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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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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.

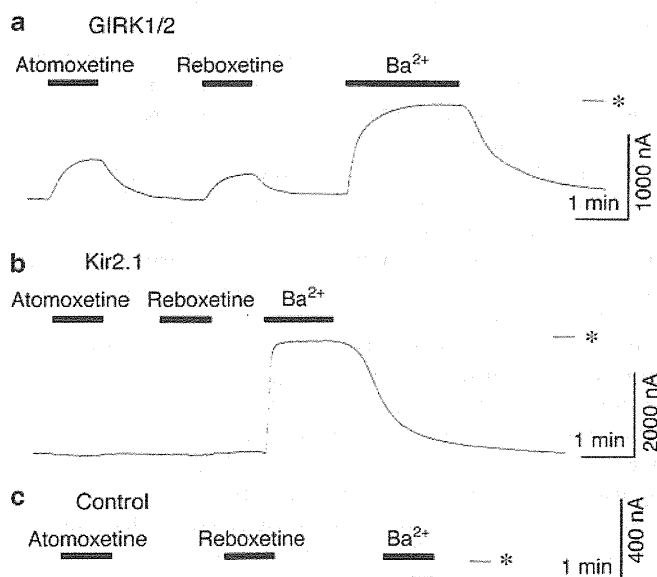

Figure 1 Inhibitory effects of atomoxetine and reboxetine on GIRK channels expressed in *Xenopus* oocytes. (a) In an oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 10 μM atomoxetine, 10 μM reboxetine, and 3 mM Ba^{2+} are shown. (b) In an oocyte injected with Kir2.1 mRNA, current responses 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

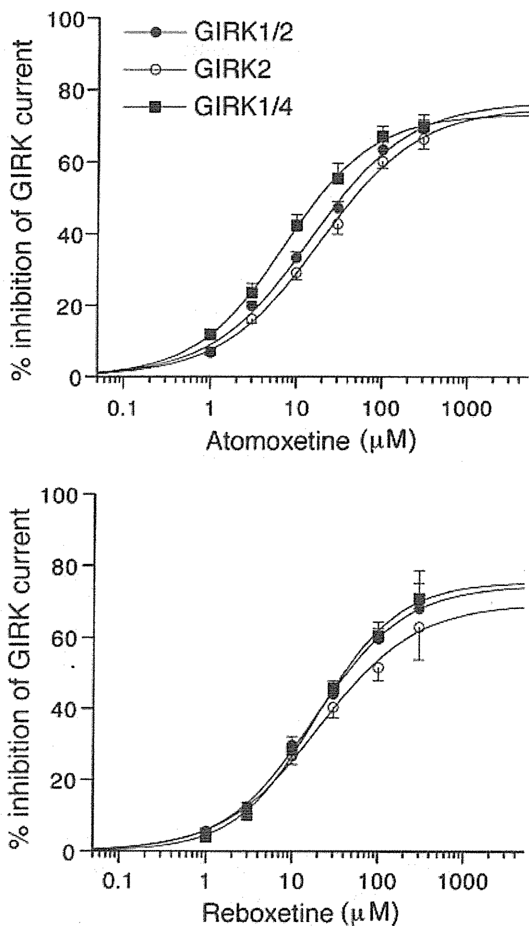

Figure 2 Concentration–response relationships for the inhibitory effects of atomoxetine and reboxetine on GIRK1/2, GIRK2, and GIRK1/4 channels. The magnitudes of inhibition of GIRK currents by the drugs were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing GIRK1/2, GIRK2, 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.

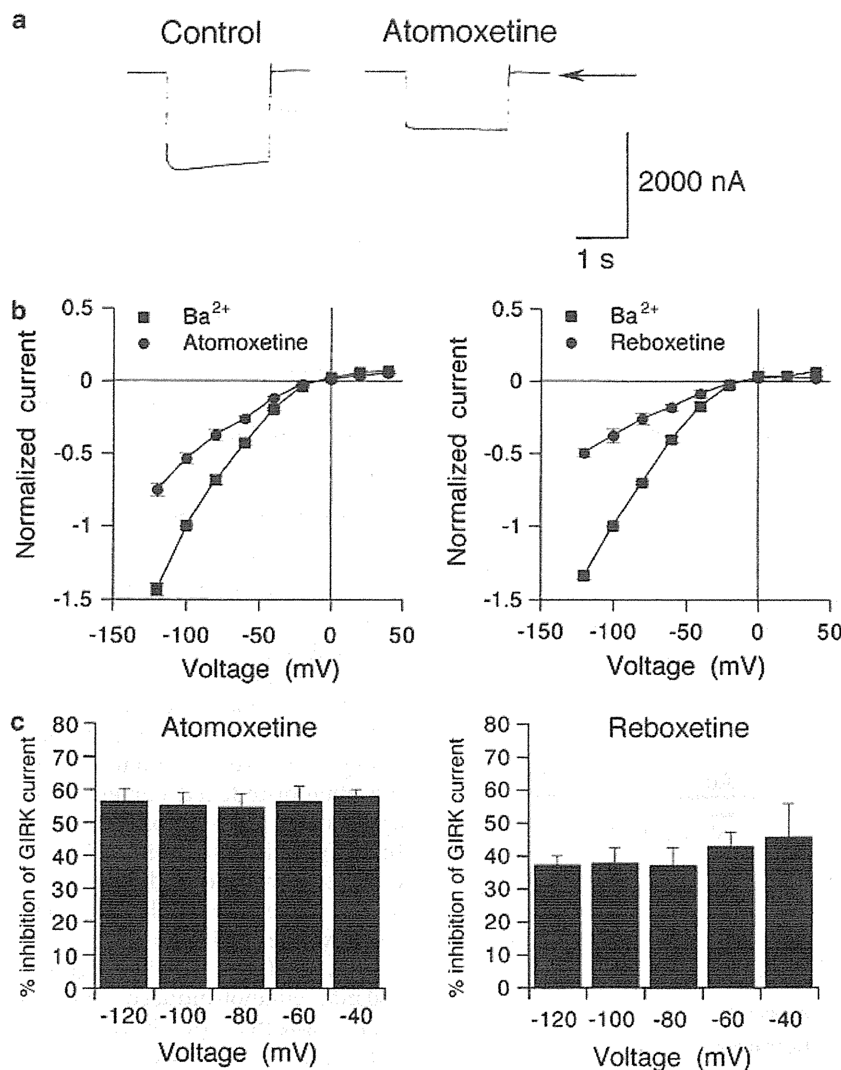

Figure 3 Characteristics of the inhibitory effects of atomoxetine and reboxetine on GIRK currents. (a) Representative GIRK1/2 currents elicited by a voltage step to −100 mV for 2 s from a holding potential of 0 mV in the presence or absence of 30 μM atomoxetine applied for 3 min. Current responses were 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

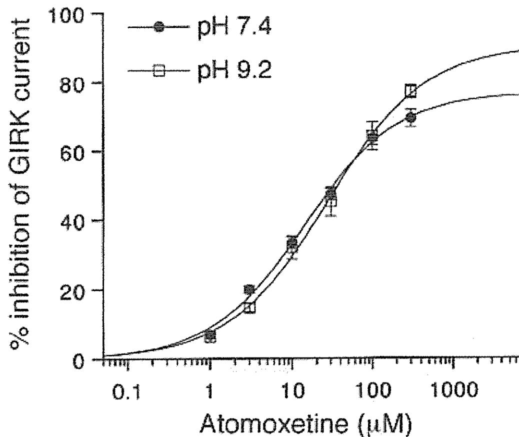

Figure 4 Concentration–response relationships for inhibition of GIRK channels by atomoxetine at different pH values. The magnitudes of inhibition of GIRK currents by atomoxetine were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing GIRK1/2 channels (1021.5 ± 100.8 nA, 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.

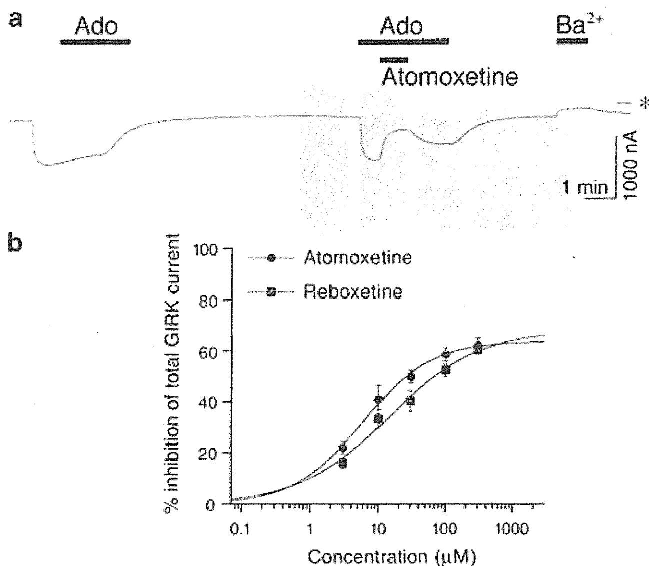

Figure 5 Effects of atomoxetine and reboxetine on GIRK channels activated by a G-protein-coupled receptor. (a) Current responses to 100 nM adenosine (Ado), 30 μM atomoxetine during application of 100 nM Ado, and 3 mM Ba^{2+} in an oocyte co-injected with mRNAs for GIRK1 and GIRK2 channels and the A_1 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

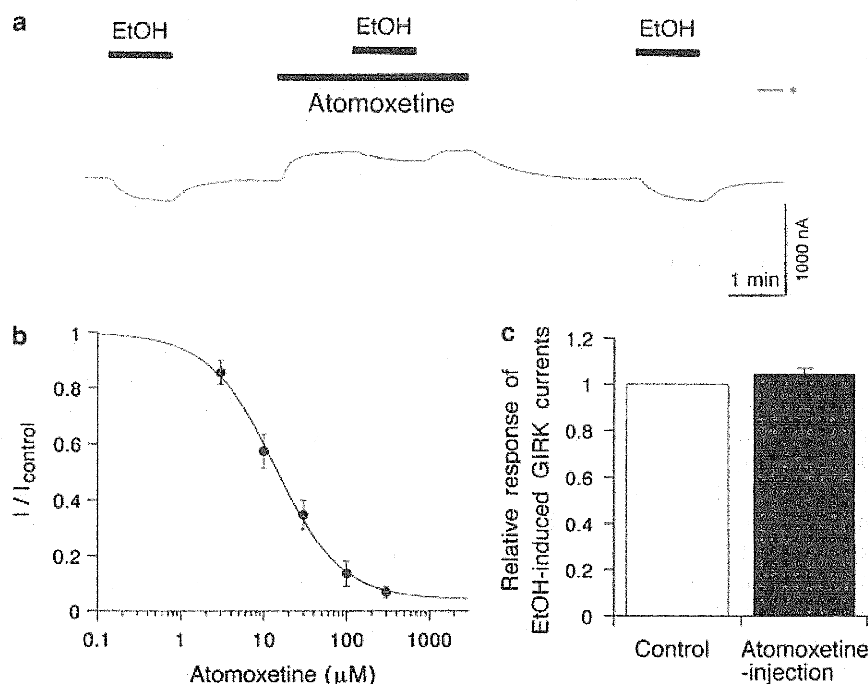

Figure 6 Effects of atomoxetine on ethanol-induced GIRK currents. (a) Current responses to 100 mM ethanol (EtOH), 100 mM EtOH in the presence of 30 μM atomoxetine, and 100 mM EtOH in an oocyte injected with GIRK1 and GIRK2 mRNAs. Asterisk indicates the zero current level. Bars show the duration of application. (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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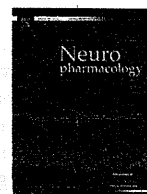

Reduced emotional and corticosterone responses to stress in μ-opioid receptor knockout mice

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Anxiety
Depression

ABSTRACT

The detailed mechanisms of emotional modulation in the nervous system by opioids remain to be elucidated, although the opioid system is well known to play important roles in the mechanisms of analgesia and drug dependence. In the present study, we conducted behavioral tests of anxiety and depression and measured corticosterone concentrations in both male and female μ-opioid receptor knockout (MOP-KO) mice to reveal the involvement of μ-opioid receptors in stress-induced emotional responses. MOP-KO mice entered more and spent more time in the open arms of the elevated plus maze compared with wild-type mice. MOP-KO mice also displayed significantly decreased immobility in a 15 min tail-suspension test compared with wild-type mice. Similarly, MOP-KO mice exhibited significantly decreased immobility on days 2, 3, and 4 in a 6 min forced swim test conducted for 5 consecutive days. The increase in plasma corticosterone concentration induced by tail-suspension, repeated forced swim, or restraint stress was reduced in MOP-KO mice compared with wild-type mice. Corticosterone levels were not different between wild-type and MOP-KO mice before stress exposure. In contrast, although female mice tended to exhibit fewer anxiety-like responses in the tail-suspension test in both genotypes, no significant gender differences were observed in stress-induced emotional responses. These results suggest that MOPs play an important facilitatory role in emotional responses to stress, including anxiety- and depression-like behavior and corticosterone levels.

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1. Introduction

Stress is hypothesized to be one of the triggering factors that causes mental illness, including anxiety and depression. Several brain areas are hypothesized to be involved in stress-induced emotional responses via corticosterone release by the hypothalamic-pituitary-adrenal (HPA) axis. Although several neurotransmitter systems, such as serotonin and catecholamines, have been hypothesized to be involved in these mechanisms, the precise molecular mechanisms are still unclear. Endogenous opioid peptides, such as endorphins, have been shown to modulate serotonergic and catecholaminergic neurotransmission (Chen et al., 2001; Hung et al., 2003; Ukai and Lin, 2002). Furthermore, pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in mice in a forced swim test (Amir, 1982). Chronic morphine facilitated immobility in a forced swim test (Molina et al., 1994). Opioids have also been reported to increase stress-related hormone levels (Mellon and Bayer, 1998). These previous reports indicate that the endogenous opioid system impacts behavioral responses to stress.

Opioid receptors have been classified into at least three subtypes, μ, δ, and κ (MOP, DOP, and KOP, respectively). Endomorphin-1 and -2, endogenous peptides that are selective for MOP, reportedly decreased immobility time in both the forced swim and tail-suspension tests (Fichna et al., 2007). A DOP selective agonist, SNC80, also decreased immobility time in a forced swim test (Broom et al., 2002). Furthermore, the KOP selective agonist U69593 increased, and the KOP selective antagonist nor-binaltorphimine decreased, immobility time in a forced swim test (Mague et al., 2003). Although three opioid receptor subtypes may be involved in stress-induced emotional responses, even the most selective ligands for a specific subtype (i.e., β-funaltrexamine for

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MOP, naltrindole for DOP, and nor-binaltorphimine for KOP) possess certain affinities for other subtypes (Newman et al., 2002) which may contribute to the discrepant findings about the role of opioid receptor subtypes in stress responses. Therefore, the precise molecular mechanisms underlying stress-induced emotional responses have not yet been clearly delineated by traditional pharmacological studies that use only selective ligands.

Recent success in developing knockout (KO) mice with MOP gene deletion has revealed the central role of MOPs, rather than other opioid receptor subtypes, in various opioid effects, including analgesia, reward, and tolerance (Ide et al., 2004; Kieffer, 1999; Loh et al., 1998; Sora et al., 2001, 1997). Although several compensatory changes might occur in KO animals, these animals have potential utility in investigating the *in vivo* roles of specific proteins. Opioid receptors have been shown to modulate responses to stress, including depression-like behavior (Filliol et al., 2000; McLaughlin et al., 2003). Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms underlying stress-induced emotional responses. Both the forced swim test (Porsolt et al., 1977) and tail-suspension test (Steru et al., 1985) have been widely used to assess depression-like behavior, with several modifications. Many reports using these two tests have shown that the inescapable stress of swimming or suspending a mouse by its tail can provide valuable information about emotional responses in stressful situations. The present study investigated the contributory role of the MOP in emotional responses to height, tail-suspension, repeated forced swim, and restraint stress using MOP-KO mice.

2. Materials and methods

2.1. Animals

The present study used wild-type and homozygous MOP-KO mouse littermates on a C57BL/6J genetic background (backcrossed at least 10 generations) as previously described (Sora et al., 2001). The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animal care and treatment were in accordance with our institutional animal experimentation guidelines. Naïve adult (>10 weeks old) male and female mice were group-housed in an animal facility maintained at 22 ± 2 °C and 55 ± 5% relative humidity under a 12 h/12 h light/dark cycle with lights on at 8:00 am and off at 8:00 pm. Food and water were available *ad libitum*. All behavioral tests and blood sample collections were conducted between 1:00 pm and 6:00 pm.

2.2. Elevated plus maze

The testing apparatus was a white plastic plus-shaped maze, elevated 80 cm from the floor. The maze consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 50 cm) without a roof. During testing, the time spent in the open arms and the number of entries into the open arms were recorded for 5 min. A mouse was considered to have entered an arm only if all four paws entered that arm.

2.3. Locomotor activity

Locomotor activity was assessed with an animal activity-monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co., Tokyo, Japan). Mice were placed individually in 30 × 45 × 30 cm plastic cages, to which they had not been previously exposed, under dim light and sound-attenuated conditions. Locomotor activity was monitored for 3 h.

2.4. Tail-suspension test

For tail-suspension testing, mice were suspended by their tail which was taped on a metal hook in test chambers (20 × 20 × 25 cm) constructed of white plastic walls and floor. Each hook was connected to a computerized strain gauge that was adjusted to detect animal movements (Tail-suspension System, Neuroscience Inc., Osaka, Japan). The total duration of immobility was measured for 15 min per day for 2 consecutive days.

2.5. Forced swim test

For forced swim testing, animals were forced to swim in a cylindrical Plexiglas tank (30 cm height × 30 cm diameter) containing 20 cm deep water for 6 min per day for 5 consecutive days. The water temperature was maintained at approximately 25 °C. Immobility time was recorded with an animal activity-monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co., Tokyo, Japan). After each session, the mice were immediately removed from the cylinder, dried with a towel, and kept under a heating lamp until completely dry, before being returned to their home cages.

2.6. Stress procedures and corticosterone enzyme immunoassay

After the 2 day tail-suspension test or 5 day forced swim test, blood samples (50 μl) were obtained from the tail vein. For restraint stress, mice were placed in a 50 ml conical centrifuge tube with multiple ventilation holes. Mice were restrained vertically in the tube for 12 h, followed by a 12 h rest with food and water available *ad libitum*. Mice were restrained again for 12 h, and then blood samples were obtained. All blood samples were immediately centrifuged for 20 min at 1000 × *g*. Plasma samples were stored at −80 °C until analysis. Plasma corticosterone levels were determined with a Corticosterone Enzyme Immunoassay Kit (Assay Design Inc., Ann Arbor, MI, USA).

2.7. Statistical analysis

Entry counts and time spent on the open arms of the elevated plus maze and stress-induced changes in plasma corticosterone concentrations were analyzed with Student's *t*-test. The results of other analyses were statistically evaluated with analysis of variance (ANOVA) followed by the Tukey–Kramer test. Values of $p < 0.05$ were considered statistically significant.

3. Results

We first assessed basal anxiety-like behavior of both mouse genotypes in the elevated plus maze (Fig. 1). Compared with wild-type mice, MOP-KO mice had significantly higher entry counts ($p < 0.05$, Student's *t*-test) and a longer time spent on the open arms ($p < 0.01$, Student's *t*-test) in both male and female mice. Although female mice tended to have more entry counts and more time spent in the open arms than male mice in both genotypes, no significant differences were observed.

When spontaneous locomotor activity of both wild-type and MOP-KO mice was analyzed (Fig. 2), MOP-KO mice displayed normal locomotor activity, similar to wild-types, during the 3 h test. A three-way, mixed-design ANOVA of spontaneous locomotor activity with two within-subjects factors (genotype and gender) showed no significant interactions (genotype: $F_{1,30} = 1.56$, $p = 0.221$; gender: $F_{1,30} = 0.08$, $p = 0.784$).

To test the influence of MOP-KO in stress-induced responses, immobility time in a 15 min tail-suspension test was analyzed every minute in wild-type and MOP-KO mice (Fig. 3). A three-way, mixed-design ANOVA of immobility time with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes in the tail-suspension test ($F_{1,22} = 6.92$, $p < 0.05$), although both genotypes showed time-dependent increases (Fig. 3a). The ANOVA also revealed that immobility time was not significantly different between male and female mice ($F_{1,22} = 3.01$, $p = 0.097$), although female mice tended to show less immobility than males. When the data of male and female mice were combined (Fig. 3b), significant differences were found in immobility time between genotypes ($F_{1,24} = 5.45$, $p < 0.05$, two-way, repeated-measures ANOVA). *Post hoc* tests revealed that MOP-KO mice had significantly less immobility time compared with wild-type mice from 7 to 9, 12 and 13 min after the tail-suspension test commenced. These differences in immobility time between wild-type and MOP-KO mice were not found during the second trial of the tail-suspension test on the next day (data not shown).

To test another type of stress stimulus, immobility time during the 6 min, 5-consecutive-day forced swim test was also analyzed in wild-type and MOP-KO mice (Fig. 4). Both genotypes and both male and female mice showed time-dependent increases in immobility time (Fig. 4a–d). Furthermore, immobility time during the 6 min forced swim test significantly increased, or tended to increase, in

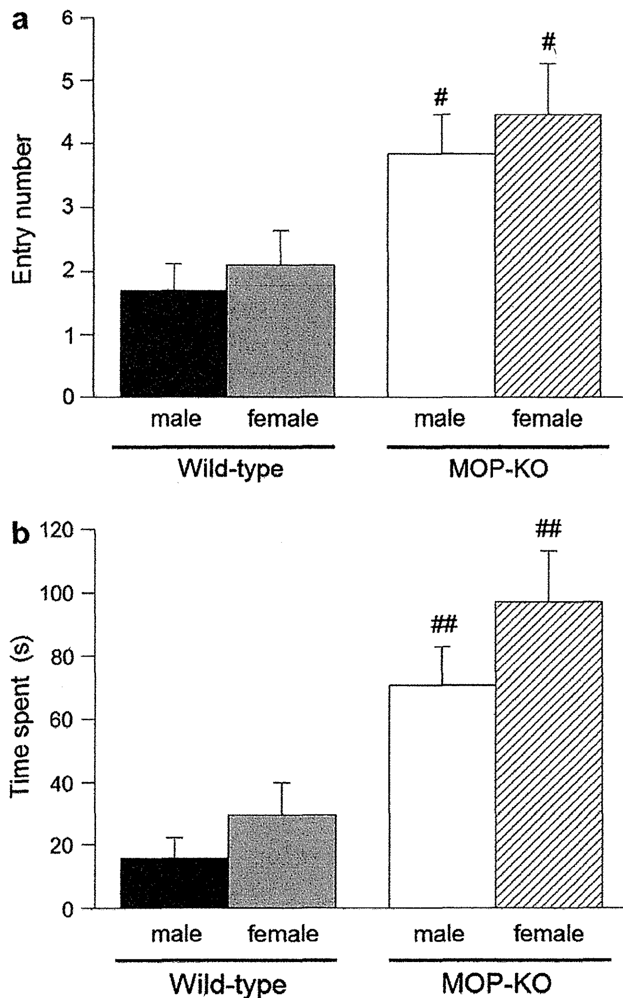


Fig. 1. Anxiety-like behavior in wild-type and MOP-KO mice in the elevated plus maze. The (a) number of entries and (b) time spent in the open arms of the elevated plus maze were measured for 5 min in wild-type mice (male, $n = 10$; female, $n = 9$) and MOP-KO mice (male, $n = 12$; female, $n = 13$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

a day-dependent manner (wild-type male mice: $F_{4,45} = 8.07$, $p < 0.001$; wild-type female mice: $F_{4,40} = 11.9$, $p < 0.001$; MOP-KO male mice: $F_{4,30} = 2.35$, $p = 0.077$; MOP-KO female mice: $F_{4,30} = 7.00$, $p < 0.001$; two-way, repeated-measures ANOVA). *Post hoc* comparisons revealed that immobility time on days 2–5 significantly increased compared with day 1 in both wild-type male and female mice ($p < 0.05$). Immobility time significantly increased on day 5 compared with day 1 in MOP-KO male mice and on days 4 and 5 compared with day 1 in MOP-KO female mice ($p < 0.05$). A three-way, mixed-design ANOVA of total immobility time during the 6 min tests on each of the 5 days with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes ($F_{1,29} = 10.9$, $p < 0.005$) but was not significantly different between genders ($F_{1,29} = 1.39$, $p = 0.248$) (Fig. 4e). Thus, when the male and female data were combined (Fig. 4f), MOP-KO mice showed significantly less immobility time compared with wild-type mice on days 2, 3, and 4.

We then analyzed stress-induced changes in plasma corticosterone concentrations in wild-type and MOP-KO mice (Fig. 5). The three types of stress significantly increased plasma corticosterone

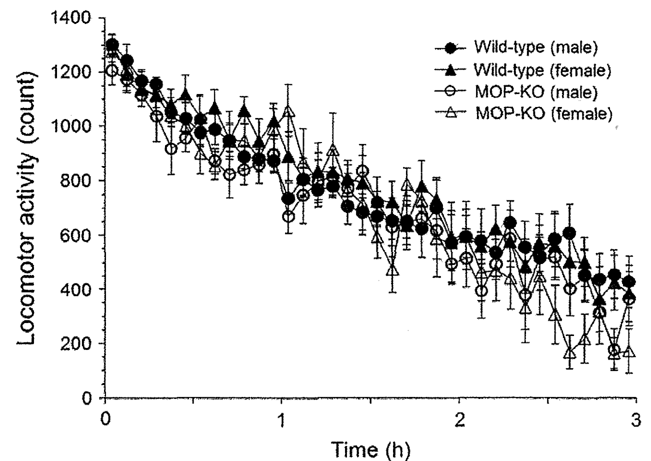
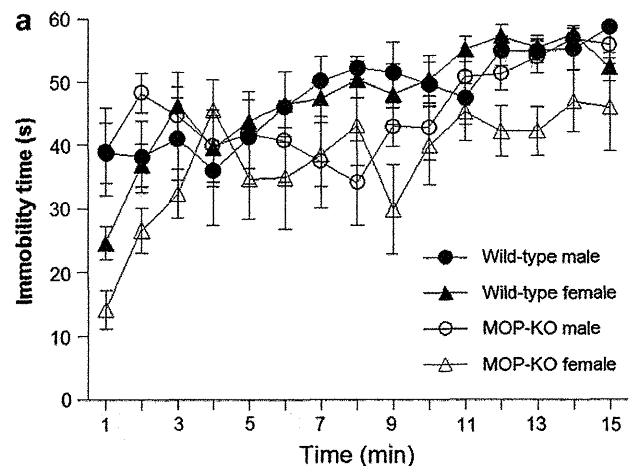


Fig. 2. Spontaneous locomotion in wild-type and MOP-KO mice. Spontaneous locomotion during 3 h habituation to a novel environment in wild-type mice (male, $n = 12$; female, $n = 9$) and MOP-KO mice (male, $n = 6$; female, $n = 7$). Each point represents the sum of 5 min locomotor activity. Data are expressed as mean ± SEM.



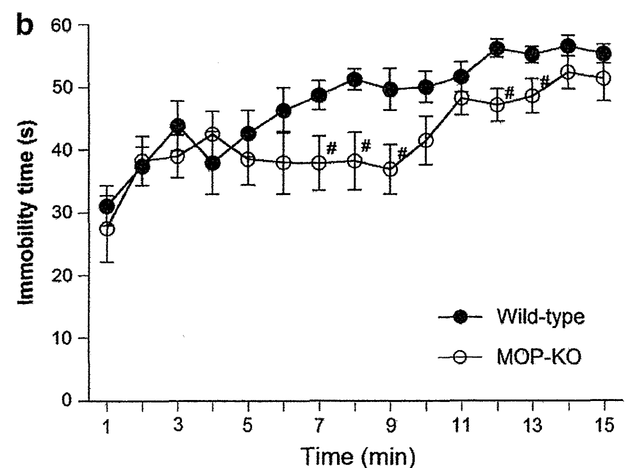


Fig. 3. Immobility in wild-type and MOP-KO mice in the 15 min tail-suspension test. (a) Immobility time was measured in wild-type mice (male, $n = 6$; female, $n = 7$) and MOP-KO mice (male, $n = 7$; female, $n = 6$). (b) Combined data of male and female mice in the 15 min tail-suspension test. $^{\#}p < 0.05$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

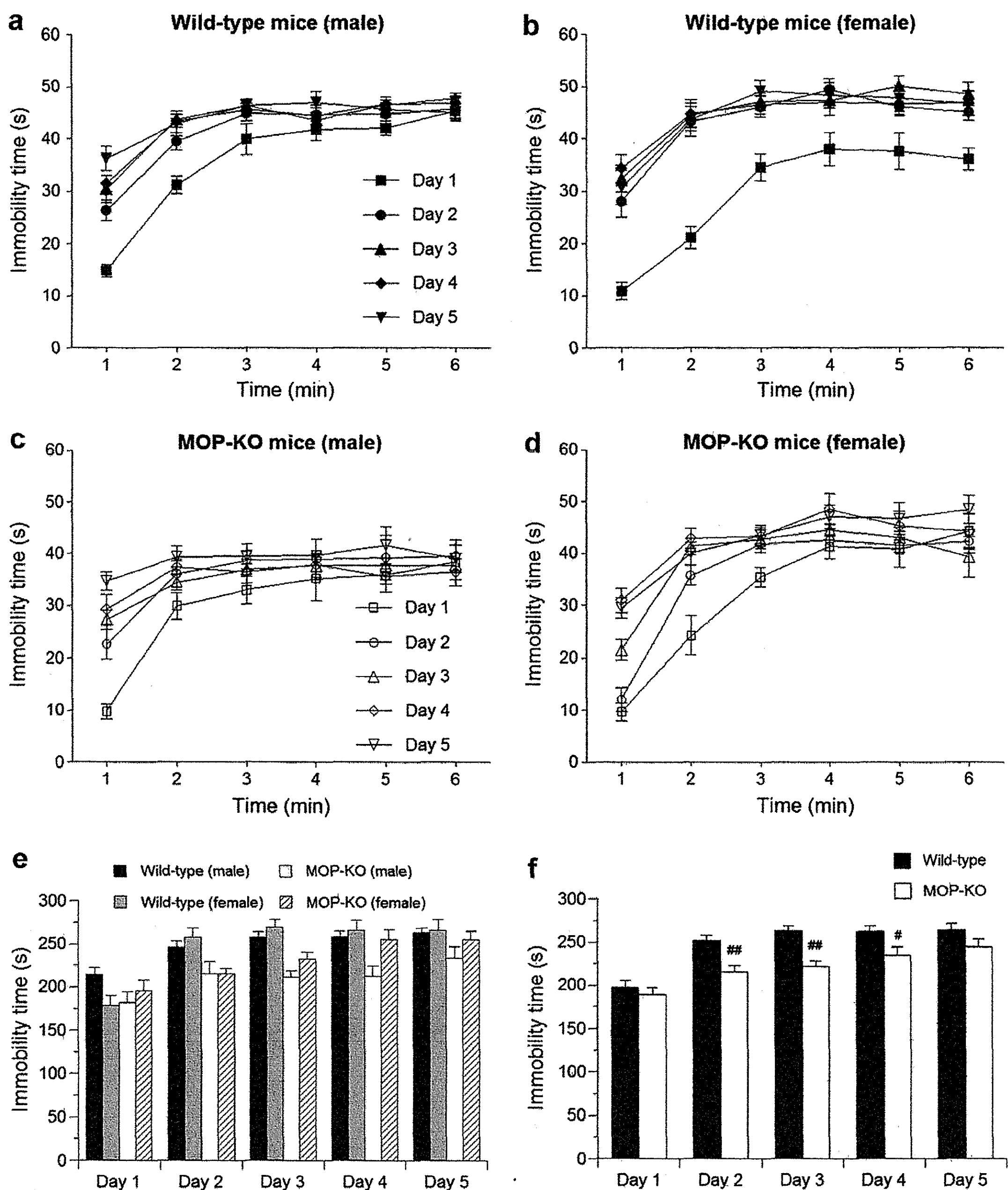


Fig. 4. Immobility in wild-type and MOP-KO mice in the 6 min, 5-consecutive-day forced swim test. Immobility time was measured in (a) wild-type male mice ($n = 10$), (b) wild-type female mice ($n = 9$), (c) MOP-KO male mice ($n = 7$), and (d) MOP-KO female mice ($n = 7$). (e) Sum of 6 min immobility time over 5 days. (f) Combined data of male and female mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

concentrations in both genotypes and in both male and female mice ($p < 0.05$, Student's t-test). Although no significant differences were observed in basal plasma corticosterone concentrations in naive mice, the stress-induced increases in plasma corticosterone concentrations were significantly different ($p < 0.05$, Student's t-test), or tended to be significantly different (restraint stress in female mice: $p = 0.065$, Student's t-test), between genotypes in both male and female mice. Both male and female MOP-KO mice had significantly lower plasma corticosterone concentrations compared with wild-type mice after the stress procedures. Although female mice tended to have slightly higher corticosterone concentrations than male mice (i.e., naive or after tail-suspension or restraint stress), no significant differences were observed (Student's t-test). Contrary to these findings, female mice tended to exhibit lower corticosterone concentrations than male mice after forced swim stress in both genotypes, although no significant differences were observed (Student's t-test).

4. Discussion

In the present study, MOP-KO mice displayed significantly decreased immobility time in both the tail-suspension and repeated forced swim tests and significantly reduced stress-induced increases in plasma corticosterone concentrations compared with wild-type mice. Moreover, MOP-KO mice also entered more, and spent more time in, the open arms of the

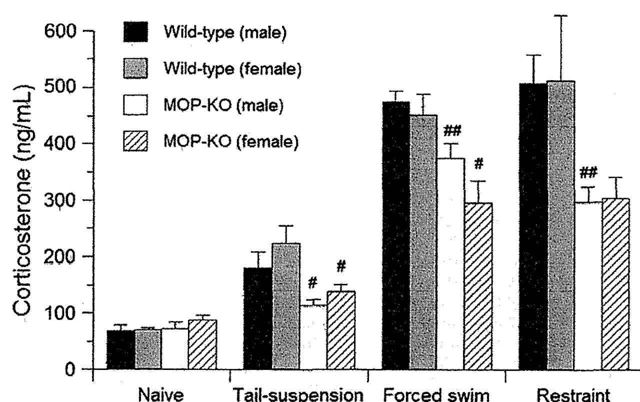


Fig. 5. Stress-induced increase in plasma corticosterone concentrations in wild-type and MOP-KO mice. Plasma corticosterone levels were analyzed (i) in naive wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (ii) after the 2 day tail-suspension test in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (iii) after the 5 day forced swim test in wild-type mice (male, $n = 10$; female, $n = 8$) and MOP-KO mice (male, $n = 7$; female, $n = 7$), and (iv) after restraint stress in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

elevated plus maze. These results suggest that MOP-KO mice are resistant to stress exposure and exhibit fewer stress-induced emotional responses (i.e., anxiety- and depression-like behaviors) compared with wild-type mice, although the influences of other factors (e.g., response to novelty) should be considered in future studies.

No significant differences were observed in locomotor activity between wild-type and MOP-KO mice, although MOP-KO mice exhibited a slight tendency toward decreased locomotion. These results indicate that the present behavioral effects in MOP-KO mice were not attributable to variations in locomotor activity. MOP-KO mice entered more, and spent more time in, the open arms of the elevated plus maze in the present study. Similar results have been reported with another MOP-KO mouse strain in both the elevated plus maze test and light–dark box test (Filliol et al., 2000). This anxiolytic-like state of MOP-KO mice is consistent with a previous report in which the MOP-selective agonist DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) induced anxiogenic-like activity in the elevated plus maze (Calenco-Choukroun et al., 1991). In contrast, several contradictory studies have reported an anxiolytic-like effect of morphine and MOP agonists (Asakawa et al., 1998; Koks et al., 1999). One of the reasons for this discrepancy using MOP-selective ligands might involve other opioid receptor subtypes. The most selective ligands for a specific opioid receptor subtype possess certain affinities for other subtypes (Newman et al., 2002). Although further studies using our and other MOP-KO mouse strains in various paradigms to assess anxiety-like responses (e.g., open field test) might be needed, the present results suggest that MOPs are involved in anxiety-like responses to height stress.

The decrease in immobility time in MOP-KO mice compared with wild-type mice in both the tail-suspension and repeated forced swim tests is consistent with previous reports. The decrease in immobility time in the forced swim test has been reported using another MOP-KO mouse strain (Filliol et al., 2000). These results suggest that MOP activation facilitates stress-induced, depression-like behavioral responses. Additionally, Fichna et al. (2007) reported contradictory findings in which intracerebroventricular treatment with endomorphin-1 and -2, endogenous MOP-selective peptides, decreased immobility time in both the forced swim and tail-suspension tests. Codeine, a relatively weak MOP agonist, also decreased immobility time in tail-suspension tests in mice (Berrocoso and Mico, in press). Although these reports might suggest that the MOP modulates depression-like behavior in contrast to our present results, other reports are consistent with our results. Chronic morphine facilitated immobility time in a rat forced swim test (Molina et al., 1994). Pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in a forced swim test in mice (Amir, 1982). Furthermore, intraperitoneal treatment of morphine enhanced immobility time in rats in a naloxone-sensitive manner (Zurita and Molina, 1999). These discrepant results might be attributable to differences in animals, mouse strains, time course, injection route, or other experimental conditions. Notably, different mouse strains have exhibited differential responses in forced swim tests (David et al., 2003). Further studies may reveal the reasons for these discrepant results.

To study the involvement of the MOP in emotional responses to repeated stress, the present study used both the 6 min forced swim test conducted for 5 consecutive days and the 15 min tail-suspension test conducted for 2 consecutive days, two regimens which were modified from typically used procedures in mice (Porsolt et al., 1977; Steru et al., 1985). When we analyzed immobility time from day 1 at 3–6 min in the forced swim test (excluding the data from the first 2 min), no significant differences were found between wild-type and MOP-KO mice. Additionally, no significant differences in immobility time were observed from day 1 for the first 6 min between wild-type and MOP-KO mice in the tail-suspension test. Although standard procedures for the analysis of depression-like behavior did not reveal significant differences, MOP-KO mice showed significant differences in depression-like behavior after repeated or longer stress exposure in the forced swim and tail-suspension tests. Our present results might suggest that MOPs facilitate emotional responses to repeated or longer stress exposure. In the present procedures, MOP-KO mice exhibited significantly decreased immobility time in the repeated forced swim test only on days 2, 3, and 4, and they only showed a tendency toward decreased immobility on day 5. In the tail-suspension test, MOP-KO mice had significantly decreased immobility time only after the first 5 min from the beginning of the test during the first trial, and no significant differences were observed during the second trial. Interestingly, the increase in plasma corticosterone concentrations in MOP-KO mice was still significantly lower than wild-type mice after the differences in behaviors between wild-type and MOP-KO mice in both tests disappeared. MOPs may facilitate the early behavioral responses to stress but are not necessary to fully express the behavioral responses after chronic stress procedures. Other neuronal systems might regulate the expression of stress-induced behavioral responses, and MOPs might facilitate this regulation.

At the hormonal level, one of the major responses to stress is an increase in corticosterone secretion caused by stimulation of the HPA axis. In the present study, plasma corticosterone concentration significantly increased after stress exposure in both wild-type and MOP-KO mice. The increased corticosterone levels after both forced swim and restraint stress were higher than after the tail-suspension test. This finding might be attributable to differences in the intensity of the stressors, although variations in the duration and frequency of these stressors might modify these levels. Additionally, the stress-induced increases in plasma corticosterone concentration were less in MOP-KO mice compared with wild-type mice. Our present results are consistent with previous reports. Endogenous opioids have been reported to have facilitatory effects on the HPA axis (Douglas et al., 1998). The increase in plasma corticosterone levels by morphine indicated activation of the HPA axis by MOP (Coventry et al., 2001; Ignar and Kuhn, 1990). In a different MOP-KO mouse strain, morphine- and restraint

stress-induced increases in plasma corticosterone levels were also reduced (Roy et al., 2001; Wang et al., 2002). Stress is well known to activate the HPA axis and increase norepinephrine release in the locus coeruleus. Moreover, stress-induced norepinephrine release in the locus coeruleus is partially regulated by both opioid and noradrenergic mechanisms (Nakai et al., 2002; Nestler et al., 1999; Valentino and Van Bockstaele, 2001), suggesting that MOPs may be involved in the activation of the HPA axis and locus coeruleus.

Knockout animals may be hypothesized to have potential utility in investigating the *in vivo* roles of specific proteins. Previous reports using gene mutant mice suggest that MOPs play an important role in various effects of opioids, such as antinociception, tolerance, reward, and locomotion (Ide et al., 2004; Matthes et al., 1996; Sora et al., 2001, 1997). Our present results also demonstrated the involvement of MOPs in stress-induced emotional responses. However, although no differences in DOP and KOP expression were evident in MOP-KO mice in the present study (Sora et al., 1997), several compensatory changes might occur in MOP-KO mice. These possible compensatory changes, especially with regard to neurotransmitter release and hormonal valence, could elicit changes in stress-induced emotional responses. Future studies, such as behavioral analyses using MOP-KO mice with viral expression of MOPs, may reveal the influences of compensatory changes in stress-induced emotional responses.

Gender differences in emotional responses may also exist (Toufexis, 2007; Toufexis et al., 2006). In the present study, several differences were found between male and female mice in stress-induced emotional responses, although these differences were not significant. In the elevated plus maze, female mice showed less anxiety-like behavior than male mice of both genotypes. These results are consistent with previous reports using rodents (Fernandes et al., 1999; Steenbergen et al., 1990) and suggest the presence of gender differences in anxiety-like behavior. However, no differences in immobility time were found between male and female wild-type mice in either the tail-suspension or forced swim tests. A previous report found that male and female C57BL/6J mice, the genetic background strain used in the present study, exhibited no differences in immobility time in either the tail-suspension or forced swim tests (Caldarone et al., 2003). Interestingly, female MOP-KO mice tended to exhibit less immobility in the tail-suspension test and more immobility in the forced swim test compared with male MOP-KO mice. Although the present study found no significant differences between genders, and additional studies may be required, MOPs may differentially modulate depression-like responses in both tests, especially in female mice.

In conclusion, we found decreased anxiety-like behavior in the elevated plus maze, decreased immobility in both the tail-suspension and forced swim tests, and reduced stress-induced plasma corticosterone concentrations in MOP-KO mice compared with wild-type mice. These results suggest that MOPs play an important facilitatory role in stress sensitivity and/or stress-induced emotional responses, including anxiety- and depression-like responses.

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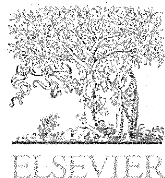

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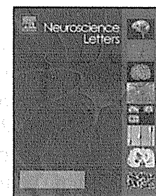

Association between 5-hydroxytryptamine 2A receptor gene polymorphism and postoperative analgesic requirements after major abdominal surgery

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ABSTRACT

Although the serotonin (5-hydroxytryptamine (5-HT)) 2A receptor has been reported to be associated with pain, no relationship has been found between single nucleotide polymorphisms in the 5-HT2A receptor gene and analgesic requirements. To clarify the mechanism of individual differences in analgesic requirements, we investigated the relationship between the 5-HT2A 102T/C gene polymorphism and analgesic requirements in 135 patients who underwent major open abdominal surgery and were managed with continuous epidural analgesia with opioids after surgery. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism. We found that the 102T/C polymorphism had significant main effects with regard to analgesic requirements. In addition, significant interaction effects were found between the 102T/C polymorphism and sex in terms of analgesic requirements. Among female subjects, patients with the T/T genotype of the 102T/C polymorphism had more analgesic requirements than those with the other genotypes. This finding suggests that the linkage disequilibrium block, which includes the 102T/C polymorphism of the 5-HT2A receptor gene, is involved in individual differences in analgesic requirements in women.

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Postoperative pain can contribute to various problems: atelectasis, hypoxemia, ileus, and circulatory and metabolic dysfunctions including a marked increase in cardiac output, blood pressure, metabolism, and oxygen consumption [4]. Although patient-controlled analgesia is widely used, the optimal method for management of postoperative analgesia has not yet been established [9].

Nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids are used as analgesics in postoperative pain treatment. The dose of opioid differs with the age and sex of the patient, operative procedure, previous opioid requirement, etc. [11]. Because opioids are known to cause severe adverse effects (e.g., nausea, vomiting, and respiratory depression), they must be used with close monitoring. In addition, sensitivity to opioid analgesics is known to differ among individuals [11].

Tissue injury as a result of the operation causes inflammation and the production and release of algesic substances (e.g., bradykinin, substance P, and serotonin (5-hydroxytryptamine: 5-HT)) and prostaglandin, which enhances this action. As a result, nociceptors are excited, and the patient experiences postoperative pain [11]. The relationship between the receptor subtypes of 5-HT and pain has been studied. For example, Abbott et al. reported that pain response was blocked by pretreatment with the 5-HT2A/2C antagonists ketanserin and ritanserin and the 5-HT2A antagonist spiperone [1]. Tokunaga et al. also reported that pretreatment with the 5-HT2A receptor antagonist ketanserin attenuated the behavioral response normally observed after the injection of 5-HT [23]. From these results, it is thought that the pain is caused by mediation of the 5-HT2A receptor activation.

The 5-HT2A receptor gene consists of three exons spanning approximately 63 kb and is located in 13q14–21. Several polymorphisms in this gene have been reported. In particular, the −1438A/G

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single nucleotide polymorphism (SNP) in the promoter region and 102T/C SNP in exon 1 show strong linkage disequilibrium (LD) [12]. Furthermore, many association studies with psychiatric disorders have been reported to date [2,5,16,22,25]. Some studies on whether 5-HT2A receptor gene polymorphisms influence pain suggest that among patients with irritable bowel syndrome (IBS) or fibromyalgia, individuals with the T/T genotype of 102T/C have a lower pain threshold than those with the T/C and C/C genotypes [3,7,17]. Since it has been reported that the expression of the C allele of 102T/C in the temporal cortex of healthy individuals was lower than the expression of the T allele at both the mRNA and the protein levels [18], it is possible that this polymorphism is responsible for the individual differences in pain sensitivity and influences analgesic requirements. However, no relationship has been reported between the SNPs in the 5-HT2A receptor gene and analgesic requirements. The present study was undertaken to clarify the mechanism that causes individual differences in analgesic requirements.

In this study, to elucidate whether the SNP in the 5-HT2A receptor gene influences analgesic requirements, we examined the relationship between genetic variations in the 5-HT2A receptor gene and individual differences in analgesic requirements.

The study protocol was approved by the institutional review boards at the Institute of Medical Science, The University of Tokyo (Tokyo, Japan), Toho University Sakura Medical Center (Chiba, Japan), the Tokyo Institute of Psychiatry (Tokyo, Japan), and Azabu University (Kanagawa, Japan).

The study subjects were 135 patients who underwent major open abdominal surgery with combined general and epidural anesthesia at Research Hospital, Institute of Medical Science, The University of Tokyo, or at Toho University Sakura Medical Center (78 men and 57 women; mean age ± standard deviation (SD), 65.1 ± 8.6 years and 62.3 ± 10.7 years, respectively). The surgical procedures were mostly gastrectomy for gastric cancer and colectomy for colorectal cancer. We obtained written informed consent from all subjects. Postoperative pain was managed primarily with continuous epidural anesthesia with fentanyl or morphine. Fentanyl or morphine was diluted with 0.25% bupivacaine in a total volume of 100 ml and infused at a constant rate of 2 ml/h through a catheter placed in the lower thoracic or upper lumbar epidural space. In case a patient complained of significant postoperative pain despite the administration of continuous epidural analgesic, appropriate doses of opioids, including morphine, buprenorphine, pentazocine, and pethidine, and/or NSAIDs, including diclofenac and flurbiprofen, were systemically administered as rescue analgesics at the discretion of the surgeons. In addition, some NSAIDs were used as antipyretics. To allow intersubject comparisons of rescue analgesic doses required during the first 24-h postoperative period, the doses of opioids and NSAIDs administered as rescue analgesics during this period were converted to the equivalent dose of systemic pentazocine [8,15]. The study subjects were also asked to rate their pain intensity at rest during the first 24-h postoperative period using a 5-point verbal numerical rating scale (NRS pain score: 0 = no pain, 1 = mild pain, 2 = moderate pain, 3 = severe pain, 4 = extremely severe pain). Peripheral blood or oral mucosa samples were collected from these subjects for gene analysis, and we performed extraction and purification of genomic DNA by the phenol/chloroform method.

The indexes of analgesic requirements were as follows: the frequency of use of rescue analgesics during the first 24-h postoperative period (RA-24 h), the total frequency of use of rescue analgesics and antipyretics during the first 24-h postoperative period (TRA-24 h), values of the total dose of the administered rescue analgesics and antipyretics converted into the equivalent dose of systemic pentazocine (TP-with antipyretics), and values of the total dose of the administered rescue analgesics (excluding antipyretics) converted into the equivalent dose of systemic pentazocine (TP-without antipyretics).

To evaluate the effect of operative procedures after analysis of the association between the SNP and indexes of analgesic requirements, the patients were divided into (1) those who underwent gastrectomy and (2) those who underwent enterectomy. We only included patients for who underwent operative procedures involving the same degree of postoperative pain; thus, patients who underwent minimally invasive surgical procedures were excluded because their degree of pain was extremely different from that of most of the other patients. In addition, we excluded patients who underwent multiorgan surgery (e.g., gastrectomy and colectomy, gastrectomy and splenectomy).

The 102T/C polymorphism of the 5-HT2A receptor gene was genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) according to the methods reported by Warren et al. [24]. After amplifying 372-bp fragments that included position 102T/C, the PCR products were digested with MspI. The genotype was identified by gel electrophoresis with 2% agarose gel.

The χ^2 test was used to assess compliance with the Hardy–Weinberg equilibrium (HWE). Analysis of covariance (ANCOVA) was performed to examine the contribution of the SNP to the indexes of analgesic requirements using SPSS 12.0J for Windows (SPSS Japan Inc., Tokyo, Japan). In the analyses, SNP and sex were independent variables; the indexes of analgesic requirements and NRS pain scores were dependent variables; and age, height, and weight were covariates. Bonferroni multiple comparisons were used as *post hoc* tests. When the variables showed significant main effect and/or interaction effect according to the results of two-way ANCOVA, they were subjected to multiple comparisons using *post hoc* Bonferroni correction wherein the mean values, adjusted for age, height, and weight, were compared among different genotypes with the 120T/C polymorphism. Pearson's correlation coefficient (r) was calculated to examine the correlation between the indexes of analgesic requirements and NRS pain scores. Student's/Welch's t test was used to evaluate the effect of operative procedures. Statistical significance was defined as $p < 0.05$.

Genotype and allele frequencies of the 102T/C gene polymorphism were as follows: T/T, 44 (32.6%); T/C, 66 (48.9%); C/C, 25 (18.5%); T allele, 57.0%; C allele, 43.0%. The genotype distribution of polymorphism in the total subjects was in agreement with the HWE (χ^2 (1) = 0.000, p = 1.000, with Yates' continuity correction).

The means (± standard error (SE)) of the indexes of analgesic requirements by the genotypes of the 102T/C polymorphism are shown in Table 1. The results of two-way ANCOVA with age, height, and weight as covariates showed significant main effects of the 102T/C polymorphism for all indexes of analgesic requirements: RA-24 h, TRA-24 h, TP-with antipyretics, and TP-without antipyretics ($F(2,126)$ = 5.352, p = 0.006; $F(2,126)$ = 5.793, p = 0.004; $F(2,126)$ = 4.840, p = 0.009; $F(2,126)$ = 4.861, p = 0.009, respectively). However, three of the main effects in the indexes of analgesic requirements (i.e., RA-24 h, TP-with antipyretics, and TP-without antipyretics) were qualified by significant interaction effect between the 102T/C polymorphism and sex ($F(2,126)$ = 5.611, p = 0.005; $F(2,126)$ = 3.905, p = 0.023; and $F(2,126)$ = 5.192, p = 0.007, respectively). For these three variables, the significant simple main effects were found among genotypes in women ($F(2,51)$ = 6.160, p = 0.004; $F(2,51)$ = 4.873, p = 0.012; and $F(2,51)$ = 5.924, p = 0.005, respectively). *Post hoc* comparisons showed that the women with the T/T genotype had a significantly greater analgesic requirement than women with the T/C and C/C genotypes (Table 1). On the other hand, with regard to the TRA-24 h index, no interaction effect was observed ($F(2,126)$ = 2.589, p = 0.079), and *post hoc* comparisons showed that individuals with the T/T genotype had a significantly

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Table 1
Mean (±SE) of the indexes of analgesic requirements by genotypes of the 102T/C polymorphism.

Subjects	Genotype	RA-24 h	TRA-24 h	TP-with antipyretics	TP-without antipyretics
Total population	T/T (n=44)	1.023 ± 0.22	1.341 ± 0.25[b]	19.261 ± 4.35	17.386 ± 4.33
	T/C (n=66)	0.561 ± 0.10	0.697 ± 0.12	10.341 ± 1.75	8.977 ± 1.62
	C/C (n=25)	0.560 ± 0.13	0.840 ± 0.16	12.600 ± 3.14	9.900 ± 2.93
Male subjects	T/T (n=28)	0.679 ± 0.16	1.107 ± 0.19	14.464 ± 3.36	11.786 ± 3.42
	T/C (n=36)	0.583 ± 0.15	0.750 ± 0.18	11.458 ± 2.68	10.208 ± 2.52
	C/C (n=14)	0.786 ± 0.19	1.000 ± 0.21	16.071 ± 4.77	14.464 ± 4.48
Female subjects	T/T (n=16)	1.625 ± 0.52[a]	1.750 ± 0.60	27.656 ± 10.31[c]	27.188 ± 10.06[d]
	T/C (n=30)	0.533 ± 0.12	0.633 ± 0.13	9.000 ± 2.14	7.500 ± 1.90
	C/C (n=11)	0.273 ± 0.14	0.636 ± 0.24	8.182 ± 3.57	4.091 ± 2.74

RA-24 h: The frequency of use of rescue analgesics during the first 24-h postoperative period.
TRA-24 h: The total frequency of use of rescue analgesics and antipyretics during the first 24-h postoperative period.
TP-with antipyretics: the values of total dose of the administered rescue analgesics and antipyretics converted into the equivalent dose of systemic pentazocine.
TP-without antipyretics: the values of total dose of the administered rescue analgesics (excluding antipyretics) converted into the equivalent dose of systemic pentazocine.
The p values were corrected by Bonferroni's method.
[a] Significantly different between the T/T genotype and the T/C genotype (p=0.011) and between the T/T genotype and the C/C genotype in female patients (p=0.009).
[b] Significantly different between the T/T genotype and the T/C genotype in the total study population (p=0.003).
[c] Significantly different between the T/T genotype and the T/C genotype (p=0.016) and between the T/T genotype and the C/C genotype in the female subjects (p=0.044).
[d] Significantly different between the T/T genotype and the T/C genotype (p=0.010) and between the T/T genotype and the C/C genotype in the female subjects (p=0.014).

greater analgesic requirement than those with the T/C genotype (p=0.003).

The mean (±SD) of the NRS pain scores in the total study population, men, and women was as follows: total, 1.59 ± 1.28; male, 1.55 ± 1.20; female, 1.64 ± 1.40. Significant association was not observed between the SNP and NRS pain scores. However, significant positive correlations were shown between the indexes of analgesic requirements and NRS pain scores (RA-24 h, r=0.270, p=0.004; TRA-24 h, r=0.214, p=0.025; TP-with antipyretics, r=0.213, p=0.025; TP-without antipyretics, r=0.238, p=0.012).

The indexes of analgesic requirements by the operative procedures and genotypes of the 102T/C polymorphism are shown in Fig. 1. The results of the t test showed marginally significant differences in RA-24 h and TRA-24 h ($t(99)$=1.794, p=0.076 and $t(99)$=1.928, p=0.057, respectively), and showed significant differences in TP-with antipyretics and TP-without antipyretics ($t(76)$=2.930, p=0.014 and $t(78)$=2.569, p=0.012, respectively). To examine the influence of the SNP in each operative procedure, we performed two-way ANCOVA for TP-with antipyretics and TP-without antipyretics. The results showed significant main effects of the 102T/C polymorphism in the gastrectomy group (TP-with antipyretics, $F(2,44)$=5.435, p=0.008; TP-without antipyretics, $F(2,44)$=5.938, p=0.005). *Post hoc* comparisons showed that patients with the T/T genotype had a significantly greater analgesic requirement than those with the T/C (TP-with antipyretics, p=0.008; TP-without antipyretics, p=0.006) and C/C genotypes (TP-with antipyretics, p=0.097; TP-without antipyretics, p=0.052). However, no significant difference observed in the enterectomy group (Fig. 1).

We examined the relationship between 102T/C polymorphism in the 5-HT2A receptor gene and individual differences in analgesic requirements. The results suggest that individuals with the T/T genotype have greater analgesic requirements after major abdominal surgery than those with the other genotypes, but this result was seen only in women. In other words, it appears that females with the T/T genotype might have a lower pain threshold or analgesic sensitivity than those with the T/C and C/C genotypes. Only TRA-24 h, 1 of the 4 indexes of analgesic requirements, did not show a significant interaction effect between the 102T/C polymorphism and sex ($F(2,126)$=2.589, p=0.079). However, the tendency of this p value was low (Table 1), and women with the T/T genotype tended to have greater analgesic requirements than those with the other genotypes.

These data support the results of previous studies that pain sensitivity was significantly higher in patients with IBS or fibromyalgia who had the T/T genotype [3,7,17]. Our result has shown the possibility that the LD block, which includes the 102T/C polymorphism in the 5-HT2A receptor gene, influences pain sensitivity and causes individual differences in analgesic requirements after major abdominal surgery. According to HapMap Data Release 27, other polymorphisms also (e.g., rs6311 and rs9534511) in the LD block, which includes the 102T/C polymorphism, may be involved in the pain threshold or analgesic sensitivity. However, because it was reported that this SNP changes the expression of mRNA

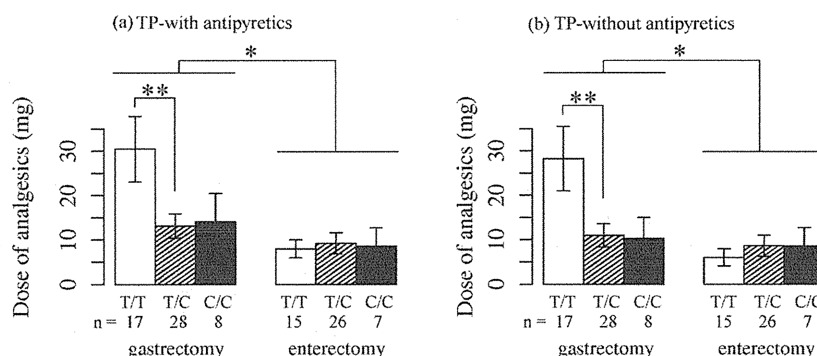

Fig. 1. Indexes of analgesic requirements by operative procedure. *Significantly greater dose of analgesic administration for the patients who underwent gastrectomy compared with those who underwent enterectomy in total population. **Significantly greater dose of analgesic administration for the T/T genotype compared with the T/C genotype in both the patients who underwent gastrectomy and enterectomy.