Introduction

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) are the most widely used first-line treatment for major depression and have also proved to be effective for many anxiety disorders (1, 2). As with other types of antidepressants, SSRIs have been shown to exert anti-nociceptive effects in humans (3) and also in animal experiments (4 – 6). Since SSRIs inhibit 5-HT reuptake transporters and thus elevate the extracellular concentrations of 5-HT, the increased efficacy of 5-HT transmission in the central nervous system (CNS) is thought to primarily contribute to the analgesic effects of SSRIs.

Fluvoxamine was the first SSRI to be developed and generates relatively potent analgesic effects in comparison with other SSRIs (5). Such analgesic effects are evident in the rat formalin test (7 – 9) and in acute heat (5, 10) and mechanical nociception (4) in mice. Fluvoxamine relieves chronic neuropathic pain in animal models (4, 7, 11 – 14). It is likely that fluvoxamine generates its analgesic effects by acting at both the
supraspinal and spinal levels. Intracerebroventricularly injected fluvoxamine has been shown to generate analgesic effects on acute heat nociception (10) and formalin-induced acute pain (7). In addition, most previous studies have demonstrated its analgesic effects on acute and chronic pain after intrathecal injection (10–13), in relation with the bulbospinal descending 5-HT pathway (15). However, despite the various behavioral studies that have assessed its analgesic effects on acute and chronic pain, there has been no information about the effects of fluvoxamine on nociceptive synaptic transmission, which could be of crucial importance for understanding the analgesic mechanisms of SSRIs.

In the study presented here, we focused on spinal nociceptive transmission and investigated the effects of fluvoxamine on monosynaptic A-fiber- and C-fiber-mediated excitatory postsynaptic currents (EPSCs) recorded from whole-cell voltage-clamped superficial dorsal horn neurons in spinal slices with attached dorsal roots prepared from naïve adult mice. The results of fluvoxamine on spontaneous and miniature EPSCs (sEPSCs and mEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) were also studied. The results suggested that fluvoxamine reduced A-fiber- and C-fiber-mediated EPSCs via presynaptic mechanisms involving 5-HT1A and/or 5-HT3 receptors.

Materials and Methods

All of the experimental protocols used in the present study were approved by the Animal Care and Use Committee of Nagoya City University, and carried out in accordance with the guidelines of the National Institutes of Health and The Japanese Pharmacological Society.

Slice preparation

Transverse slices of the spinal cord were made as reported previously (16). Briefly, 5–6-week-old male ddY-strain mice (SLC, Shizuoka) were anesthetized with urethane (1.6 g/kg, i.p.) and α-chloralose (15 mg/kg, i.p.), and a dorsal laminectomy was performed in the lumbosacral region. After the spinal cord had been isolated, spinal cord slices (450 μm) with the L4 or L5 dorsal root left intact were prepared using a vibratome (DSK-1000; Dosaka, Kyoto) in ice-cold low-sodium artificial cerebrospinal fluid (low-sodium ACSF, pH 7.4, containing 113 mM NaCl, 3 mM KCl, 1 mM NaH2PO4, 25 mM NaHCO3, 11 mM β-glucose, 2 mM CaCl2, and 1 mM MgCl2. The slices were then transferred to a recording chamber on the stage of a microscope (BX51-WI; Olympus, Tokyo) and superfused with standard ACSF kept at 30°C–32°C at a rate of 2.0–2.5 ml/min.

Patch-clamp electrophysiology

Whole-cell voltage-clamp recordings were made from substantia gelatinosa (SG) neurons visually identified using an upright microscope (BX51WI, Olympus) with infrared differential interference contrast (IR-DIC) optics. The patch electrodes (2.5–3 μm tip diameter) were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) and had a resistance of 2.5–4 MΩ when filled with the internal solution containing 135 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 1.1 mM EGTA, 2 mM MgCl2, 3 mM MgATP, 0.3 mM TrisGTP, pH 7.4, adjusted with KOH. Dorsal roots were orthodromically stimulated through the suction electrode (0.2–0.5 ms duration, 0.1 Hz) to evoke A-fiber- or C-fiber-mediated EPSCs at a holding potential of −70 mV. At this holding potential, close to the reversal potential for GABA_A and glycine receptors under our experimental conditions, the outward IPSCs were not usually detectable. A-fiber-mediated synaptic responses were considered to be monosynaptic if their response latency remained constant and no failures were observed during high-frequency (20 Hz for 1 s) stimulation. C-fiber-mediated synaptic responses were identified as monosynaptic when they exhibited no failures during low-frequency (1 Hz for 20 s) stimulation (16–18). When asynchronous evoked unitary EPSCs occurring after A-fiber stimulation were recorded (evoked at 0.05 Hz), extracellular calcium was replaced with 4 mM strontium (19). Spontaneous IPSCs (sIPSCs) were obtained at a holding potential of 0 mV using the patch electrodes filled with the internal solution containing 110 mM Cs2SO4, 5 mM TEA, 5 mM HEPES, 5 mM EGTA, 0.5 mM CaCl2, 2 mM MgCl2, 5 mM MgATP, pH 7.2, adjusted with CsOH. At this holding potential, close to the reversal potential for glutamate receptors under our experimental conditions, the inward IPSCs were not usually detectable.

When action potential–independent mEPSCs were recorded, tetrodotoxin (TTX) (0.5 μM) was added to the extracellular solution. Under these experimental conditions, EPSCs, sEPSCs, and mEPSCs were detected as inward deflections, while sIPSCs were detected as outward deflections (EPC-8; HEKA, Darmstadt, Germany), which were low-pass filtered at 4 kHz, and digitized at 10 kHz for computer analysis with pClamp10 software (Molecular Devices, Union City, CA, USA). The access resistance was monitored by measuring capacitive
transients obtained in response to a hyperpolarizing voltage step (10 mV, 10 ms) from a holding potential of −70 mV or 0 mV. All experiments were carried out at 30°C – 32°C.

The effects of fluvoxamine on A-fiber- or C-fiber-mediated EPSCs were evaluated by comparing averaged EPSCs taken during the peak responses to the drug (18 traces for 3 min) with those before drug application (18 traces for 3 min). Asynchronous unitary EPSCs were analyzed offline using a peak detection program (MiniAnalysis; Synaptosoft, Fort Lee, NJ, USA), and the average frequency and amplitude of unitary EPSCs occurring between 25 ms and 900 ms poststimulus to exclude synchronously released evoked events, in the presence of strontium alone and in the presence of both strontium and fluvoxamine, were compared. sEPSCs, mEPSCs, and mIPSCs were analyzed using MiniAnalysis (Synaptosoft), and the frequency and amplitude distribution of the events (ranging from 226 to 772 events) before the end of fluvoxamine application were compared with those (ranging from 295 to 879 events) obtained before application of the drug. Fluvoxamine was applied only once for each slice in this study except for the experiment where the concentration–response relationship was examined.

**Drugs**

Fluvoxamine maleate was a gift from Meiji Seika Kaisha (Tokyo). WAY100635 maleate and tropisetron hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tocris Cookson (Bristol, UK), respectively. Strontium was obtained from Nakalai (Kyoto). Drugs were dissolved in distilled water and then administered by bath application at 1000-fold dilution with standard ACSF.

**Statistical analyses**

All data are expressed as the mean ± S.E.M. Statistical significance was evaluated by a two-tailed t-test with Bonferroni correction after one-way analysis of variance (ANOVA) for multiple comparisons or a two-tailed Student’s t-test (20). When the effects of fluvoxamine on the paired-pulse ratio, asynchronous unitary EPSCs, sEPSCs, mEPSCs, and sIPSCs were evaluated, the paired t-test (two-tailed) was used. The Kolmogorov-Smirnov test was used for comparison of the cumulative probability distributions of sEPSCs, mEPSCs, and sIPSCs. Differences at $P < 0.05$ were considered significant.

**Results**

**Effects of fluvoxamine on monosynaptic A-fiber- and C-fiber-mediated EPSCs**

Monosynaptic A-fiber- and C-fiber-mediated EPSCs were evoked by electrical stimulation of a dorsal root through the suction electrode (Fig. 1A). In this study, monosynaptic A-fiber-mediated EPSCs were evoked at the stimulus threshold of $97 ± 8 \mu A$ (n = 12), suggesting that Aδ-fibers were recruited (21). The stimulus threshold to evoke monosynaptic C-fiber-mediated EPSCs was $830 ± 120 \mu A$ (n = 12).

Bath application of fluvoxamine (10, 30, and 100 μM) concentration-dependently inhibited both monosynaptic A-fiber- and C-fiber-mediated EPSCs in SG neurons voltage-clamped at a holding potential of −70 mV. Figure 1B illustrates representative time courses of EPSC suppression in response to fluvoxamine with a gradual recovery upon washout. These concentrations of fluvoxamine reduced monosynaptic A-fiber-mediated EPSCs by 10.2% ± 7.6% (10 μM, n = 5), 18.4% ± 9.7% (30 μM, n = 5), and 71.6% ± 9.1% (100 μM, n = 6) and monosynaptic C-fiber-mediated EPSCs by 7.2% ± 2.8% (10 μM, n = 5), 19.8% ± 13.8% (30 μM, n = 5), and 63.7% ± 5.4% (100 μM, n = 6) (shown as summary graphs in Fig. 1C).

**5-HT receptor subtypes mediating the fluvoxamine-induced suppression of monosynaptic A-fiber- and C-fiber-mediated EPSCs**

In our previous behavioral study, we demonstrated that the analgesic effect of fluvoxamine on acute mechanical nociception was mediated by 5-HT3 receptors, but not 5-HT1A and 5-HT2A/2C receptors (4). In addition, we also revealed that spinal 5-HT1A receptors mediated the analgesic effects on acute thermal nociception, driven by the bulbospinal 5-HT pathways upon administration of the thyrotropin-releasing hormone (TRH) analogue taltirelin (22). Therefore, WAY100635 and tropisetron, a 5-HT1A receptor antagonist and a 5-HT3 receptor antagonist, respectively, were employed to assess whether these receptor subtypes mediated the fluvoxamine-induced suppression of monosynaptic A-fiber- and C-fiber-mediated EPSCs.

As summarized in Fig. 1C, the inhibitory effect of fluvoxamine on A-fiber-mediated EPSCs was reduced by WAY100635 and tropisetron, whereas its effect on C-fiber-mediated EPSCs was decreased by WAY100635 but not tropisetron. Fluvoxamine (100 μM) reduced monosynaptic A-fiber- and C-fiber-mediated EPSCs by 16.5% ± 7.1% (n = 5) and 9.1% ± 3.3% (n = 3) in the presence of WAY100635, respectively ($P < 0.05$ vs.
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100 µM fluvoxamine alone), and by 24.7% ± 4.2% (n = 5, P < 0.05 vs. 100 µM fluvoxamine alone) and 52.2% ± 11.5% (n = 5) in the presence of tropisetron, respectively.

Effects of fluvoxamine on paired-pulse response of monosynaptic A-fiber-mediated EPSCs

Inhibition of monosynaptic A-fiber- and C-fiber-mediated EPSCs by fluvoxamine could be attributable to either decreased release of glutamate or decreased sensitivity of postsynaptic receptors. To determine the synaptic site of action of fluvoxamine, we first examined the effect of fluvoxamine on the ratio of the amplitude of the second EPSC divided by that of the first [paired-pulse ratio (PPR)] elicited by two successive stimuli of identical strength at an interval of 50 ms. A change in the PPR is considered attributable to a presynaptic change in release probability (23, 24). We applied this paired-pulse protocol to monosynaptic A-fiber-mediated EPSCs. Figure 2 illustrates a typical time course of changes in monosynaptic A-fiber-mediated EPSCs and PPR for each paired stimulation in response to fluvoxamine (100 µM). Fluvoxamine inhibited the first EPSC more potently than the second and therefore increased PPR. In 4 neurons tested, fluvoxamine (100 µM) increased the PPR from 0.72 ± 0.06 to 0.93 ± 0.04...
(P < 0.05, Fig. 2D), suggesting that fluvoxamine decreases the release probability of glutamate from presynaptic terminals of A-fibers.

**Further exploration of the synaptic locus for the inhibitory action of fluvoxamine**

We next examined the effect of fluvoxamine (100 µM) on sEPSCs recorded in 10 SG neurons voltage-clamped at a holding potential of −70 mV. Sample recordings before and during application of fluvoxamine shown in Fig. 3A indicate that fluvoxamine produced a profound increase in the frequency of sEPSCs. This was clearly evidenced by the cumulative probability distribution of sEPSCs inter-event intervals and amplitude (Fig. 3: B, C, respectively). Fluvoxamine increased the mean frequency of sEPSCs from 14.8 ± 4.2 to 31.8 ± 4.8 Hz (Fig. 3: D).
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It also exhibited a weak but statistically significant facilitation of the amplitude of sEPSCs (from $25.3 \pm 3.2$ to $29.9 \pm 2.9$ pA, $P < 0.05$; Fig. 3D). In addition, action potential–independent mEPSCs recorded in the presence of TTX (0.5 µM) showed similar sensitivity to fluvoxamine (100 µM, Fig. 4). In 10 SG neurons, fluvoxamine significantly increased the frequency of mEPSCs from $15.9 \pm 2.5$ to $25.7 \pm 3.6$ Hz ($P < 0.05$), and it also increased their amplitude from $23.9 \pm 1.3$ to $29.3 \pm 2.1$ pA ($P < 0.05$, Fig. 4D). By contrast, fluvoxamine (100 µM) had no apparent influence on sIPSCs mediated via GABA<sub>A</sub> and glycine receptors recorded in 4 SG neurons at a holding potential of 0 mV (Fig. 5). The frequency of sIPSCs before
and during application of fluvoxamine were 2.7 ± 0.5 and 2.7 ± 0.6 Hz, respectively (P < 0.05, Fig. 5D). The amplitude of sIPSCs were not changed (from 34.4 ± 8.1 to 32.7 ± 7.7 pA, P > 0.05; Fig. 5D). Together, these results alone suggest that fluvoxamine may increase glutamate release by presynaptic mechanisms. However, it is plausible that they reflect largely the effects on excitatory interneurons, since SG neurons receive glutamatergic excitatory synaptic inputs from spinal intrinsic interneurons in addition to the primary afferent fibers.

Hence, to assess precisely the effect on excitatory synaptic transmission between SG neurons and the primary afferents, unitary EPSCs arising from the primary afferent fibers were isolated. Thus, desynchronization of the quantal events underlying the evoked monosynaptic A-fiber-mediated EPSCs was elicited by strontium (25), and the effect of fluvoxamine on the amplitude and frequency of asynchronous unitary events was examined. Monosynaptic A-fiber-mediated EPSCs were first elicited in standard ACSF followed by continuous perfusion of ACSF containing 4 mM strontium instead of 2 mM calcium. Introduction of 4 mM strontium–containing external solution increased asynchronous unitary events, as illustrated by a typical time course shown in Fig. 6, and reduced the amplitude of monosynaptic A-fiber-mediated EPSCs. Similar to the effect on monosynaptic A-fiber-mediated EPSCs in normal external solution shown in Fig. 1, fluvoxamine (100 μM) further reduced the amplitude of monosynaptic A-fiber-mediated EPSCs remaining in 4 mM strontium–containing external solution (data not shown) and also reduced the frequency of asynchronous unitary events without significant influences on their amplitude (Fig. 6D). In 5 neurons, the frequency of asynchronous unitary events occurring between 25 ms and 900 ms poststimulus was increased from 9.7 ± 3.5 to 36.7 ± 3.6 Hz by substituting strontium for extracellular calcium, and this was reduced to 26.2 ± 5.8 Hz (decrease by 28.7% ± 9.1%, P < 0.05) by further adding fluvoxamine (100 μM). By contrast, the amplitude of asynchronous unitary events was slightly increased from 28.9 ± 4.1 to 32.7 ± 3.2 pA by substituting strontium for extracellular calcium, which was not reduced, but rather increased by fluvoxamine to 42.1 ± 6.4 pA (increase by 27.6% ± 12.7%, not significant).

**Discussion**

In the present study, we carried out an in vitro electrophysiological investigation of the effect of the SSRI fluvoxamine on spinal nociceptive synaptic transmission and its pharmacology in the mouse dorsal horn. To this end, we employed spinal slices with an attached dorsal root prepared from adult mice and focused on the effects
on monosynaptic excitatory synaptic transmission mediated via A-fibers and/or C-fibers stimulated electrically through a dorsal root. Fluvoxamine inhibited both monosynaptic A-fiber- and C-fiber-mediated EPSCs, to which 5-HT$_{1A}$ and 5-HT$_3$ receptors were found to contribute differently. Moreover, analysis of synaptic sites of action using paired stimuli and afferent-derived asynchronous unitary events revealed that fluvoxamine modulated synaptic release of glutamate rather than its postsynaptic receptors. By contrast, fluvoxamine increased glutamate release from spinal interneurons without affecting GABA and glycine release, as we demonstrated in the study of spontaneous and/or miniature postsynaptic currents in the presence of fluvoxamine, which may hamper its inhibitory effect on pain signaling.

SSRIs blocks primarily reuptake of 5-HT and thus increases the extracellular level of endogenous 5-HT, which is therefore thought to be a likely first step leading to amelioration of depressive diseases and also other pharmacological effects including pain relief. In particular, the analgesia induced by SSRIs appears to involve increased 5-HT levels in the supraspinal region and/or spinal cord, as supported by several previous behavioral studies involving local intracerebroventricular or intrathecal injection (7, 10 – 13) and depletion of supraspinal (6) and bulbospinal (4) 5-HT levels.

Exogenously increased 5-HT has been shown to suppress both monosynaptic A-fiber- and C-fiber-mediated EPSCs in spinal slice preparations from adult rats (26, 27), which may partly contribute to the antinociceptive effects demonstrated in rats given 5-HT intrathecally (28 – 30). However, few previous studies have addressed the relationship between endogenously increased 5-HT and nociceptive synaptic transmission. As we demonstrated here in spinal slice preparations from adult mice, fluvoxamine also suppressed both monosynaptic A-fiber- and C-fiber-mediated EPSCs, suggesting that endogenously increased 5-HT can also exert similar influences on nociceptive synaptic transmission (Fig. 1).

In this study, fluvoxamine was superfused in the range of 10 – 100 $\mu$M to reduce A- and C-fiber-mediated EPSCs. By contrast, fluvoxamine was shown to produce 50% inhibition of 5-HT uptake measured in synaptosomal suspension of rat cerebral at 0.3 $\mu$M (31). We consider that higher concentrations of fluvoxamine were needed to approach and sufficiently block the 5-HT transporter in the slice preparation. In fact, the finding that the inhibition of A- and C-fiber-mediated EPSCs by fluvoxamine involved activation of 5-HT receptors supports the idea that superfusion of these concentrations of fluvoxamine really increased the extracellular level of endogenous 5-HT. However, we should bear in mind that fluvoxamine could affect some molecular targets other than the 5-HT transporter in the micromolar range. Lee et al. (32) demonstrated that fluvoxamine inhibited the voltage-gated K$^+$ channel Kv1.5 transfected in Chinese hamster ovary (CHO) cells with an IC$_{50}$ value of 2.0 $\mu$M. Therefore, it is plausible that some undefined effects other than 5-HT reuptake inhibition could contribute to any effects demonstrated in the present study. The 5-HT receptor subtypes mediating suppression of monosynaptic A-fiber- and C-fiber-mediated EPSCs remain undetermined in rat spinal slices (26, 27). Here, we further demonstrated that suppression of monosynaptic A-fiber-mediated EPSCs by fluvoxamine involved activation of 5-HT$_{1A}$ and 5-HT$_3$ receptors, whereas suppression of monosynaptic C-fiber-mediated EPSCs by fluvoxamine was mediated by 5-HT$_{1A}$ but not 5-HT$_3$ receptors (Fig. 1), keeping in mind that both pronociceptive and antinociceptive roles have been demonstrated for spinal 5-HT$_{1A}$ and 5-HT$_3$ receptors (15). Nevertheless, although we focused only on monosynaptic nociceptive synaptic transmission in the spinal dorsal horn, the present electrophysiological and pharmacological experiments employing specific antagonists of 5-HT$_{1A}$ and 5-HT$_3$ receptors may at least partly support our previous behavioral studies that addressed the relationship between endogenously released 5-HT and receptor subtypes mediating its antinociceptive effects assessed using acute heat and mechanical stimuli; the analgesic effect of fluvoxamine on acute mechanical nociception was mediated by 5-HT$_3$ receptors (4), and the analgesic effect on acute thermal nociception, driven by the bulbospinal 5-HT pathways upon administration of the TRH analogue taltirelin, was mediated by 5-HT$_{1A}$ receptors (22). In addition, 5-HT$_3$ receptors have been shown to be involved in the antinociceptive effect of the SSRI paroxetine on acute nociception elicited by thermal stimuli (33).

The paired-pulse protocol and analysis of miniature synaptic currents such as mEPSCs are the most widely used approaches for assessing the synaptic sites of drug action. Fluvoxamine increased the PPR concurrently with a reduction of monosynaptic A-fiber-mediated EPSCs (Fig. 2), which is considered to reflect a presynaptic change in release probability (23, 24). More importantly, fluvoxamine reduced the frequency of A-fiber-evoked asynchronous unitary events without having any significant influence on their amplitude (Fig. 6). In the spinal cord, dorsal horn neurons receive excitatory inputs from a variety of different origins, and thus mEPSCs may reflect largely unitary synaptic events derived from spinal intrinsic interneurons, which hampers precise analysis of the unitary currents arising from nociceptive afferents. Indeed, we demonstrated here that the mean frequency of sEPSCs and mEPSCs...
was profoundly increased by fluvoxamine (Figs. 3 and 4), which we did not explore further in this study. Therefore, it is of crucial importance to isolate these unitary synaptic currents, as we recorded asynchronous A-fiber-evoked unitary events in strontium-containing ACSF. These results strongly suggest a presynaptic locus for the inhibitory effect of fluvoxamine on monosynaptic A-fiber-mediated EPSCs. Moreover, the finding that fluvoxamine did not exert postsynaptic modulation, as evidenced by the absence of significant influence on the amplitude of asynchronous events, also suggests that fluvoxamine reduced monosynaptic C-fiber-evoked EPSCs through presynaptic mechanisms as well. These electrophysiological results are consistent with studies demonstrating the presence of both 5-HT1A and 5-HT3 receptors on the primary afferent terminals (see ref. 34 for review). In addition, the finding that blockade of 5-HT3 receptors did not affect the inhibitory effect of fluvoxamine on monosynaptic C-fiber-mediated EPSCs suggests distinct roles of 5-HT3 receptors on A-fiber and C-fiber terminals in modulating nociceptive synaptic transmission. Moreover, intrinsic GABAergic neurons also express 5-HT3 receptors (35, 36), activation of which has been shown to increase the release of GABA (37). More recently, Xie et al. have shown that the 5-HT3 receptor agonist mCPBG increases both the amplitude and frequency of GABAergic and glycergic sIPSCs in SG neurons of rat spinal cord slices (38). Thus, increased GABA and/or glycine could act on primary afferent terminals to reduce nociceptive synaptic transmission. However, endogenously increased 5-HT is unlikely to reach such 5-HT3 receptors located on presynaptic terminals of inhibitory interneurons, since sIPSCs were not influenced by fluvoxamine (Fig. 5).

In conclusion, we have demonstrated that the SSRI fluvoxamine exerts presynaptic inhibition of monosynaptic nociceptive excitatory synaptic transmission from primary afferents. Although endogenously increased 5-HT can potentially activate multiple subclasses of 5-HT receptors, the present study revealed that activation of 5-HT1A and 5-HT3 receptors can reduce release probability at primary afferent terminals and therefore suppress nociceptive synaptic transmission, which may account for the synaptic mechanism underlying the antinociceptive effect of fluvoxamine. Since diverse receptor subtypes for 5-HT are present in the spinal dorsal horn (15, 39, 40), any possible contribution of subtypes other than 5-HT1A and 5-HT3 receptors to the inhibitory effect of fluvoxamine on either A-fiber- and C-fiber-mediated nociceptive transmission needs to be further investigated. Moreover, further attempts to explore whether subtype-specific agonists for 5-HT receptors mimic the effects of fluvoxamine here may strengthen our understanding of the roles of 5-HT in modulating pain signaling in the spinal dorsal horn.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (C) (20602004, 23590720) from the Japan Society for the Promotion of Science, Tokyo, Japan (M.T.). There are no financial or other relationships that might lead to a conflict of interest.

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