK currents by a drug at the highest concentrations tested (300 μM). The number of *Xenopus* oocytes tested (*n*) is indicated in parentheses. The n_H values indicate the mean ± SEM of Hill coefficients.

channel types (Figure 2b). Furthermore, inhibition of GIRK1/4 channels by 10 μM atomoxetine was more effective than 10 μM reboxetine ($P < 0.05$, Tukey–Kramer *post hoc* test), whereas the effects of atomoxetine on GIRK1/2 and GIRK2 channels were similar to reboxetine ($P > 0.05$ at each concentration, Tukey–Kramer *post hoc* test).

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 atomoxetine applied for 3 min (Figure 3a). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by atomoxetine 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 reboxetine, similar results were observed ($n = 7$). These results suggest that the channels were inhibited by atomoxetine and reboxetine primarily at the holding potential of 0 mV and time-independently during each voltage pulse. Similar to the 3 mM Ba^{2+}-sensitive current components corresponding to the magnitudes of basal GIRK currents, the magnitudes of currents reduced by 30 μM atomoxetine in oocytes expressing GIRK1/2 channels increased with negative membrane potentials, and the current–voltage relationships showed strong inward rectification ($n = 9$; Figure 3b), indicating a characteristic of GIRK currents. The percentage inhibition of GIRK1/2 currents by 30 μM atomoxetine showed no significant difference across voltages between −120 and −40 mV (no significant atomoxetine effect × membrane potential effect interaction, $P > 0.1$, one-way ANOVA; $P > 0.1$ across voltages, Tukey–Kramer *post hoc* test; Figure 3c). For reboxetine, similar results were observed ($n = 7$; Figure 3b and c). The results suggest that the inhibition of GIRK channels by atomoxetine and reboxetine was voltage-independent. Furthermore, similar results were obtained in oocytes expressing GIRK1/4 channels ($n = 5$ for atomoxetine and $n = 4$ for reboxetine; data not shown). Therefore, atomoxetine and reboxetine may have similar actions as GIRK channel inhibitors.

Atomoxetine possesses a secondary amine group with a pK_a value of 9.23 (Eli Lilly and Company Data Sheet; Supplementary Figure S1). At physiological pH or below, atomoxetine exists mainly in a protonated form, ~98.5% at pH 7.4, 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 atomoxetine. However, in oocytes expressing GIRK1/2 channels, the percentage inhibition of GIRK channels by atomoxetine at the same concentrations was not significantly affected by extracellular pH 7.4 and 9.2 (no significant pH × atomoxetine interaction, $P > 0.5$, two-way ANOVA; $P > 0.1$ at each concentration, Tukey–Kramer *post hoc* test; Figure 4). The results indicate that a marked increase in the proportion of the uncharged form may not significantly affect all of the effects on GIRK channels, suggesting that GIRK channel inhibition may be mediated by both forms of atomoxetine with similar effectiveness.

Effects of Atomoxetine and Reboxetine on GIRK Channels Activated by a G-Protein-Coupled Receptor or GTPγS

We examined the effects of atomoxetine and reboxetine on GIRK channels activated by a G-protein-coupled receptor.

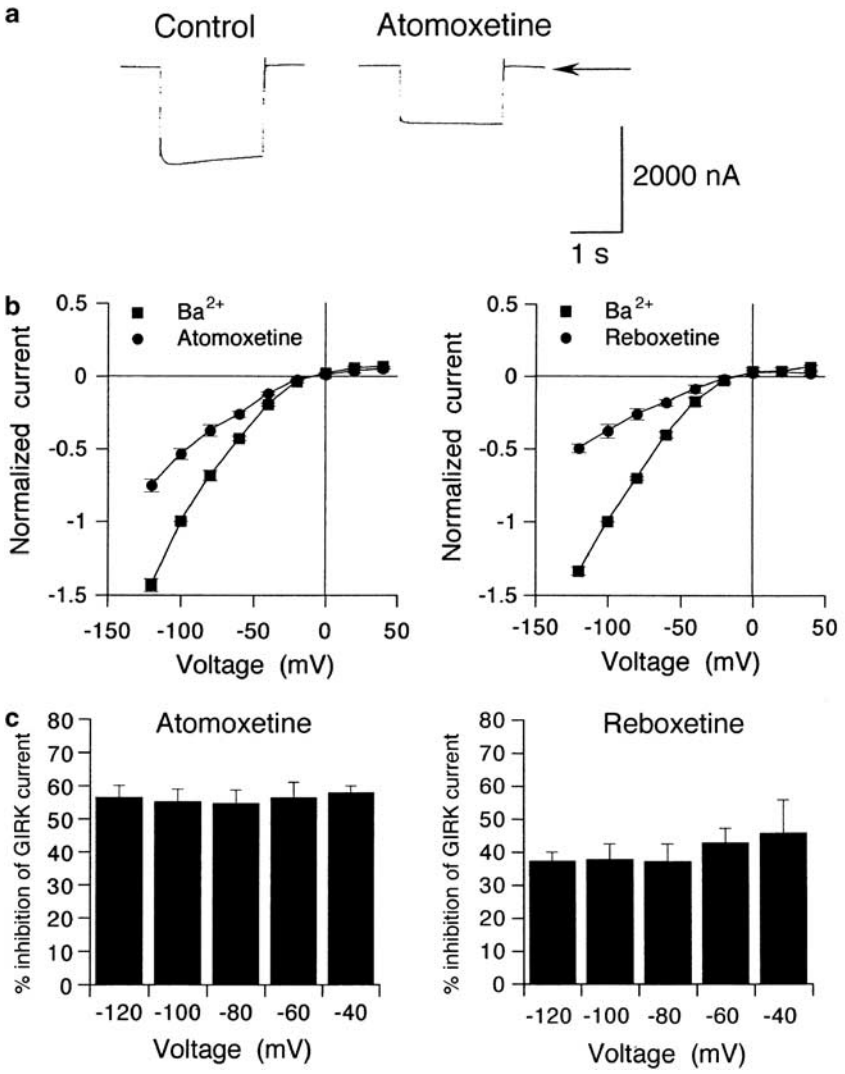

Figure 3 Characteristics of the inhibitory effects of atomoxetine and reboxetine on GIRK currents. (a) Representative GIRK1/2 currents elicited by a voltage step to −100 mV for 2 s from a holding potential of 0 mV in the presence or absence of 30 μM atomoxetine applied for 3 min. Current responses were recorded in an hK solution containing 96 mM K^+. Arrow indicates the zero current level. (b) Current–voltage relationships of the magnitudes of the current component sensitive to 3 mM Ba^{2+} and the magnitudes of currents reduced by 30 μM atomoxetine (left) or 30 μM reboxetine (right) in oocytes expressing GIRK1/2 channels. Current responses to a drug were normalized to the 3 mM Ba^{2+}-sensitive current component measured at a membrane potential of −100 mV (1219.7 ± 79.2 nA, $n = 14$). (c) Percentage inhibition of GIRK1/2 channels by atomoxetine or reboxetine over the voltage range of −120 to −40 mV. The magnitudes of inhibition of GIRK currents by 30 μM atomoxetine (left, $n = 8$) or 30 μM reboxetine (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.

In oocytes co-expressing GIRK1/2 channels and A_1Rs (Kobayashi *et al*, 2002), 100 nM adenosine significantly induced inward GIRK currents (1000.7 ± 76.9 nA, $n = 10$; Figure 5a), and 300 μM atomoxetine or reboxetine alone consistently inhibited basal GIRK currents (3 mM Ba^{2+}-sensitive current components, 157.2 ± 31.3 nA, $n = 10$). The current responses to 100 nM adenosine were reduced by the addition of atomoxetine or reboxetine ($n = 5$ for each NRI; Figure 5a). These results suggest that atomoxetine and reboxetine inhibited total GIRK currents through the GIRK channels activated by the A_1R and the basally active GIRK channels. The percentage inhibition of total GIRK currents by atomoxetine or reboxetine ($IC_{25} = 4.5 \pm 1.6$ and 8.6 ± 1.7 μM; $IC_{50} = 42.7 \pm 12.3$ and 55.1 ± 16.4 μM; $n_H = 0.93 \pm 0.04$ and 0.79 ± 0.13; $n = 5$, respectively; Figure 5b) was not significantly different from that of basal GIRK currents in oocytes injected with GIRK1 and GIRK2 mRNAs ($P > 0.05$, IC_{25} and IC_{50} values for each NRI, Student's *t*-test; $P > 0.05$ at each concentration, Tukey–Kramer *post hoc* test), suggesting that the effects of the NRIs on A_1R-activated GIRK channels were similar to those on GIRK channels activated by basally free G-protein $\beta\gamma$ subunits present in oocytes.

GIRK channels are activated by various G-protein-coupled receptors through the direct action of G-protein $\beta\gamma$ subunits released from the heterotrimeric G-protein complex (Dascal, 1997; Kobayashi and Ikeda, 2006). The effects of the NRIs on GIRK channels activated by G-protein-coupled signaling mechanisms were further examined using GTPγS, a nonhydrolyzable GTP analogue that maintains G-proteins in an activated state. Injection of GTPγS into *Xenopus* oocytes injected with GIRK1 and

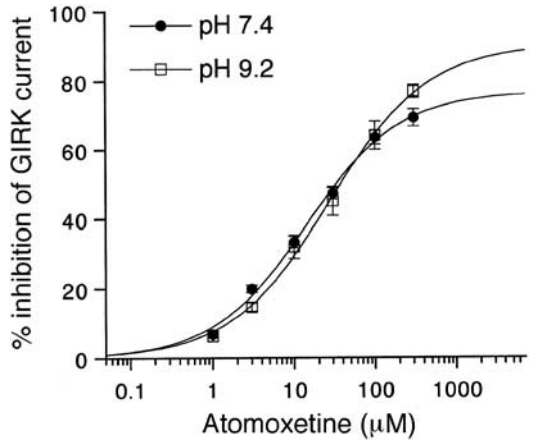

Figure 4 Concentration–response relationships for inhibition of GIRK channels by atomoxetine at different pH values. The magnitudes of inhibition of GIRK currents by atomoxetine were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing GIRK1/2 channels (1021.5 ± 100.8 nA, pH 7.4, $n = 8$; 852.4 ± 141.4 nA, pH 9.2, $n = 6$). Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+. Each point and error bar represents the mean ± SEM of the percentage responses.

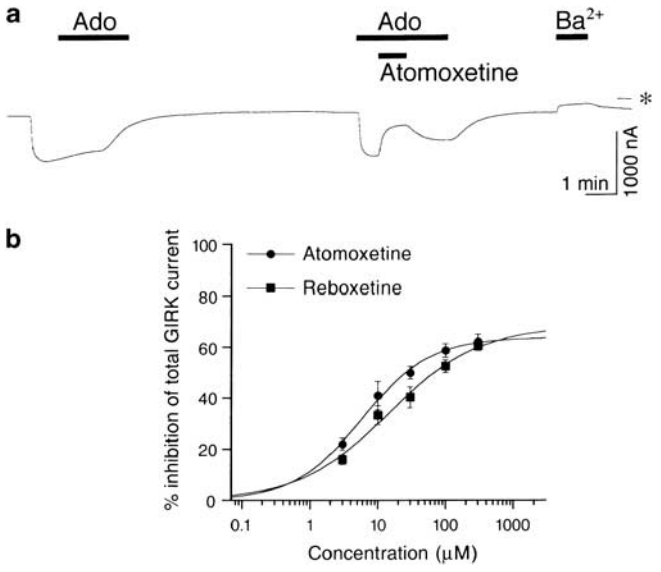

Figure 5 Effects of atomoxetine and reboxetine on GIRK channels activated by a G-protein-coupled receptor. (a) Current responses to 100 nM adenosine (Ado), 30 μM atomoxetine during application of 100 nM Ado, and 3 mM Ba^{2+} in an oocyte co-injected with mRNAs for GIRK1 and GIRK2 channels and the A_1 adenosine receptor (A_1R) are shown. Bars show the duration of application. Asterisk indicates the zero current level. (b) Concentration–response relationships for the inhibitory effects of atomoxetine and reboxetine on total GIRK currents composed of Ado-induced GIRK currents and basal GIRK currents. Each point and error bar represents the mean ± SEM of the percentage responses. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+.

GIRK2 mRNAs increased inward currents with time and reached a steady-state level (938.9 ± 119.2 nA, $n = 18$) as reported earlier (Kovoor *et al*, 1995). 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, $n = 9$). Increased GIRK currents composed of basal GIRK currents and GTPγS-induced GIRK currents were inhibited by atomoxetine or reboxetine ($IC_{50} = 29.0 \pm 6.2$ and 52.3 ± 10.1 μM; $n_H = 1.28 \pm 0.04$ and 1.14 ± 0.06; $n = 6$ and 12, respectively). The percentage inhibition of total GIRK currents by atomoxetine or reboxetine was not significantly different from that of basal GIRK currents in GTPγS-untreated oocytes injected with GIRK1 and GIRK2 mRNAs ($P > 0.05$, IC_{50} value for each NRI, Student's *t*-test; $P > 0.05$ at each concentration, Tukey–Kramer *post hoc* test), suggesting that the effects of the NRIs on basally active GIRK channels and GIRK channels activated by G-protein activation induced by GTPγS were similar.

Atomoxetine Inhibits Ethanol-Induced GIRK Currents

GIRK channels are also activated by ethanol independent of G-protein signaling pathways (Kobayashi *et al*, 1999). Atomoxetine was shown to reduce cumulative heavy drinking days in the treatment of psychiatric patients with comorbid alcohol use disorders (Wilens *et al*, 2008). Therefore, we also examined the effects of atomoxetine on GIRK channel activation induced by ethanol. The effects of atomoxetine were evaluated by measuring the amplitude of the ethanol-induced current response during extracellular application of atomoxetine at different concentrations. In oocytes injected with GIRK1 and GIRK2 mRNAs, the GIRK currents induced by 100 mM ethanol (420.0 ± 32.5 nA, $n = 5$) were reversibly attenuated in the presence of atomoxetine ($IC_{25} = 5.8 \pm 1.1$ μM; $IC_{50} = 15.4 \pm 3.1$ μM; $n_H = 1.22 \pm 0.22$; $n = 5$; Figure 6a and b). However, 100 mM ethanol-induced GIRK currents were not significantly affected by intracellularly applied atomoxetine (104.3 ± 2.8% of untreated control current, paired *t*-test, $P > 0.1$, $n = 5$; Figure 6c). Moreover, in oocytes expressing GIRK channels, the basal currents were not significantly affected by intracellularly applied atomoxetine (103.0 ± 2.2% of untreated control current, paired *t*-test, $P > 0.1$, $n = 5$). The results indicate that intracellular atomoxetine could not inhibit GIRK channels. In contrast, GIRK channel inhibition induced by extracellularly applied atomoxetine, which is mainly protonated at pH 7.4, was reversible with washout (Figures 1a and 6a). As the protonated form may not readily permeate the cell membrane, extracellularly applied atomoxetine may exist mainly on the extracellular side. Altogether, extracellular atomoxetine may inhibit GIRK channels activated by ethanol.

DISCUSSION

In this study, we showed that atomoxetine and structurally related reboxetine, clinically used selective NRIs, inhibited brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. However, Kir1.1 and Kir2.1 channels in other Kir channel subfamilies were insensitive to both NRIs. The inhibitory effects on GIRK channels were concentration-dependent, but voltage-independent, and time-independent during each voltage pulse. The present results suggest that the site of action on

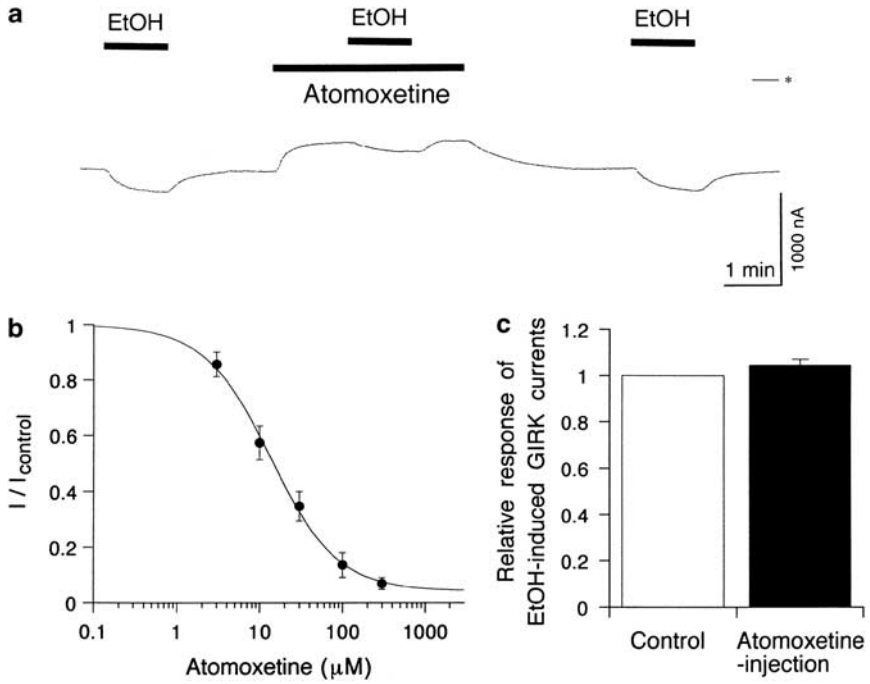

Figure 6 Effects of atomoxetine on ethanol-induced GIRK currents. (a) Current responses to 100 mM ethanol (EtOH), 100 mM EtOH in the presence of 30 μM atomoxetine, and 100 mM EtOH in an oocyte injected with GIRK1 and GIRK2 mRNAs. Asterisk indicates the zero current level. Bars show the duration of application. (b) Concentration-dependent inhibition of EtOH-induced GIRK currents by atomoxetine. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM EtOH (420.0 ± 32.5 nA, $n = 5$), and I is the current amplitude in the presence of atomoxetine. (c) Lack of effect of intracellular atomoxetine on 100 mM EtOH-induced GIRK currents. The amplitude of EtOH-induced GIRK currents after atomoxetine injection (black bar) was compared with the amplitude of EtOH-induced GIRK currents before the injection (control, white bar) in the same oocyte expressing GIRK channels ($n = 5$). Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+. All values are expressed as mean ± SEM.

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, shows a concentration-dependence, a voltage-dependence, and a time-dependence with a comparatively small effect on the instantaneous current but a marked inhibition of the steady-state current at the end of the voltage pulses (Lesage *et al*, 1995). These observations suggest that atomoxetine and reboxetine may cause an allosteric conformational change in GIRK channels even before the voltage pulses, rather than simple occlusion of the open channel. Interestingly, GIRK channels are also inhibited by the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Kobayashi *et al*, 2003; Takahashi *et al*, 2006), despite a great difference in the pharmacological profiles for monoamine transporters between the two NRIs and fluoxetine. The chemical structures of atomoxetine and reboxetine are related to fluoxetine (Boot *et al*, 2005; Supplementary Figure S1), suggesting that the common moiety of the structures may play a key role in interacting with GIRK channels. Additionally, the *Xenopus* oocyte expression assay with a conventional two-electrode voltage clamp is generally conducted using defolliculated oocytes, which are still covered over the plasma membrane with the vitelline membrane, at room temperature (Fraser and Djamgoz, 1992; Weber, 1999; Ikeda *et al*, 2003). Further studies using mammalian cells, including neurons and cardiac myocytes, at physiological temperature may be useful for advancing our understanding of the effects of NRIs on GIRK channels.

Atomoxetine is predominantly metabolized by the genetically polymorphic cytochrome P450 2D6 (CYP2D6) pathway, and its pharmacokinetics and metabolism are characterized by two distinct activities of CYP2D6: active or poor metabolic capability (Witcher *et al*, 2003; Simpson and Plosker, 2004). The maximum plasma concentrations during treatment with atomoxetine at therapeutic doses ranged from ~0.7–4.8 μM in CYP2D6 active metabolizers (Witcher *et al*, 2003), whereas those in CYP2D6 poor metabolizers (~7% of the Caucasian population) were six-fold higher than those in CYP2D6 active metabolizers (Simpson and Plosker, 2004). Additionally, co-administration of the SSRI paroxetine, a potent inhibitor of CYP2D6, increased the plasma concentrations of atomoxetine by 3.5-fold, with a pharmacokinetic profile similar to CYP2D6 poor metabolizers (Belle *et al*, 2002), suggesting a significant increase in atomoxetine concentrations with concomitant treatment with CYP2D6 inhibitors. The maximum plasma concentrations of reboxetine at therapeutic doses in depressed patients ranged from 0.5 to 2.1 μM (Poggesi *et al*, 2000). Additionally, increases in doses of the NRIs are associated with increases in plasma concentrations (Öhman *et al*, 2001; Witcher *et al*, 2003), and the concentration in a fatal case of atomoxetine overdose was reported to be up to 32.5 μM (Garside *et al*, 2006). Recent studies using radiolabeled NRI ligands have indicated that NRIs are extensively distributed in most tissues (Kiyono *et al*, 2004, 2008; Kanegawa *et al*, 2006). Indeed, brain and heart levels of NRIs were ~4.7- to 6.5-fold and 9- to 12-fold higher for

atomoxetine (Kiyono *et al*, 2004) and ~15- to 16-fold and 21- to 32-fold higher for reboxetine than corresponding blood levels, respectively (Kanegawa *et al*, 2006; Kiyono *et al*, 2008). Therefore, brain and heart concentrations during treatment with therapeutic doses of atomoxetine and reboxetine, as well as after overdose, overlap with their effective concentrations in inhibiting brain- and cardiac-type GIRK channels (Figure 2). GIRK channels in the brain and heart may be inhibited by atomoxetine and reboxetine, particularly with the use of atomoxetine with CYP2D6 poor metabolizers or co-administration of CYP2D6 inhibitors. Inhibition of GIRK channels causes a depolarization of membrane potential, resulting in an increase in cell excitability (Kuzhikandathil and Oxford, 2002). GIRK channels have an important function in regulating neuronal excitability, synaptic transmission, and heart rate (Lüscher *et al*, 1997; Kovoor *et al*, 2001). Therefore, even partial inhibition of GIRK channels by atomoxetine and reboxetine may affect certain brain and heart functions.

Interestingly, GIRK2 knockout mice exhibit reduced anxiety-related behavior (Blednov *et al*, 2001). In clinical studies, reboxetine and atomoxetine were effective in the treatment of panic disorder and comorbid anxiety disorder, respectively (Versiani *et al*, 2002; Geller *et al*, 2007), suggesting their anxiolytic properties. Although their therapeutic effects are generally thought to be primarily attributable to inhibition of norepinephrine reuptake in the brain (Hajós *et al*, 2004; Simpson and Plosker, 2004), inhibition of GIRK channels may also contribute to improvement of anxiety symptoms.

GIRK2 knockout mice exhibit spontaneous seizures and are more susceptible to seizures induced by pentylenetetrazol than wild-type mice (Signorini *et al*, 1997). In animal studies using atomoxetine or reboxetine, convulsions were observed only at extremely high doses (Wong *et al*, 2000; Wernicke *et al*, 2007). The incidence of seizures during treatment with NRIs has been reportedly rare (Montgomery, 2005; Wernicke *et al*, 2007). Brain levels of the drugs in overdose cases may be considerably higher than levels during treatment at therapeutic doses (Poggesi *et al*, 2000; Kiyono *et al*, 2004, 2008; Garside *et al*, 2006; Kanegawa *et al*, 2006), suggesting that potent inhibition of neuronal GIRK channels by atomoxetine and reboxetine after overdose may contribute to increased seizure activity. However, the NRIs simultaneously increase extracellular levels of norepinephrine in the brain (Hajós *et al*, 2004; Simpson and Plosker, 2004), and norepinephrine has anticonvulsant effects (Ahern *et al*, 2006). The enhancement of norepinephrine by NRIs may be involved in the rare incidence of seizures. Although atomoxetine and reboxetine are generally well tolerated and have a benign side effect profile (Hajós *et al*, 2004; Simpson and Plosker, 2004), the inhibitory effects on GIRK channels may be partly related to the occurrence of other neurological side effects, such as insomnia and dizziness.

In the heart, GIRK channels cause slowing of heart rate in response to activation of M_2 muscarinic receptors through acetylcholine release from the stimulated vagus nerve (Kubo *et al*, 1993b; Krapivinsky *et al*, 1995). GIRK1 or GIRK4 knockout mice exhibit slightly elevated resting heart rates (Bettahi *et al*, 2002). Atomoxetine and reboxetine are associated with modest increases in heart rate (Hajós *et al*, 2004; Simpson and Plosker, 2004) and tachycardia in cases of toxicity (LoVecchio and Kashani, 2006). The binding affinities of atomoxetine and reboxetine for the muscarinic receptor are in the low micromolar range (Cusack *et al*, 1994; Wong *et al*, 2000; Hajós *et al*, 2004). Inhibition of norepinephrine reuptake enhances sympathetic nerve activity (Keller *et al*, 2004). The present results indicate that atomoxetine and reboxetine inhibit cardiac-type GIRK1/4 channels at clinically relevant heart concentrations. Altogether, an increase in heart rate during treatment with the drugs may be related to not only enhancement of sympathetic nerve activity and antagonism of the muscarinic receptor but also inhibition of atrial GIRK channels. Additionally, QT interval prolongation in two cases with atomoxetine overdose was reported (Barker *et al*, 2004; Sawant and Daviss, 2004). Recently, atomoxetine at micromolar concentrations was shown to inhibit cloned human *ether-a-go-go*-related gene (hERG) channels underlying rapidly activating delayed rectifier K^+ currents using the *Xenopus* oocyte expression assay (Scherer *et al*, 2009). Inhibition of delayed rectifier K^+ currents induces QT prolongation (Scherer *et al*, 2009), and QT prolongation after atomoxetine overdose may be related to inhibition of hERG channels but not GIRK channels among cardiac K^+ channels. Furthermore, GIRK4 knockout mice are resistant to atrial fibrillation caused by vagal stimulation without showing any changes in atrioventricular nodal function and ventricular arrhythmias (Kovoor *et al*, 2001). Tertiapine, a selective GIRK blocker in the heart, terminates atrial fibrillation, the most common arrhythmia (Hashimoto *et al*, 2006). Atomoxetine and reboxetine may therefore have an advantage in treating psychiatric patients with comorbid atrial fibrillation.

Atomoxetine was shown to reduce cumulative heavy drinking days in the treatment of psychiatric patients with comorbid alcohol use disorders (Wilens *et al*, 2008). Interestingly, GIRK2 knockout mice show reduced ethanol-induced conditioned taste aversion and conditioned place preference and are less sensitive than wild-type mice to some of the acute effects of ethanol, including anxiolysis, habituated locomotor stimulation, and acute handling-induced convulsions (Hill *et al*, 2003). In the present study, atomoxetine inhibited ethanol-induced GIRK1/2 currents, suggesting that it may suppress some GIRK-related effects of ethanol. Furthermore, GIRK knockout mice also show reduced cocaine self-administration (Morgan *et al*, 2003) and attenuation of the morphine withdrawal syndrome (Cruz *et al*, 2008). In the nervous system, GIRK channels are activated by μ-opioid and CB_1 cannabinoid receptors (North, 1989; Dascal, 1997; Kobayashi and Ikeda, 2006). Reboxetine and atomoxetine have also been shown to be useful in the treatment of cocaine dependence and marijuana users, respectively (Tirado *et al*, 2008; Szerman *et al*, 2005). Inhibition of GIRK channels by atomoxetine and reboxetine may have a role in the treatment of drug addiction.

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