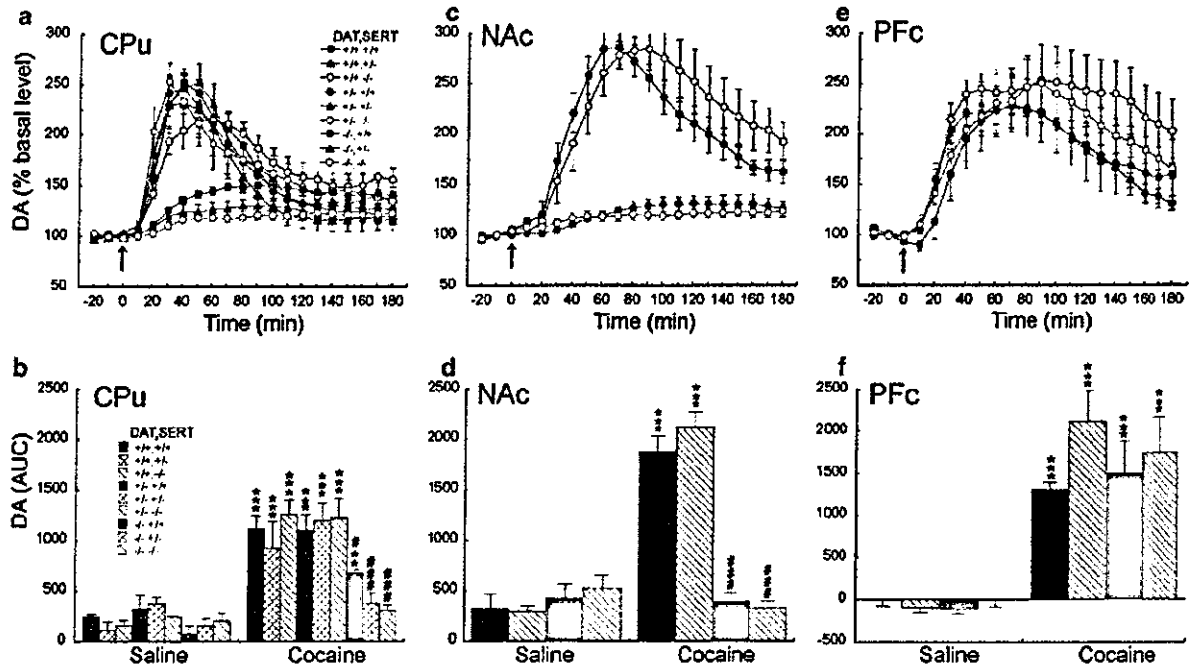

Figure 2 (a, c, and e) Temporal pattern of DA response to cocaine (10 mg/kg, s.c.) in CPu, NAc, and PFc, respectively. Each point represents the mean (±SEM) of the percentage of DA_{ex} baselines. The time of injections is indicated with an arrow. (b, d, and f) Histogram represents the mean AUC (±SEM) of DA response to saline or cocaine in CPu, NAc, and PFc during 180 min interval after injection. $^{**}P<0.01$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

DAT-KO mice (DAT−/−SERT+/+), peaking at about 90 min (Figure 2a). This pattern is not observed in NAc, where DAT−/−SERT+/+ mice do not exhibit any larger increments in DA_{ex} levels (Figure 2c). In further contrast, wild-type, DAT−/−SERT+/+, and DAT−/−SERT−/− mice each exhibit indistinguishable cocaine-induced DA responses in PFc (Figure 2e).

Drug effects on DA_{ex} levels can be assessed by studying AUCs (Figure 2b, d, and f). ANOVAs of mean AUC (±SEM) for drug effects on DA_{ex} levels reveal that drugs have significant effects on DA AUC in CPu ($F(1,62)=132.32$, $P<0.0001$), NAc ($F(1,34)=80.60$, $P<0.0001$), and PFc ($F(1,28)=67.59$, $P<0.0001$). Genotype and drug × genotype interactions were significant for DA AUC in CPu ($F(8,62)=5.45$, $P<0.0001$; $F(8,62)=3.41$, $P<0.01$; respectively) and NAc ($F(3,34)=23.82$, $P<0.0001$; $F(3,34)=36.09$, $P<0.0001$; respectively), but not in PFc ($F(3,28)=0.89$, $P=0.46$; $F(3,28)=0.94$, $P=0.43$; respectively). In CPu (Figure 2b), DAT-KO mice exhibit statistically significant cocaine-induced increments in DA_{ex} levels, although these increases are less than those found in wild-type mice. By contrast, in DAT−/−SERT+/− and DAT−/−SERT−/− mice, the same genotypes that do not exhibit rewarding effects of cocaine also do not exhibit cocaine-induced increases in DA_{ex} in CPu. No significant differences are observed in cocaine-induced DA AUC increases in CPu between the DAT+/+ and DAT+/− mice. In NAc (Figure 2d), cocaine fails to increase DA_{ex} in DAT−/− SERT+/+ or in DAT−/−SERT−/− mice. There are no significant differences in cocaine-induced DA increases in NAc between wild-type and DAT+/+ SERT−/− mice. In PFc (Figure 2f), cocaine produces significant increases in DA_{ex} in all genotypes.

Systemic Cocaine Effects on 5-HT_{ex} in CPu, NAc, and PFc

The temporal patterns of 5HT responses to cocaine in CPu, NAc, and PFc are shown in Figure 3a, c, and e. DAT+/+ SERT−/− and DAT+/−SERT−/− mice show gradual 5HT responses to cocaine in CPu (Figure 3a) and NAc (Figure 3c), but not in PFc (Figure 3e). 5-HT response curves produced by cocaine are observed in CPu (Figure 3a) and NAc (Figure 3c) in all genotypes except DAT−/− SERT−/− mice. The peak of 5-HT response are smaller for SERT−/− mice than for either SERT+/+ or SERT+/− mice. SERT−/− mice exhibit no 5-HT response to cocaine in PFc (Figure 3c), while wild-type mice exhibit robust increases.

Drug effects on 5-HT_{ex} levels are expressed as mean AUC (±SEM) in Figure 3b, d, and f. Two-way ANOVAs of the AUC for 5-HT responses to cocaine show significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,62)=181.49$, $P<0.0001$; $F(8,62)=5.01$, $P<0.0001$; $F(8,62)=4.88$, $P<0.0001$; respectively), NAc ($F(1,34)=31.57$, $P<0.0001$; $F(3,34)=6.44$, $P<0.0001$; $F(3,34)=8.41$, $P<0.0001$; respectively) and PFc ($F(1,28)=57.74$, $P<0.0001$; $F(3,28)=11.55$, $P<0.0001$; $F(3,28)=15.59$, $P<0.0001$; respectively). In CPu (Figure 3b) and NAc (Figure 3d), multiple AUC comparisons reveal that cocaine significantly increases 5-HT_{ex} in DAT+/+SERT−/− and DAT+/−SERT−/− mice, but

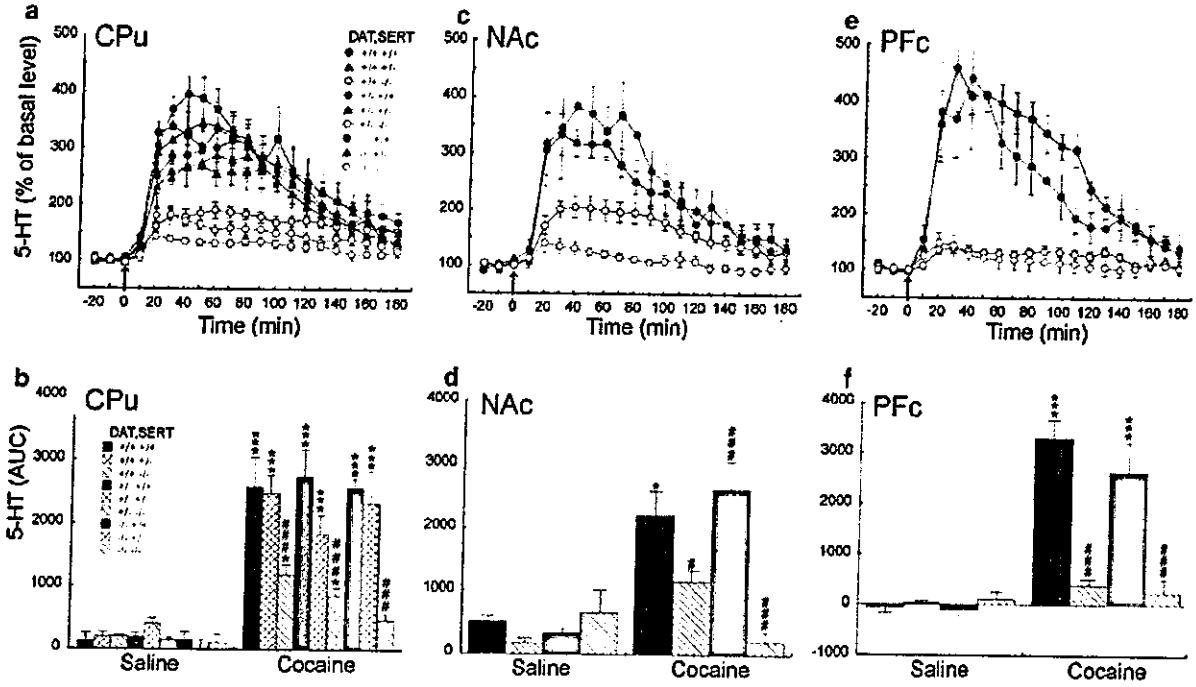

Figure 3 (a, c, and e) Temporal pattern of 5-HT response to cocaine (10 mg/kg, s.c.) in CPu, NAc, and PFc, respectively. The time of injections is indicated with an arrow. (b) Histogram represents the mean AUC (±SEM) of 5-HT response to saline or cocaine in CPu, NAc, and PFc during the 180 min interval after injection. $^{*}P<0.05$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

not in DAT−/−SERT−/− mice. SERT+/− mice display cocaine-induced increases in 5-HT_{ex} in CPu that are similar to those found in wild-type values. PFc 5-HT_{ex} levels are not altered significantly by cocaine in SERT−/− mice (Figure 3f).

Systemic Fluoxetine Effects on DA_{ex} in CPu and NAc

The temporal patterns of DA response to fluoxetine in CPu and NAc of DAT+/+ SERT+/+, DAT−/−SERT+/+, DAT+/+SERT−/−, and DAT−/−SERT−/− mice are shown in Figure 4a and c. In CPu (Figure 4a), DAT−/− SERT+/+ mice exhibit gradual DA responses to fluoxetine that display time courses similar to those of cocaine and persist for at least 3 h (Figure 4a). Two-way ANOVAs of DA AUC responses show significant effects of Drug ($F(1,33)=9.62$, $P<0.01$) and Drug × Genotype interactions ($F(1,33)=4.94$, $P<0.01$). Multiple comparisons reveal that fluoxetine significantly increases DA AUC only in the CPu of DAT−/−SERT+/+ mice (Figure 4b). In NAc, DA responses to fluoxetine display no significant effects of either Drug ($F(1,29)=0.0076$, $P=0.93$), Genotype ($F(1,29)=0.49$, $P=0.69$), genotype ($F(1,29)=0.69$, $P=0.41$) or Drug × Genotype interaction ($F(3,29)=1.55$, $P=0.22$) (Figure 4d).

Systemic GBR12909 Effects on 5-HT_{ex} in CPu

The temporal pattern of CPu 5-HT response to GBR12909 is shown in Figure 5a. DAT+/+SERT−/− mice exhibit remarkable 5-HT_{ex} increases after administration of GBR12909, which are not seen in WT mice. These SERT-KO mice continue to display elevated CPu 5-HT_{ex} levels for at least 3 h. Two-way ANOVA of the AUC of the DA response to GBR 12909 shows significant effects of Drug ($F(1,13)=14.43$, $P<0.01$), Genotype ($F(1,13)=7.63$, $P<0.05$), and Drug × Genotype interactions ($F(1,13)=5.74$, $P<0.05$). Multiple comparisons show that GBR12909 administration significantly increases CPu 5-HT_{ex} in DAT+/+SERT−/−, but not in wild-type mice (Figure 5b).

Local Cocaine Effects on DA_{ex} and 5-HT_{ex} in CPu

DA_{ex} and 5-HT_{ex} level changes in CPu following local cocaine infusion are shown in Figure 6a and c. Local cocaine cannot induce DA response curve in CPu of DAT−/− SERT+/+ and DAT−/−SERT−/− mice, but produces gradual 5-HT response curve in DAT+/+SERT−/− mice.

ANOVAs of mean AUC (±SEM) for DA responses reveal significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,24)=161.46$, $P<0.0001$; $F(3,24)=48.20$, $P<0.0001$; $F(3,24)=47.30$, $P<0.0001$; respectively). Multiple AUC comparisons show that local cocaine fails to increase DA_{ex} in CPu of DAT−/− SERT+/+ or in DAT−/−SERT−/− mice (Figure 6b). ANOVAs of mean AUC (±SEM) for 5-HT responses also reveal significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,24)=43.26$, $P<0.0001$; $F(3,24)=9.55$, $P<0.0001$; $F(3,24)=9.70$, $P<0.0001$; respectively). Multiple comparisons reveal that local cocaine significantly increases 5-HT_{ex} in wild-type, DAT+/+SERT−/− and DAT−/−SERT+/+ mice, but not in DAT−/−SERT−/− mice (Figure 6d). Moreover, there were no significant changes in NAc DA_{ex} in DAT−/−

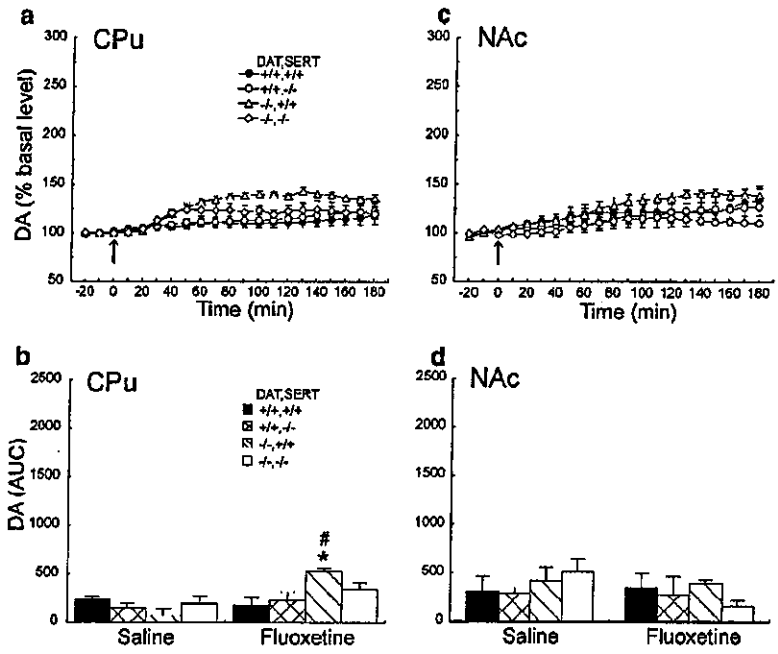

Figure 4 (a and c) Temporal pattern of DA response to fluoxetine (20 mg/kg, s.c.) in CPu and NAc, respectively. The time of injections is indicated with an arrow. (b and d) The histogram represents the mean AUC (±SEM) of DA response to saline or fluoxetine in CPu and NAc during the 180 min interval after injection. $^{*}P<0.05$ compared to the saline group of the same genotype; $^{\#}P<0.05$ compared to the fluoxetine-treated wild-type group.

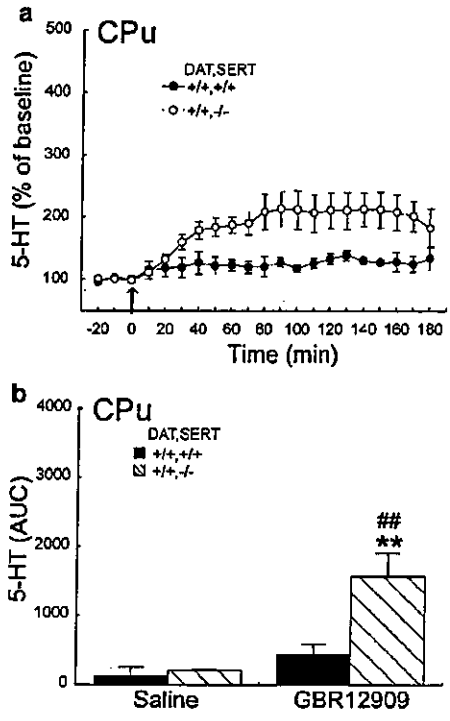

Figure 5 (a) Temporal pattern of 5-HT response to GBR12909 (10 mg/kg, s.c.) in CPu. The time of injections is indicated with an arrow. (b) The histogram represents the mean AUC (±SEM) of 5-HT response to saline or GBR12909 in CPu during 180 min interval after injection. $^{**}P<0.01$ compared to the saline group of the same genotype; $^{\#\#}P<0.01$ compared to the GBR12909-treated wild-type group.

SERT+/+ and DAT−/−SERT−/− mice after local cocaine infusions (data not shown).

DISCUSSION

These microdialysis results reveal parallels with and differences from the patterns of KO effects on reward elicited by cocaine and fluoxetine that we have previously reported in these mouse strains. We can thus evaluate hypotheses about the pharmacological profiles and brain localization of processes hypothesized to mediate cocaine reward with regard to their convergence or divergence with this microdialysis data.

Differential DA Responses in to Cocaine in CPu, NAc, and PFc and Correlations with Assessments of Cocaine Reward

The current data do not provide simple correlations with models that postulate that enhanced NAc DA_{ex} levels alone are necessary and sufficient for cocaine reward. Although this hypothesis has been supported by data from microinjection and lesion studies (Kuhar *et al*, 1991; Koob and Nestler, 1997; Bardo, 1998; Kelley and Berridge, 2002), many results from gene KO studies fail to support the simple hypothesis that DA alone mediates the rewarding effects of cocaine. Our current observations that cocaine does not increase DA_{ex} in NAc of homozygous DAT-KO mice contrasts with the nearly-intact cocaine reward found in these animals (Rocha *et al*, 1998; Sora *et al*, 1998). These *in vivo* microdialysis data are also consistent with studies which document failure of cocaine to block DA uptake in

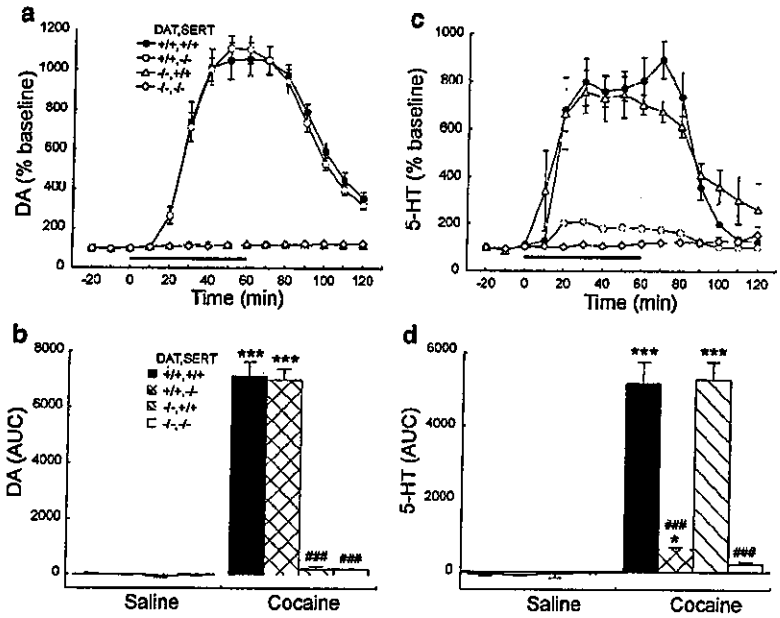

Figure 6 (a and c) Temporal pattern of DA and 5-HT response to local cocaine infusion (100 μM) in CPu, respectively. Horizontal bar indicates the time of infusions. (b and d) The histogram represents the mean AUC (±SEM) of DA and 5-HT response to saline or cocaine in CPu during 120 min interval after injection. $^{*}P<0.05$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

NAc samples taken from DAT homozygous mice in *in vitro* experiments (Budygin *et al*, 2002; Moron *et al*, 2002).

The current data also fail to provide simple correlations with models that postulate that enhanced PFc DA levels are necessary and sufficient for cocaine reward. This hypothesis has also been supported by a substantial body of lesion and microinjection data (Goeders and Smith, 1983; Goeders *et al*, 1986; Bardo, 1998; Tzschentke, 2001). Cocaine increases DA_{ex} in PFc of both wild-type and homozygous DAT-KO mice that exhibit cocaine reward and DAT/SERT double homozygous KO mice that do not display cocaine reward.

Intriguingly, the current results for DA in CPu appear to provide the best fit with studies of cocaine-induced place preferences. Although intra-CPu cocaine does not affect DA_{ex} levels in DAT-KO mice, systemic cocaine causes about 1.5-fold increase in peak DA_{ex} concentrations in CPu dialysate from DAT-KO mice that are rewarded by cocaine, but not from DAT/SERT double homozygous KO mice that lack cocaine CPP. Systemic fluoxetine also increases CPu DA_{ex} levels in homozygous DAT-KO mice in which this compound is rewarding, but not in wild-type mice or homozygous SERT-KO mice in which fluoxetine does not produce a place preference.

Differential 5-HT Responses to Cocaine in CPu, NAc, and PFc and Correlations with Assessments of Cocaine Reward

Although cocaine-induced increases in CPu and NAc $5\text{-}HT_{ex}$ are found in SERT-KO mice that exhibit enhanced cocaine CPP, the magnitude of the increases in $5\text{-}HT_{ex}$ after cocaine administration is attenuated when it is compared with wild-type mice. Interestingly, chronic SERT blockade with fluoxetine can also potentiate cocaine reward (Cunningham and Callahan, 1991; Kleven and Koek, 1998). It is conceivable that the attenuation of cocaine-induced $5\text{-}HT_{ex}$ rise may lead mice more sensitive to the reward effect of cocaine. These sorts of data, and the current results, continue to point to possible roles for 5-HT in cocaine reward, especially in light of the more complex hypotheses of the basis of cocaine reward discussed below.

$5\text{-}HT_{ex}$ Clearance by DAT, DA_{ex} Clearance by NET, and opportunities for 'Promiscuous Uptake'

Removal of a transporter that usually provides inactivation, re-accumulation, and recycling of a released monoamine neurotransmitter provides opportunities for greater diffusion of the monoamine, documented by higher extracellular dialysate concentrations noted here. Removal of a cognate transporter also enhances the opportunities for transmitter uptake by a transporter that normally recognizes another monoamine. The presence of the same vesicular transporter in DAT-, SERT-, and NET-expressing neurons provides the opportunity for the monoamine that has been taken up by a non-cognate plasma membrane transporter to be accumulated into vesicles, and to be re-released as a 'false transmitter' (Liu and Edwards, 1997; Uhl *et al*, 2000). DA accumulation by NET-expressing neurons also provides the opportunity for DA to be subjected to β-hydroxylation to produce norepinephrine, providing a 'true' transmitter for noradrenergic neurons. It is interesting to note that elimination of monoamine transporters has different effects on basal monoamine levels in different brain regions, supporting ideas that factors that mediate DA and 5-HT clearance from synaptic clefts may differ substantially from one terminal field to another.

Many of the present and previously reported results appear to provide evidence for uptake by non-cognate transporters, and even for possible 'false transmission' in these transporter-KO mice. Cocaine and the selective DAT blocker GBR12909 produces a substantial increase in dialysate 5-HT in SERT-KO mice that is not found in wild-type animals. These findings were supported by previous reports that have documented 5-HT uptake by cultured neurons from SERT-KO mice that could be blocked by selective DAT blockers (Pan *et al*, 2001), and 5-HT-like immunoreactivity in substantia nigra and ventral tegmental area dopaminergic neurons (Zhou *et al*, 2002). False transmission may be region-dependent, with differences in the relative densities of DAT- SERT- and NET-expressing neural elements providing differential opportunities for such processes.

Moreover, our observations of virtually identical PFc DA_{ex} baselines in each of these KO strains appear to support a relatively reduced prominence of DAT-mediated DA uptake in this region even in wild-type mice. These observations are compatible with the relatively sparse distribution of PFc DAT in several species (Freed *et al*, 1995; Sesack *et al*, 1998), in contrast with more prominent NET and SERT expression. They are also in accord with pharmacological and other evidence for significant NET-mediated DA uptake in rodent PFc (Di Chiara *et al*, 1992; Tanda *et al*, 1997; Yamamoto and Novotney, 1998). DA may thus be accumulated by NET in PFc of both wild-type and DAT-KO mice.

The current observations in DA response to cocaine and fluoxetine in CPu of DAT-KO mice may provide a different picture. Although systemic cocaine and fluoxetine increase significantly CPu DA_{ex} in DAT-KO mice, local cocaine fails to change it. These results demonstrate that SERT does nt play a role of 'promiscuous uptake' in DA clearance. Systemic cocaine- or fluoxetine-induced DA increase in CPu of DAT-KO mice may result from DA release from activated DA neuron rather than local clearance by SERT.

Comparisons with Other Results

Observations that CPu dialysate monoamine levels apparently provide the best parallel with the loss of cocaine CPP found in current results could be consistent with a previously underappreciated role for CPu structures in mediating some of the 'learned' features of cocaine reward that are manifest in conditioned place preference testing (White and McDonald, 2002). These structures can be critical for stimulus-response 'habit' learning, including that related to reward (Jog *et al*, 1999; Reynolds *et al*, 2001). It is conceivable that this structure may play an even greater role in DAT-KO mice that lack cocaine-induced DA_{ex} elevations in NAc.

The failure of dialysis results for DA alone in NAc or PFc to parallel cocaine reward effects of various KOs and the apparent parallel in CPu should not prevent further consideration of: (a) multiple compensating contributions of monoamines to the rewarding effects of cocaine; (b) contributions of cocaine effects on monoamines in other brain regions, for example, ventral pallidum (Gong *et al*, 1996, 1997), ventral tegmental area (Roberts and Koob, 1982; Ranaldi and Wise, 2001) for cocaine reward; (c) effects of nonmonoaminergic adaptations to the retained cocaine reward in the transporter KO mouse strains that retain such reward. Monoamine actions in brain regions such as the ventral tegmental area have been postulated to be central to the rewarding actions of major drug classes, such as opiates (Wise, 1989; Garzon and Pickel, 2001) and stimulants. It is quite conceivable that monoamine actions in areas not sampled in the current studies could play roles in normal cocaine reward mechanisms, and in adaptations that may underlie the retention of cocaine reward in DAT- and in SERT-KO mice. Mice with single or multiple transporter deletions display many adaptive alterations, as assessed through behavioral, neurochemical, per- or post-synaptic receptor binding, gene expression, and other analytical approaches. None of the current data should hinder attempts to add more explanatory power for the remarkable behavioral pharmacological profiles displayed by these KO mice through use of any or all of these alternative approaches.

The current results in NAc and CPu DA response to cocaine in DAT-KO mice produced in our laboratory, while highly reproducible in our hands, differ from those obtained in reports from another line of DAT-KO mice that which showed that systemic cocaine and reboxetine (NET blocker) increased DA_{ex} remarkably in NAc of DAT-KO mice (Carboni *et al*, 2001). The different DA response to cocaine in NAc and CPu between Carboni's and our DAT KOs may be due to the different DNA construction which was used to disrupt DAT gene. Moreover, our findings are consistent with other reports which demonstrated that cocaine could not affect DA clearance in NAc of DAT-KO mice via *in vitro* experiments. It is noteworthy that (1) DA_{ex} baseline in NAc of DAT-KO mice is about 10 times greater than that in wild-type mice, and that (2) the capacity for DA uptake of NET is far weaker than that of DAT (Giros *et al*, 1994; Gu *et al*, 1994). These may be the reasons why NET cannot show redundancy for DAT in NAc.

In summary, the present work adds to previous data concerning the behavioral consequences of DAT and SERT deletion, by suggesting that cocaine CPP does not necessarily correlate with simple elevations of DA the NAc or PFc. It points out unanticipated correlations with DA_{ex} elevations in CPu. It is interesting that the CPu findings parallel behavioral observations of the rewarding profiles of not only cocaine but also of fluoxetine in these varying mouse strains. While these correlations do not prove causation, the data support careful re-examination of CPu roles in psychostimulant reward (or reward learning) in both wild-type and DAT-KO mice, including both the dorsal and ventral CPu regions likely to be sampled with our microdialysis approaches. Another view of the current results is that the double homozygous DAT/SERT combined KO mice that failed to display either cocaine-induced DA_{ex} or $5\text{-}HT_{ex}$ elevations in NAc also failed to exhibit cocaine CPP, suggesting perhaps that either DA_{ex} or $5\text{-}HT_{ex}$ elevation can mediate cocaine reward and that the absence of both effects is required to eliminates the cocaine CPP. The current data also add to the growing body of evidence that may indicate uptake of released monoamines by non-cognate transporters when their cognate transporters are deleted, and provide evidence for the brain-region specificity of these processes in wild-type and in transporter KO

mice. Each of these findings adds pieces to the complex puzzle of the mediation of cocaine reward by monoaminergic brain systems.

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Inhibition of G Protein-Activated Inwardly Rectifying K^+ Channels by Various Antidepressant Drugs

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G protein-activated inwardly rectifying K^+ channels (GIRK, also known as Kir3) are activated by various G protein-coupled receptors. GIRK channels play an important role in the inhibitory regulation of neuronal excitability in most brain regions and the heart rate. Modulation of GIRK channel activity may affect many brain functions. Here, we report the inhibitory effects of various antidepressants: imipramine, desipramine, amitriptyline, nortriptyline, clomipramine, maprotiline, and citalopram, on GIRK channels. In *Xenopus* oocytes injected with mRNAs for GIRK1/GIRK2, GIRK2 or GIRK1/GIRK4 subunits, the various antidepressants tested, except fluvoxamine, zimelidine, and bupropion, reversibly reduced inward currents through the basal GIRK activity at micromolar concentrations. The inhibitions were concentration-dependent with various degrees of potency and effectiveness, but voltage- and time-independent. In contrast, Kir1.1 and Kir2.1 channels in other Kir channel subfamilies were insensitive to all of the drugs. Furthermore, GIRK current responses activated by the cloned A_1 adenosine receptor were similarly inhibited by the tricyclic antidepressant desipramine. The inhibitory effects of desipramine were not observed when desipramine was applied intracellularly, and were not affected by extracellular pH, which changed the proportion of the uncharged to protonated desipramine, suggesting its action from the extracellular side. The GIRK currents induced by ethanol were also attenuated in the presence of desipramine. Our results suggest that inhibition of GIRK channels by the tricyclic antidepressants and maprotiline may contribute to some of the therapeutic effects and adverse side effects, especially seizures and atrial arrhythmias in overdose, observed in clinical practice.
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Keywords: antidepressant; GIRK; Kir channel; ethanol; *Xenopus* oocyte

INTRODUCTION

Imipramine and other similar compounds: desipramine, amitriptyline, nortriptyline, and clomipramine, were the first successful antidepressants and have been widely used for the treatment of depression and other psychiatric disorders, such as panic and obsessive–compulsive disorders, bulimia nervosa, and chronic pain disorder (Baldessarini, 2001). Owing to their chemical structures with a three-ring molecular core, they are referred to as the tricyclic antidepressants. Later, a series of newer antidepressants have been developed: tetracyclic antidepressants, for example, maprotiline; atypical antidepressants, for example, bupropion; and selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, fluvoxamine, zimelidine, and citalopram (Baldessarini, 2001). Inhibition of norepinephrine and/or serotonin (5-hydroxytryptamine (5-HT)) transporters by antidepressants in the brain is generally thought to have important implications in their therapeutic effects (Baldessarini, 2001). In contrast, the interaction of antidepressants with muscarinic, α_1 adrenergic, and H_1 histamine receptors is involved in some of the adverse side effects (Baldessarini, 2001). It has also been shown that antidepressants inhibit the functions of several other receptors and ion channels, such as 5-HT_{2C} (Ni and Miledi, 1997) and 5-HT_3 receptors (Fan, 1994), nicotinic acetylcholine receptors (García-Colunga *et al*, 1997; Maggi *et al*, 1998), *N*-methyl-D-aspartate (NMDA) receptor channels (Sernagor *et al*., 1989), $P2X_2$ receptors (Nakazawa *et al*, 1999), voltage-gated Ca^{2+}, Na^+, and K^+ channels (Ogata *et al*, 1989; Mathie *et al*, 1998; Pancrazio *et al*, 1998; Teschemacher *et al*, 1999; Yeung *et al*, 1999; Deák *et al*, 2000; Choi *et al*, 2001; Cuellar-Quintero *et al*, 2001), Ca^{2+}-activated K^+ channels (Kamatchi and Ticku, 1991; Lee *et al*, 1997; Dreixler *et al*, 2000; Terstappen *et al*, 2001), and Cl^- channels (Maertens *et al*, 1999, 2002). The effects might also be involved in the molecular and cellular mechanisms underlying some of the therapeutic effects and side effects of various antidepressants.

G protein-activated inwardly rectifying K^+ (GIRK) channels (also known as Kir3 channels) are members of a family of inward-rectifier K^+ (Kir) channels that include

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seven subfamilies (Doupnik *et al*, 1995; Reimann and Ashcroft, 1999). Four GIRK channel subunits have been identified in mammals (Kubo *et al*, 1993b; Lesage *et al*, 1995; Wickman *et al*, 1997). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions (Kobayashi *et al*, 1995; Lesage *et al*, 1995; Karschin *et al*, 1996; Liao *et al*, 1996) or homomultimers composed of GIRK2 subunits in the substantia nigra (Inanobe *et al*, 1999), whereas atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky *et al*, 1995). Various G protein-coupled receptors, such as M_2 muscarinic, α_2 adrenergic, D_2 dopaminergic, 5-HT_{1A}, opioid, nociceptin/orphanin FQ and A_1 adenosine receptors, activate GIRK channels (North, 1989; Ikeda *et al*, 1995, 1996, 1997) through direct action of G protein $\beta\gamma$ subunits (Reuveny *et al*, 1994). In addition, ethanol activates GIRK channels independent of G protein-coupled signaling pathways (Kobayashi *et al*, 1999; Lewohl *et al*, 1999). Activation of GIRK channels causes membrane hyperpolarization, and thus the channels play an important role in the inhibitory regulation of neuronal excitability and heart rate (North, 1989; Signorini *et al*, 1997; Wickman *et al*, 1998). Therefore, modulators of GIRK channel activity may affect many brain and cardiac functions. Using the *Xenopus* oocyte expression system, we demonstrated that various antipsychotic drugs including thioridazine and clozapine and an SSRI antidepressant drug, fluoxetine, inhibited GIRK channels (Kobayashi *et al*, 1998, 2000, 2003). Imipramine, a tricyclic antidepressant of the dibenzazepines, is chemically similar to the antipsychotic drugs thioridazine, a phenothiazine, and clozapine, a dibenzodiazepine. Therefore, we hypothesized that tricyclic antidepressants might interact with GIRK channels. In the present study, we examined the effects of various antidepressants including tricyclic antidepressants on brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels by 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 (XA1) receptor, were obtained by using the polymerase chain reaction method as described previously (Kobayashi *et al*, 1995, 2000, 2002). In addition, cDNAs for rat Kir1.1 in pSPORT and mouse Kir2.1 in pcDNA1 were provided by Dr Steven C Hebert and Dr Lily Y Jan, respectively. These plasmids were linearized by digestion with an appropriate enzyme as described previously (Ho *et al*, 1993; Kubo *et al*, 1993a; Kobayashi *et al*, 2000), and the specific mRNAs were synthesized *in vitro* by using the mMESSAGE mMACHINE™ *In vitro* Transcription Kit (Ambion, Austin, TX, USA).

Electrophysiological Analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until used. Frogs were anesthetized by immersion in water containing 0.15% tricaine (Sigma Chemical Co., St Louis, MO, USA). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely killed after the final collection. Oocytes (Stages V and VI) were isolated manually from the ovary and maintained in Barth's solution (Kobayashi *et al*, 2002). *Xenopus laevis* oocytes were injected with mRNA(s) for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each ~0.4 ng), GIRK2 (~5 ng), Kir1.1 (~5 ng) or Kir2.1 (~0.5 ng) and/or XA1 (~10 ng). The oocytes were incubated at 19°C in Barth's solution, and defolliculated following treatment with 0.8 mg ml^{-1} collagenase as described previously (Kobayashi *et al*, 2002). Whole-cell currents of the oocytes were recorded from 2 to 10 days after the injection with a conventional two-electrode voltage clamp (Kobayashi *et al*, 1999; Ikeda *et al*, 2003). The membrane potential was held at −70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-potassium (hK) solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.5, and HEPES 5, pH 7.4 with KOH) or a K^+-free high-sodium (ND98) solution (composition in mM: NaCl 98, $MgCl_2$ 1, $CaCl_2$ 1.5, and HEPES 5, pH 7.4 with NaOH) at a flow rate of 2.5 ml min^{-1}. For examining the effect of intracellular desipramine, 23 nl of 30 mM desipramine or 30 mM lidocaine *N*-ethyl bromide (QX-314) dissolved in distilled water was injected into an oocyte by using a Nanoliter injector (World Precision Instruments, Sarasota, FL, USA) as described previously (Kobayashi *et al*, 2003), and the oocyte currents were continuously recorded for approximately 30–40 min. Owing to a volume of ~1 µl in the oocyte, the intracellular concentration of desipramine was presumed as ~674 µM. In the hK solution, the K^+ equilibrium potential (E_K) was close to 0 mV, and inward K^+ current flow through Kir channels was observed at negative holding potentials. Data were fitted to a standard logistic equation by using KaleidaGraph (Synergy Software, Reading, PA, USA) for analysis of concentration–response relationships. The EC_{50} value, which is the concentration of a drug that produces 50% of the maximal current response for that drug, the IC_{25} and IC_{50} values, which are the concentrations of a drug that reduces control current responses by 25 and 50%, respectively, and the Hill coefficient (n_H) were obtained from the concentration–response relationships.

Statistical Analysis of Results

The values obtained are expressed as the mean ± SEM, and *n* is the number of oocytes tested. Statistical analysis of differences between groups was carried out by using paired *t*-test, Student's *t*-test, one-way ANOVA or two-way factorial ANOVA, followed by Bonferroni/Dunn *post hoc* test. A probability of 0.05 was taken as the level of statistical significance.

Compounds

All the antidepressants tested, except fluvoxamine (Tocris Cookson, Bristol, UK), and adenosine were purchased from Research Biochemicals Inc (Natick, MA, USA). Maprotiline,

fluvoxamine, and citalopram were dissolved in dimethyl sulfoxide (DMSO). Other drugs were dissolved in distilled water. The stock solutions of all of the compounds were stored at −30°C until used. Ethanol was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

RESULTS

Inhibition of GIRK Channels by Antidepressants

To investigate whether various antidepressants interact with brain-type GIRK1/2 and cardiac-type GIRK1/4 channels, we conducted *Xenopus* oocyte expression assays. In oocytes co-injected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which are known to depend on free G protein $\beta\gamma$ subunits present in the oocytes because of the inherent activity of G proteins (Dascal, 1997), were observed under the conditions of a hK solution containing 96 mM K^+ and negative membrane potentials (Kobayashi *et al*, 2003; Figure 1a). Application of 300 μM imipramine, desipramine, amitriptyline or nortriptyline, tricyclic antidepressants, immediately and reversibly caused a reduction of the inward currents through the expressed GIRK channels in the hK solution (Figure 1a). The current responses were abolished in the presence of 3 mM Ba^{2+}, which blocks the Kir channel family including GIRK channels ($n=3$; data not shown). None of the antidepressants produced a significant response in the K^+-free ND98 solution containing 98 mM Na^+ instead of the hK solution ($n=3$; data not shown), suggesting that the antidepressant-sensitive current components show K^+ selectivity. In uninjected oocytes, the antidepressants tested, even at the highest concentrations used, and 3 mM Ba^{2+} caused no significant response (Figure 1c; $n=4$ and 10, respectively), suggesting no effect of the antidepressants and Ba^{2+} on intrinsic oocyte channels. These results suggest that the tricyclic antidepressants inhibit GIRK1/2 channels. In addition, since the magnitudes of inhibition of basal GIRK currents by 3 mM Ba^{2+} were almost equal to those by 5 mM Ba^{2+} in oocytes expressing GIRK channels, the 3 mM Ba^{2+}-sensitive current components (1048.6±60.2 nA, $n=66$) were considered to correspond to the magnitudes of GIRK1/2 currents. Maprotiline, which is a tetracyclic antidepressant related structurally to tricyclic antidepressants, also inhibited basal GIRK currents by 44.8±4.9% at 100 μM ($n=8$), whereas citalopram, an SSRI, and bupropion had a small or no effect on the currents (21.7±2.9% inhibition and 4.7±0.6% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=6$ and 3, respectively). In addition, application of DMSO, the solvent vehicle, at the highest concentration (0.3%) used had no significant effect on the current responses in oocytes co-injected with GIRK1 and GIRK2 mRNAs ($n=4$; data not shown). Similarly, in oocytes co-injected with GIRK1 and GIRK4 mRNAs (Figures 1b), basal GIRK currents were observed under the same conditions, and the current components sensitive to 3 mM Ba^{2+} were 880.2±71.3 nA ($n=64$). The tricyclic antidepressants tested and maprotiline inhibited basal GIRK1/4 currents (Figures 1b, 2b), suggesting that the antidepressants also inhibit GIRK1/4 channels. However, citalopram and bupropion had little or no effect on basal GIRK1/4 currents (6.5±1.7% inhibition and 3.7±3.2% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=3$ and 8, respectively).

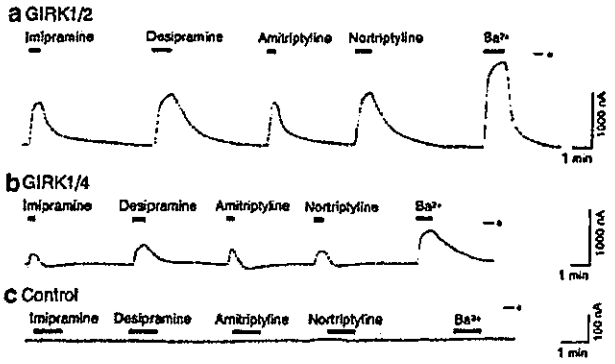

Figure 1 Inhibition by tricyclic antidepressants of brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. (a) In an oocyte co-injected with GIRK1 and GIRK2 mRNAs, current responses to imipramine, desipramine, amitriptyline, nortriptyline, and 3 mM Ba^{2+} are shown. The concentration of each antidepressant tested was 300 μM. (b) In an oocyte co-injected with GIRK1 and GIRK4 mRNAs, current responses to 100 μM of the same antidepressants as in (a) and 3 mM Ba^{2+} are shown. (c) In an uninjected oocyte, no significant current responses to 300 μM of the same antidepressants as in (a) and 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution containing 96 mM K^+. Asterisks show the zero current level. Bars show the duration of application.

We next investigated the concentration–response relationship of the inhibitory effects of the various antidepressants on GIRK channels expressed in *Xenopus* oocytes, compared with the current components sensitive to 3 mM Ba^{2+}, which fully blocked basal GIRK currents (Kobayashi *et al*, 2002). Figure 2 shows that inhibition of GIRK1/2 and GIRK1/4 channels by the antidepressants is concentration-dependent with distinctive potency and effectiveness at micromolar concentrations. All the antidepressants inhibited both types of GIRK channels to a limited extent even at high concentrations. Table 1 shows the EC_{50} and n_H values obtained from the concentration–response relationships for the antidepressants tested and the percentage inhibition of the GIRK currents by the drugs at the highest concentrations tested. The rank order of the inhibition of GIRK1/2 and GIRK1/4 channels by 100 μM of these drugs was as follows: desipramine > clomipramine ≥ imipramine > nortriptyline = amitriptyline ≥ maprotiline ≫ citalopram ≫ bupropion for GIRK1/2 channels and desipramine > clomipramine ≥ amitriptyline > maprotiline ≥ nortriptyline > imipramine ≫ citalopram = bupropion for GIRK1/4 channels. To further compare the effects of the antidepressants on GIRK channels, we also calculated the drug concentrations required to inhibit the GIRK currents by 25 or 50% (Table 1). Among these antidepressants, desipramine, a tricyclic antidepressant, was the most effective inhibitor of GIRK1/2 and GIRK1/4 channels. The effects of imipramine and clomipramine on GIRK1/2 channels were similar, and those of amitriptyline, nortriptyline, and maprotiline were similar on GIRK1/2 channels and GIRK1/4 channels. The effects of desipramine, amitriptyline, and maprotiline

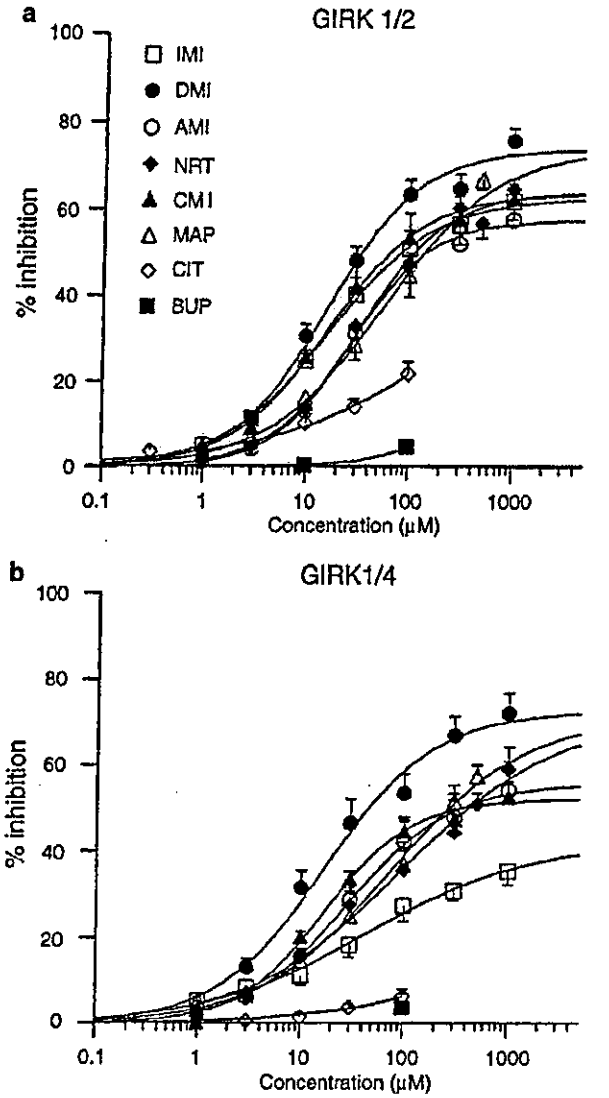

Figure 2 Concentration–response relationships for various antidepressants: imipramine (IMI), desipramine (DMI), amitriptyline (AMI), nortriptyline (NRT), clomipramine (CMI), maprotiline (MAP), citalopram (CIT), and bupropion (BUP), with regard to their effects on GIRK1/2 channels (a) and GIRK1/4 channels (b). The magnitudes of inhibition of GIRK current by antidepressants were compared with the 3 mM Ba^{2+}-sensitive current components, which were 1048.6±60.2 nA ($n=66$) in oocytes expressing GIRK1/2 channels and 880.2±71.3 nA ($n=64$) in oocytes expressing GIRK1/4 channels. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution containing 96 mM K^+. Each point and error bar represents the mean and SEM of the percentage responses obtained from 3–14 oocytes. Data points were fitted by using a logistic equation.

on GIRK1/2 channels were similar to those on GIRK1/4 channels, respectively, although inhibition of GIRK1/2 channels by maprotiline at high concentrations was more effective than that of GIRK1/4 channels. In addition, inhibition of GIRK1/2 channels by imipramine, nortriptyline, clomipramine, and citalopram was more effective than that of GIRK1/4 channels, respectively (Figure 2 and Table 1).

Although GIRK channels in the brain are predominantly GIRK1/2 heteromultimers (Liao *et al*, 1996), GIRK channels in the substantia nigra and ventral tegmental area are GIRK2 homomultimers (Karschin *et al*, 1996; Inanobe *et al*, 1999). To further address the functional relationship between various antidepressants and neuronal GIRK channels, we investigated the effects of the antidepressants on the homomeric channels (Figure 3). The inhibitory effects of the tricyclic antidepressants used and maprotiline on GIRK2 channels in oocytes expressing GIRK2 channels were less effective than those on GIRK1/2 channels ($p<0.01$, significant interaction between the channel effect and the effect of the antidepressants, two-way factorial ANOVA; and $p<0.01$, significant differences between the effects of each antidepressant on GIRK1/2 and GIRK2 channels, Bonferroni/Dunn *post hoc* test). For SSRIs, the inhibitory effect of citalopram on GIRK2 channels was similar to that on GIRK1/2 channels (23.9±6.9% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=4$), whereas fluvoxamine and zimelidine had little effect on GIRK2 channels (5.3±2.7% inhibition and 2.9±0.3% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=3$ and 3, respectively), as were the cases for GIRK heteromeric channels (Kobayashi *et al*, 2003). In addition, bupropion had little effect on GIRK2 channels (3.9±1.8% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=4$).

Furthermore, we examined whether the various antidepressants could interact with Kir1.1, an ATP-regulated inwardly rectifying K^+ channel, and Kir2.1, a constitutively active inward rectifier K^+ channel, in other Kir channel subfamilies (Figure 3). In oocytes expressing Kir1.1 or Kir2.1 channels, application of each antidepressant at 100 μM had no significant effect on the inward currents through the channels in the hK solution ($n\geq 3$ for Kir1.1 and $n=3$ for Kir2.1).

Characteristics of Antidepressant Inhibition of GIRK Channels

As the various tricyclic antidepressants tested acted as inhibitors at GIRK channels in the present study, we further investigated the effect of the tricyclic antidepressants, particularly desipramine, in more detail. The instantaneous GIRK1/2 currents elicited by the voltage step to −100 mV from a holding potential of 0 mV diminished in the presence of 100 μM desipramine (Figure 4a). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by desipramine was not significantly different from that of the instantaneous current (paired *t*-test, $p>0.05$; $n=4$ at −80, −100, and −120 mV, respectively). These results suggest that the channels were inhibited by desipramine primarily at the holding potential of 0 mV in a time-independent manner. Furthermore, similar results were obtained for imipramine, amitriptyline, nortriptyline, and clomipramine (data not shown).

Like 3 mM Ba^{2+}-sensitive currents corresponding to basal GIRK currents, desipramine-sensitive currents in oocytes expressing GIRK channels increased with negative membrane potentials, and the current–voltage relationships showed strong inward rectification (Figure 4b), indicating a characteristic of GIRK currents. Furthermore, similar

Table 1 Inhibitory Effects of Various Antidepressant Drugs on GIRK Channels

Compound	Imipramine	Desipramine	Amitriptyline	Nortriptyline	Clomipramine	Maprotiline	Citalopram	Bupropion
GIRK1/2								
EC_{50}	22.3±2.8	24.2±3.8	30.8±2.2	50.5±12.8	18.0±6.7	47.1±10.1	ND	ND
IC_{25}	18.4±3.8	11.7±1.9	24.4±3.5	23.6±3.3	17.6±8.7	36.6±10.6	ND	ND
IC_{50}	47.0±10.5	36.4±7.6	110.8±12.7	132.3±32.7	39.8±15.6	113.0±14.3	ND	ND
% max	62.0±4.0	75.9±2.9	57.7±3.8	64.8±7.2	62.8±4.5	67.0±2.0	21.7±2.9	4.7±0.6
(μM; *n*)	(1000; 14)	(1000; 10)	(1000; 8)	(1000; 8)	(1000; 9)	(500; 8)	(100; 6)	(100; 3)
n_H	0.83±0.07	0.89±0.05	1.10±0.04	1.06±0.10	1.10±0.12	0.70±0.02	ND	ND
GIRK1/4								
EC_{50}	30.4±9.9	26.3±6.3	33.4±12.0	84.1±3.9	24.0±3.8	70.8±16.6	ND	ND
IC_{25}	84.8±32.0	9.4±1.6	37.3±14.7	46.8±4.4	18.8±2.5	46.2±12.1	ND	ND
IC_{50}	ND	53.9±17.1	274.3±16.5	392.9±122.7	253.5±69.6	290.5±93.3	ND	ND
% max	35.5±3.2	72.2±4.8	54.3±6.3	59.2±5.3	52.8±3.6	57.6±2.4	6.5±1.7	3.7±3.2
(μM; *n*)	(1000; 8)	(1000; 9)	(1000; 10)	(1000; 10)	(1000; 8)	(500; 8)	(100; 3)	(100; 8)
n_H	0.98±0.13	0.88±0.09	1.07±0.09	0.89±0.09	1.02±0.08	0.83±0.07	ND	ND

The mean±SEM of the EC_{50} values and the drug concentrations required to reduce basal GIRK currents by 50 and 25% (IC_{50} and IC_{25}) are shown in μM. The values of % max indicate the mean±SEM% inhibition of basal GIRK currents by the drugs at the highest concentrations tested. The highest concentrations tested (μM) and the number of oocytes tested (*n*) are indicated in parantheses. The n_H values indicate the mean±SEM of the Hill coefficients. ND means that the value was not determined because of a low effectiveness of the drug.

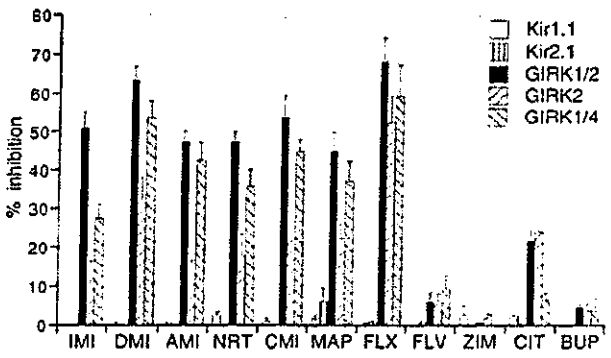

Figure 3 Comparison of the effects of various antidepressants: imipramine (IMI), desipramine (DMI), amitriptyline (AMI), nortriptyline (NRT), clomipramine (CMI), maprotiline (MAP), fluoxetine (FLX), fluvoxamine (FLV), zimelidine (ZIM), citalopram (CIT), and bupropion (BUP), on GIRK, Kir1.1 and Kir2.1 channels. The concentration of each antidepressant tested was 100 μM. The magnitudes of inhibition of Kir currents by the various antidepressants were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing Kir1.1, Kir2.1, GIRK1/2 channels, GIRK2 channels or GIRK1/4 channels (949.3±234.1 nA, $n=6$; 1452.2±274.7 nA, $n=26$; 1168.2±80.4 nA, $n=84$; 493.6±30.2 nA, $n=40$; 961.4±90.6 nA, $n=70$, respectively). Data on FLX are cited from our previous report (Kobayashi *et al*, 2003). The bars represent the means and SEM of the percentage responses obtained from 3–20 oocytes. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution.

results were obtained for imipramine, amitriptyline, nortriptyline, clomipramine, and maprotiline (data not shown).

The percentage inhibition of GIRK currents by 100 μM desipramine was measured at membrane potentials between −100 and −20 mV. For GIRK1/2 and GIRK1/4 channels, the percentage inhibition showed no significant difference across the voltages ($p>0.05$, one-way ANOVA; Figure 4c). These results suggest that inhibition of GIRK channels by desipramine was voltage-independent. Furthermore, similar results were obtained for imipramine, amitriptyline, nortriptyline, clomipramine, and maprotiline (data not shown).

At physiological pH or below, desipramine exists mainly in a protonated form, and the proportion of the uncharged form increases with an increase in pH, because of a pK_a value of 10.2 (Green, 1967). We examined whether changes in pH affect the inhibition by desipramine of GIRK channels expressed in oocytes prepared from the same donor. For GIRK1/2 channels, no effect of pH on the inhibition was observed in the concentration–response relationships for desipramine ($p>0.05$ at each concentration, $n=5$, Student's *t*-test, Figure 5). For GIRK1/4 channels, similar results were obtained at 30 and 100 μM desipramine ($p>0.05$, $n=4$, Student's *t*-test). These results suggest that the inhibition may be mediated by both forms of desipramine with almost the same effectiveness. It also appears unlikely that the inhibition by desipramine is caused by hydrophobic interactions with GIRK channels within the membrane bilayer.

Moreover, we examined the effects of desipramine on GIRK channels activated by G protein-coupled receptors. In oocytes co-expressing GIRK1/2 channels and XA1 receptors (Kobayashi *et al*, 2002), application of 10 nM adenosine induced inward GIRK currents (Figure 6a). The effects of desipramine were evaluated by measuring the amplitude of the adenosine-induced current response during application of desipramine at different concentrations. The current responses to 10 nM adenosine were reversibly inhibited by desipramine with an IC_{50} value of 38.6±8.7 μM and an n_H value of 0.76±0.06 ($n=6$, Figure 6a, b). The percentage inhibition by desipramine was similar to that of basally active GIRK1/2 channels ($p>0.1$ at each concentration,

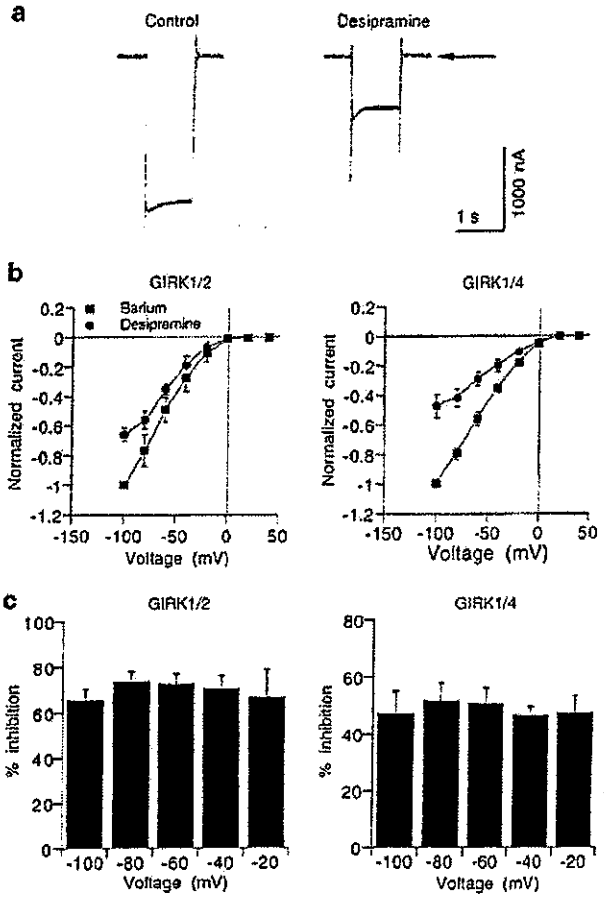

Figure 4 Characteristics of the inhibitory effects of desipramine on GIRK currents. (a) Representative GIRK1/2 currents elicited by a voltage step to −100 mV for 1 s from a holding potential of 0 mV in the absence and presence of 100 μM desipramine. Current responses were recorded in a high-potassium solution containing 96 mM K^+. Arrow indicates the zero current level. (b) Current–voltage relationships of 3 mM Ba^{2+}-sensitive inward currents and 100 μM desipramine-sensitive inward currents in oocytes expressing GIRK1/2 channels or GIRK1/4 channels. Current responses were normalized to the 3 mM Ba^{2+}-sensitive current component measured at a membrane potential of −100 mV. The Ba^{2+}-sensitive current components were 1019.4 ± 162.4 nA ($n=4$) in oocytes expressing GIRK1/2 channels and 615.0 ± 103.2 nA ($n=4$) in oocytes expressing GIRK1/4 channels. (c) Percentage inhibition of GIRK channels by desipramine over the voltage range of −100 to −20 mV. There was no significant interaction between the desipramine effect and the membrane potential effect ($p>0.05$ for GIRK1/2, $n=4$ for each group, and $p>0.05$ for GIRK1/4, $n=4$ for each group; one-way ANOVA). All values are the mean and SEM.

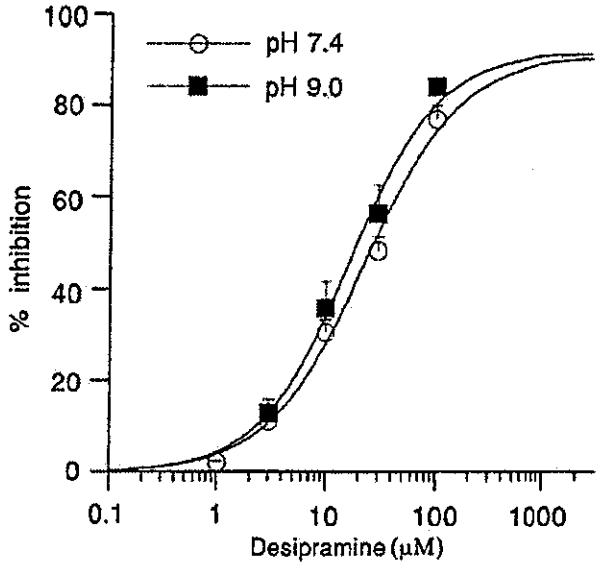

Figure 5 Concentration–response relationships for inhibition of GIRK channels by desipramine at different pH values. The magnitudes of inhibition by desipramine of GIRK current in oocytes expressing GIRK1/2 channels were compared with the 3 mM Ba^{2+}-sensitive current components, which were 865.3 ± 61.0 nA at pH 7.4 ($n=5$) and 927.4 ± 72.6 nA at pH 9.0 ($n=5$). Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. Each point and error bar represents the mean and SEM of the percentage responses obtained. Data points were fitted by using a logistic equation.

Student *t*-test), suggesting the interaction of desipramine with GIRK channels. In addition, the adenosine-induced GIRK currents were not significantly affected by intracellularly applied desipramine (90.8 ± 10.6% of pretreated control current, paired *t*-test, $p>0.1$, $n=5$, Figure 6c), whereas the GIRK currents were significantly inhibited by intracellularly applied QX-314 as reported previously (Zhou *et al*, 2001; Kobayashi *et al*, 2003). The results, therefore, suggest that extracellular desipramine can directly inhibit GIRK channels activated by the XA1 receptors.

GIRK channels are also activated by ethanol independent of G protein signaling pathways (Kobayashi *et al*, 1999). We next examined the effect of desipramine on ethanol-induced GIRK currents. In oocytes expressing GIRK1/2 channels, the GIRK currents induced by ethanol were attenuated in the presence of desipramine, with an IC_{50} value of 34.6 ± 13.4 μM and an n_H value of 1.10 ± 0.08, in a reversible manner (62.3 ± 4.9% inhibition at 100 μM, $n=5$; Figure 7). The results, therefore, suggest that desipramine can inhibit GIRK channels activated by ethanol.

DISCUSSION

We demonstrated that all of the tricyclic antidepressants tested, namely, desipramine, imipramine, amitriptyline, nortriptyline, and clomipramine, inhibited brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels at micromolar concentrations in a distinctive manner. Furthermore, maprotiline, which also possesses a similar tricyclic structure, inhibited GIRK channels. These results indicate that tricyclic moiety of these antidepressants may be involved in GIRK channel inhibition. Among SSRIs, fluoxetine significantly inhibited GIRK channels even at low micromolar levels, whereas fluvoxamine and zimelidine had little effect on the channels (Kobayashi *et al*, 2003). In the present study, citalopram weakly inhibited brain-type GIRK1/2 and GIRK2 channels, but not cardiac-type GIRK1/4 channels. These distinctive effects on GIRK channels may be due to the diverse chemical structures of SSRIs. In addition, both types of GIRK channels were insensitive to bupropion, an atypical antidepressant, suggesting that the drugs with the chemical

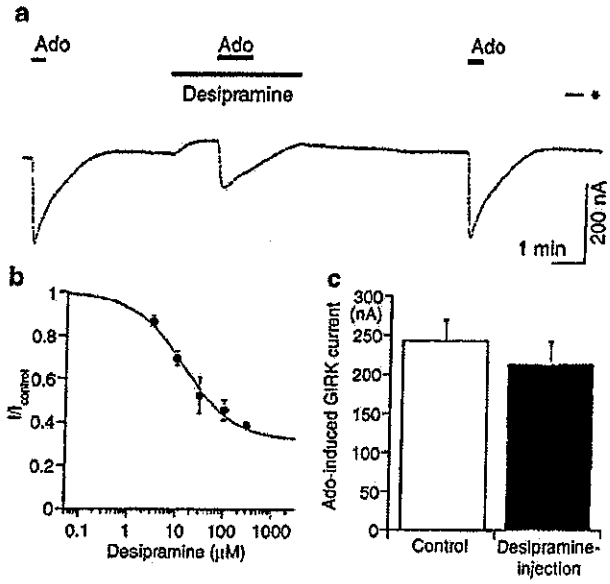

Figure 6 Inhibitory effect of desipramine on GIRK channels activated by a G protein-coupled receptor. (a) In an oocyte co-injected with mRNAs for GIRK1 and GIRK2 channels and XA1 receptor, current responses to 10 nM adenosine (Ado), 10 nM Ado in the presence of 30 μM desipramine and 10 nM Ado are shown. Bars show the duration of application. Asterisk indicates the zero current level. (b) Concentration-dependent inhibition by desipramine of Ado-induced GIRK currents. $I_{control}$ is the amplitude of GIRK currents induced by 10 nM Ado (213.8 ± 30.3 nA, $n = 6$) and I is the current amplitude in the presence of desipramine. Data points were fitted by using a logistic equation. (c) Lack of effect of intracellular desipramine on Ado-induced GIRK currents. There was no significant difference in Ado-induced GIRK currents between the groups before and after intracellular injection of desipramine ($p > 0.05$, $n = 5$, paired t-test). Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. All values are the mean and SEM.

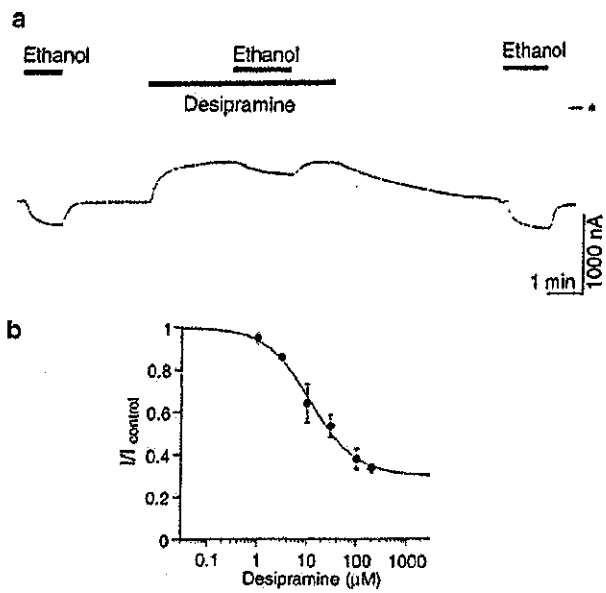

Figure 7 Inhibitory effect of desipramine on the ethanol-induced GIRK currents in *Xenopus* oocytes expressing GIRK1/2 channels. (a) In an oocyte co-injected with GIRK1 and GIRK2 mRNAs, current responses to 100 mM ethanol, 100 mM ethanol in the presence of 30 μM desipramine, and 100 mM ethanol are shown. Asterisk indicates the zero current level. Bars show the duration of application. (b) Concentration-dependent inhibition by desipramine of ethanol-induced GIRK currents. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM ethanol (309.0 ± 24.3 nA, $n = 5$) and I is the current amplitude in the presence of desipramine. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. Each point and error bar represents the mean and SEM of the relative responses. Data points were fitted by using a logistic equation.

structure similar to bupropion may have no significant effect on GIRK channels.

The inhibition of GIRK channels by the tricyclic and tetracyclic antidepressants tested was concentration-dependent but voltage- and time-independent. The GIRK currents were not completely blocked by the antidepressants even at high concentrations. Our results also suggest that desipramine, a tricyclic antidepressant, acts at the channels from the extracellular side of the cell membrane. On the other hand, blockade by extracellular Ba^{2+} and Cs^{+}, typical of Kir channel blockers that occlude the pore of the open channel, shows a concentration dependence, a strong voltage dependence and a time dependence with a comparatively small effect on the instantaneous current, but a marked inhibition on the steady-state current at the end of voltage pulses (Lesage *et al*, 1995). These observations suggest that the antidepressants may probably cause a conformational change in the channels, but not act as open channel blockers of Kir channels like Ba^{2+} and Cs^{+}. The difference in action mechanism between the antidepressants and Ba^{2+} may be involved in the incomplete blockade of GIRK channels by the antidepressants. In addition, the inhibitory effects of the tricyclic and tetracyclic antidepressants tested on GIRK2 homomeric channels were weaker than those on GIRK1/2 heteromeric channels. Moreover, Kir1.1 and Kir2.1 channels among members of the Kir channel family were insensitive to all of the antidepressants. Further studies using GIRK/Kir1.1 and GIRK/Kir2.1 chimeric channels and mutant GIRK channels may clarify the critical sites mediating the effects of the antidepressants on GIRK channels.

The therapeutic plasma concentrations of various antidepressants range approximately from 0.7 to 1.1 μM for imipramine, 0.47 to 1.1 μM for desipramine, 0.36 to 0.9 μM for amitriptyline, 0.23 to 0.57 μM for nortriptyline, 0.48 to 1.6 μM for clomipramine, 0.7 to 1.4 μM for maprotiline, and 0.23 to 0.46 μM for citalopram (Baldessarini, 2001). The plasma concentrations after their overdoses become significantly higher than those in the therapeutic use (Prouty and Anderson, 1990; Barbey and Roose, 1998; Rosenstein *et al*, 1993). As most antidepressants have highly lipophilic properties, they accumulate in the brain (Prouty and Anderson, 1990): namely, the brain-to-plasma ratios are approximately 13–20:1 for imipramine (Besret *et al*, 1996), 15–25:1 for desipramine (Baumann *et al*, 1983), 10–35:1 for amitriptyline (Glotzbach and Preskorn, 1982; Baumann *et al*, 1984; Miyake *et al*, 1990), 8–15:1 for nortriptyline (Baumann *et al*, 1984; Miyake *et al*, 1990), 10–48:1 for clomipramine (Friedman and Cooper, 1983; Eschalier *et al*, 1988), 44–59:1 for maprotiline (Miyake *et al*, 1991), and 4–8:1 for citalopram (Kugelberg *et al*, 2001). In addition, the mean brain concentrations of the

tricyclic antidepressants in postmortem humans were approximately 20 times higher than the corresponding blood levels (Prouty and Anderson, 1990). Therefore, the present findings suggest that GIRK channels in the brain may be inhibited by the various tricyclic antidepressants and maprotiline at clinically relevant brain concentrations.

Interestingly, GIRK2-deficient mice showed reduced anxiety and an increase in motor activity (Blednov *et al*, 2001). Although it is generally thought that the therapeutic effects of antidepressants are primarily due to inhibition of the reuptake of norepinephrine and/or serotonin in the brain, inhibition of neuronal GIRK channels by the antidepressants may contribute to additive therapeutic effects for depression and some psychiatric disorders. Also, antidepressant drugs have various side effects, including sedation, tremor, seizures, orthostatic hypotension, tachycardia, and weight gain (Baldessarini, 2001). Among these side effects, the incidence of seizures is a serious side effect. The risk of seizures for most antidepressants increases with the dose (Rosenstein *et al*, 1993; Barbey and Roose, 1998; Baldessarini, 2001). The mean plasma concentrations of tricyclic antidepressants in several patients who experienced seizures following overdoses were reported to be approximately eight times higher than those in the therapeutic doses (Boehnert and Lovejoy, 1985; Rosenstein *et al*, 1993). Therefore, the tricyclic antidepressants and maprotiline at the brain levels after the overdoses may potently inhibit neuronal GIRK channels. As opposed to these drugs, citalopram, which only slightly inhibits GIRK channels even at toxic concentrations, has a lower seizure risk (Barbey and Roose, 1998). The inhibition of GIRK channels leads to depolarize the membrane potential, resulting in an increase in neuronal excitability (Kuzhikandathil and Oxford, 2002). In addition, GIRK2-deficient mice show spontaneous seizures (Signorini *et al*, 1997). Therefore, potent inhibition of neuronal GIRK channels by some antidepressants may contribute to the cause of the neuropsychiatric toxicity, particularly seizures.

Antidepressant drugs including tricyclic antidepressants and SSRIs show analgesic activity (Messing *et al*, 1975; Lin *et al*, 1980; Schreiber *et al*, 1996; Korzeniewska-Rybicka and Plaznik, 2000; Galeotti *et al*, 2001). Studies using GIRK2-deficient mice and *weaver* mutant mice, which have mutant GIRK2 channels insensitive to G proteins and ethanol, have shown that the analgesic effects induced by opioids or ethanol are remarkably reduced in these mice, suggesting that the GIRK channel activation induces analgesia (Kobayashi *et al*, 1999; Ikeda *et al*, 2000, 2002; Blednov *et al*, 2003; Mitrovic *et al*, 2003). However, our studies have demonstrated that various tricyclic antidepressants and fluoxetine (Kobayashi *et al*, 2003) inhibit GIRK channels. Therefore, the analgesic effects of these antidepressants may not be mediated by GIRK channels. On the other hand, it has been shown that the analgesic effects of various antidepressant drugs may be caused by interaction with other several targets: voltage-gated Na^+ channels (Pancrazio *et al*, 1998) and voltage-gated, ATP-sensitive and Ca^{2+}-activated K^+ channels (Galeotti *et al*, 2001), by the action of 5-HT (Messing *et al*, 1975; Lin *et al*, 1980; Tura and Tura, 1990) and by opioid mechanisms (Reichenberg *et al*, 1985).

In the heart, acetylcholine released from the stimulated vagus nerve opens atrial GIRK channels via activation of the M_2 muscarinic acetylcholine receptor, and ultimately causes slowing of the heart rate (Brown and Birnbaumer, 1990). Sinus tachycardia during tricyclic antidepressant treatment is frequently observed (Baldessarini, 2001). Various tricyclic antidepressants exhibit nanomolar affinity for the receptor (Stanton *et al*, 1993). The present study demonstrated that micromolar concentrations of various tricyclic antidepressants and maprotiline inhibited cardiac-type GIRK1/4 channels. The heart concentrations of the tricyclic antidepressants are approximately 20–200 times higher than the corresponding blood levels (Elonen *et al*, 1975; Jandhyala *et al*, 1977). No exact heart-to-plasma ratio for maprotiline has been reported. However, because maprotiline has a similar tricyclic structure, it may also highly accumulate in the heart. Therefore, atrial GIRK channels may also be inhibited by the antidepressants in clinical practice. Taken together, sinus tachycardia during the treatment with antidepressants may be related to not only antagonism of the M_2 muscarinic acetylcholine receptor, but also inhibition of atrial GIRK channels. Moreover, toxic plasma levels after overdoses of tricyclic antidepressants are associated with cardiac side effects including atrial premature contractions and supraventricular tachycardia (Frommer *et al*, 1987). Cardiac-type GIRK1/4 channels are abundantly present in the atrium of the heart (Krapivinsky *et al*, 1995). Therefore, greater inhibition of atrial GIRK channels by the antidepressants after overdoses may contribute to the incidence of these atrial arrhythmias in cardiac toxicity. In contrast, the cardiac side-effects of bupropion and citalopram in clinical practice are uncommon (Baldessarini, 2001). These drugs have weak antimuscarinic properties (Baldessarini, 2001) and no significant effect on GIRK1/4 channels (Figure 2b). Therefore, the minimal cardiotoxic effects of bupropion and citalopram may be partly attributable to their lack of any significant effect on atrial GIRK channels, together with their weak antimuscarinic effects.

Desipramine, a norepinephrine reuptake inhibitor, reduces ethanol consumption like SSRIs including fluoxetine (Murphy *et al*, 1985; Naranjo and Knoke, 2001), suggesting a relation between the antidepressants and ethanol effects. Recently, we demonstrated that fluoxetine inhibited ethanol-induced GIRK1/2 currents (Kobayashi *et al*, 2003). The present study also demonstrates that desipramine at micromolar concentrations can inhibit GIRK1/2 currents induced by ethanol. However, GIRK2-deficient mice displayed no significant change in ethanol consumption (Blednov *et al*, 2001). Further studies using mice treated with antisense oligodeoxynucleotides for the GIRK mRNAs, mice lacking other GIRK subunit(s) in addition to the GIRK2 subunit and mutant mice with GIRK channels insensitive to ethanol alone may help to advance the understanding of the antagonism by desipramine of ethanol-induced GIRK effects *in vivo* as well as the understanding of the functions of GIRK channels.

Lastly, our electrophysiological studies have clarified the functional actions of various psychotropic drugs on GIRK channels using *Xenopus* oocyte expression assays (Kobayashi *et al*, 1999, 2000, 2003). Recent studies using GIRK2-deficient mice suggested that GIRK activators may be useful for the treatment of epilepsy and pain (Signorini *et al*, 1997; Ikeda *et al*, 2002; Blednov *et al*, 2003). In addition, since

GIRK knockout mice show decreased cocaine self-administration (Morgan *et al*, 2003), selective GIRK channel inhibitors might be potential agents for the treatment of abusers of cocaine. Therefore, as GIRK channels are considered candidates for clinically relevant targets, it may be important to clarify the pharmacological and physiological effects of various agents interacting with GIRK channels.

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Buprenorphine Antinociception is Abolished, but Naloxone-Sensitive Reward is Retained, in μ-Opioid Receptor Knockout Mice

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Buprenorphine is a relatively nonselective opioid receptor partial agonist that is used in the management of both pain and addiction. To improve understanding of the opioid receptor subtypes important for buprenorphine effects, we now report the results of our investigation on the roles of μ-, δ-, and κ-opioid receptors in antinociceptive responses and place preferences induced by buprenorphine. Buprenorphine antinociception, assessed by hot-plate and tail-flick tests, was significantly reduced in heterozygous μ-opioid receptor knockout (MOR-KO) mice and abolished in homozygous MOR-KO mice. In contrast, buprenorphine retained its ability to establish a conditioned place preference (CPP) in homozygous MOR-KO, although the magnitude of place preference was reduced as the number of copies of wild-type μ-opioid receptor genes was reduced. The remaining CPP of buprenorphine was abolished by pretreatment with the nonselective opioid antagonist naloxone, but only partially blocked by pretreatment with either the δ-selective opioid antagonist naltrindole or the κ-selective opioid antagonist norbinaltorphimine. These data, and biochemical confirmation of buprenorphine actions as a partial δ-, μ-, and κ-agonist, support the ideas that μ-opioid receptors mediate most of analgesic properties of buprenorphine, but that μ- and δ- and/or κ-opioid receptors are each involved in the rewarding effects of this drug.
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Keywords: opioid receptor; knockout mice; buprenorphine; antinociception; reward; detoxification

INTRODUCTION

Buprenorphine is a relatively long-acting nonselective partial agonist of opioid receptors that has been widely used as an analgesic and an antiaddiction therapeutic. Previous reports suggest that systemically administered buprenorphine can produce μ-opioid receptor-mediated antinociceptive actions and also antagonize morphine antinociception (Cowan *et al*, 1977; Kamei *et al*, 1995; 1997). Intrathecal (i.t.) buprenorphine administration produces antinociception that can be antagonized by κ-opioid antagonists, and it also blocks the antinociceptive effects of κ-opioid agonists in the acetic acid writhing test (Kamei *et al*, 1995; Leander, 1988; Tejwani and Rattan, 2002). Although Neilan *et al* (1999) reported buprenorphine to be a partial δ-opioid receptor agonist, Pick *et al* (1997) did not find such an effect. Each opioid receptor subtype has thus been implicated in buprenorphine antinociception, but with several inconsistencies.

Buprenorphine is also used as a therapeutic agent for patients with opioid dependence (Cheskin *et al*, 1994; Lintzeris *et al*, 2002), even though its own abuse liability is manifest by findings including its self-administration by laboratory animals (Mello *et al*, 1988; Winger and Woods, 2001). The precise molecular mechanisms underlying the therapeutic and rewarding effects of buprenorphine have not been clearly delineated, although investigators have estimated its antinociceptive and rewarding effects by using selective agonists and antagonists. Recent success in developing knockout mice with μ-opioid receptor gene deletions have allowed definition of the loss of the analgesic and rewarding effects of morphine that occurs in mice in the absence of μ-opioid receptors (Kieffer, 1999; Loh *et al*, 1998; Sora *et al*, 1997b, 2001). DPDPE, an agonist active at δ-opioid receptors with some affinity for μ-opioid receptors, has a much weaker analgesic effect in homozygous μ-opioid receptor knockout (MOR-KO) mice (Matthes *et al*, 1998; Sora *et al*, 1997a). These observations are especially interesting since the distribution of δ- and κ-opioid receptors is nearly normal in MOR-KO mice (Loh *et al*, 1998; Matthes *et al*, 1996; Sora *et al*, 1997b).

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We now report herein the results of further investigations into the molecular mechanisms that underlie antinociceptive and rewarding effects of buprenorphine, which we conducted by using various pharmacological agents, MOR-KO mice, and cDNAs for μ-, δ-, or κ-opioid receptors. We found abolition of buprenorphine-elicited thermal analgesia in homozygous MOR-KO mice, but retention of some naloxone-sensitive buprenorphine rewarding effects in these animals. These observations are supplemented by *in vitro* data that document partial buprenorphine agonism at δ- as well as μ- and κ-opioid receptors. Our results indicate that μ-opioid receptors play mandatory roles in buprenorphine antinociception and that δ-, κ-, and μ-opioid receptors are involved in buprenorphine reward.

METHODS

Animals

Wild-type, heterozygous, and homozygous MOR-KO mouse littermates from crosses of heterozygous/heterozygous MOR-KO mice with a C57BL/6J genetic background, as described previously (Sora *et al*, 2001), served as subjects. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines. Naive adult (>10 weeks old) mice were housed in an animal facility maintained at 24±1°C and 50% relative humidity under a 12/12 h light/dark cycle with lights on at 0800 and off at 2000. Food and water were available *ad libitum*.

Drugs

For *in vivo* assays, all drugs were dissolved in saline and injected into animals in volumes of 10 ml/kg. Buprenorphine hydrochloride, naloxone hydrochloride, naltrindole hydrochloride, and norbinaltorphimine dihydrochloride (norBNI dihydrochloride) were purchased from SIGMA Chemical Co. (St Louis, MO). Morphine hydrochloride was purchased from Sankyo Co. (Tokyo, Japan).

For *in vitro* assays, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a μ-opioid receptor-selective agonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor agonist, were purchased from Peninsula Laboratories Ltd. (Merseyside, UK). (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]benzeneacetamide (U69593), a κ-opioid receptor-selective agonist, was a gift from Upjohn (Kalamazoo, MI). [tyrosyl-3,5-^{3}H(*N*)]DAMGO (50.5 Ci/mmol), [phenyl-3,4-^{3}H]U69593 (47.5 Ci/mmol), and [tyrosyl-2,6-^{3}H(N)]DPDPE (33.0 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA).

Antinociceptive Tests

Hot-plate testing was performed according to the method of Woolfe and MacDonald (1944) with slight modifications. A commercially available apparatus consisting of acrylic resin cage (20 × 25 × 25 cm: width × length × height) and a thermo-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) were used for this test. Mice were placed on a 52±0.2°C hot plate, and latencies to paw licking were recorded with a cutoff time of 60 s. Tail-flick testing was carried out according to the method of D'Amour and Smith (1941) with slight modifications, by using a commercially available apparatus consisting of an irradiator for heat stimulation and a photosensor for the detection of the tail-flick behavior (Model MK-330A, Muromachi Kikai Co., Tokyo, Japan). Mice were loosely wrapped in a felt towel, their tails were heated, and tail-flick latencies were automatically recorded with a cutoff time of 15 s. Tail-flick and then hot-plate testing were conducted 20 min after each subcutaneous (s.c.) drug injection. Buprenorphine was administered in doses of 0.1, 0.2, 0.7, and 2.0 mg/kg, for cumulative doses of 0.1, 0.3, 1.0, and 3.0 mg/kg, respectively. Morphine was injected s.c. at a dose of 10 mg/kg. The hot-plate and tail-flick responses of each mouse in the drug-induced antinociception were converted to the percent of maximal possible effect (%MPE) according to the following formula:

$$\%\text{MPE} = \frac{(\text{post drug latency} - \text{pre drug latency})}{(\text{cut-off time} - \text{pre drug latency})} \times 100\%$$

Conditioned Place Preference (CPP) Test

CPP test was carried out according to the method of Hoffman and Beninger (1989) with some modifications. For this test, we used a two-compartment plexiglass chamber, one compartment (17.5 × 15 × 17.5 cm: width × length × height) was black with a smooth floor and the other was of the same dimensions, but white with a textured floor. For pre- and postconditioning test phases, a T-style division with double 6 × 6 cm openings allowed access to both compartments. During the conditioning phases, the openings were eliminated to restrict mice to a single compartment. Locomotion and time spent in each compartment was recorded by using an animal activity monitoring apparatus equipped with an infrared detector (Neuroscience Inc., Osaka, Japan). The compartment chamber was placed in a sound- and light-attenuated box under conditions of dim illumination (about 40 lx). Conditioned place preferences were assessed by a protocol consisting of three phases (preconditioning, conditioning, and test phases). On days 1 and 2, the mice were allowed to freely explore the two compartments through the openings for 900 s and acclimatized to the apparatus. On day 3 (preconditioning phase), the same trial was performed and the time spent in each compartment was measured for 900 s. There was no significant difference between time spent in the black compartment with a smooth floor (464±12 s, n=92) and time spent in the white compartment with a textured floor (436±12 s, n=92), indicating that there was no preference before conditioning in the apparatus itself. We selected a counterbalanced protocol in order to nullify each mouse's initial preference, as discussed previously (Tzschentke, 1998). Biased mice that spent more than 80% of the time (ie 720 s) on one side on day 3 or more than 600 s on one side on day 2 and more than 600 s on the other side on day 3 were not used for further experiments. Conditioning was conducted once daily for 4 consecutive days (days 4–7). Mice were injected with either buprenorphine (1.0 mg/kg s.c.) or saline and immediately confined to the black or