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Stimulation of serotonin transport by the cyclic GMP phosphodiesterase-5 inhibitor sildenafil

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Abstract

The serotonin (5-hydroxtryptamine, 5-HT) transporter (SERT) plays a critical role in the inactivation of synaptic 5-HT and has been implicated in multiple psychiatric and peripheral disorders. SERT regulation studies demonstrate that activation of cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-linked pathways can increase SERT activity. As cGMP actions are limited by cGMP-specific phosphodiesterase (PDEs), we investigated whether the cGMP-specific PDE5 inhibitor sildenafil (Viagra®) can stimulate 5-HT uptake and potentiate cGMP-mediated regulation. In RBL-2H3 cells, SERT activity was stimulated by sildenafil in a concentration- and time-dependent manner. Sildenafil also enhanced the stimulation of SERT triggered by the adenosine receptor agonist 5′ -*N*-ethylcarboxamidoadenosine (NECA), effects blocked by the PKG inhibitor *N*-[2-(methylamino)ethy]-5-isoquinoline-sulfonamide (H8). Sildenafil stimulation of 5-HT uptake arises from an increase in 5-HT transport Vmax and is paralleled by elevated SERT surface antagonist binding, also H8-sensitive. These findings implicate cGMP-targeted PDEs in limiting the regulation of antidepressant-sensitive 5-HT transport.

Keywords: Serotonin; Transporter; cGMP; PKG; Sildenafil; PDE5

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter in both the central and peripheral nervous systems, participates in a variety of physiological processes including gastrointestinal and platelet function, arousal, appetite and mood (Nestler et al., 2001). In turn, disrupted 5-HT signaling has been implicated in a wide range of disorders including irritable bowel syndrome, anxiety disorders, obsessive compulsive disorder, and depression (Lucki, 1998). Inactivation of 5-HT following release at synapses is mediated by the antidepressant-sensitive 5-HT transporter (SERT, 5-HTT). SERTs are members of the SLC6A gene family of Na+/Cl− dependent solute transporters, encoded in man by a single gene localized to chromosome 17q11.2 (Ramamoorthy et al., 1993). Recent studies have uncovered evidence for altered SERT gene expression or transport function in disorders linked to altered serotonergic signaling, including autism, obsessive compulsive disorder, depression and suicide (Cook and Leventhal, 1996; Flattem and Blakely, 2000; Kilic et al., 2003; Ozaki et al., 2003; Caspi et al., 2003).

Altered SERT function in disease states may arise from change in SERT structure, as encoded by genetic variants (Ozaki et al., 2003), or alternatively from compromised SERT regulation pathways. SERT-mediated transport activity is acutely regulated by multiple signaling pathways (Blakely et al., 1998). Treatments of transfected cells

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expressing SERTs with protein kinase C (PKC) activators or protein phosphatase 1/2A (PP1/2A) inhibitors leads to transporter phosphorylation and a loss of SERT cell surface density (Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy and Blakely, 1999). Protein kinase A (PKA) and protein kinase G (PKG) activators also trigger SERT phosphorylation (Ramamoorthy et al., 1998). Interestingly, RBL-2H3 cells (Miller and Hoffman, 1994) and platelets (Launay et al., 1994) have been found to support G-protein coupled receptor stimulation of SERT through a PKG linked pathway. Using RBL-2H3 cells, we (Zhu et al., 2004) have recently defined distinct trafficking-dependent and independent pathways supporting SERT stimulation downstream of adenosine receptor and guanylyl cyclase activation.

Recognizing that signaling through cGMP-dependent pathways is limited by the conversion of cGMP to 5′ GMP by phosphodiesterase (PDE) 5 (Francis et al., 2001), we noted in our initial report that adenosine receptor stimulation could be potentiated by the selective PDE5 inhibitor sildenafil (Viagra®). As a further definition of limits on SERT regulation could elucidate novel therapeutic opportunities, we further explored the ability of sildenafil on its own to modulate SERT activity and to enhance adenosine receptor-triggered SERT activation. We demonstrate that, in RBL-2H3 cells, sildenafil stimulates SERT activity in a concentration and time-dependent manner. Sildenafil is active on SERT at concentrations where it also potentiates adenosine receptor-mediated SERT stimulation. We show that SERT stimulation arises from an increase of 5-HT transport capacity (Vmax), accompanied by a commensurate increase in the surface SERT binding. As with adenosine receptor stimulation of SERT, sildenafil stimulation of 5-HT uptake and elevation of SERT surface levels can be completely abolished by PKG inhibition. Using transfected Chinese Hamster Ovary (CHO) cells, we demonstrate that these effects of sildenafil are specific for SERT and not observed for homologous norepinepherine and dopamine transporters (NET and DAT, respectively). These studies reinforce an important role for PKG-linked pathways in establishing SERT surface density, and indicate specificity among biogenic amine transporters that may be useful in future dissection of mechanisms supporting transporter regulation and in the development of therapeutics.

2. Materials and methods

5′ -*N*-ethylcarboxamidoadenosine (NECA) and *N*-[2-(methylamino)ethy]-5-isoquinoline-sulfonamide (H8) were purchased from Sigma (St. Louis, MO). Sildenafil was purified from Viagra® tablets as described earlier (Corbin et al., 2003), and part was a gift from Pfizer (Groton, CT). Paroxetine, and desipramine are obtained from Sigma (St. Louis, MO). 1-(2-Diphenylmethoxyethyl)-4-(3-phenylpropyl) piperazine dihydrochloride (GBR 12935 dihydrochloride) is a product of Tocris (Ellisville, MO). [^{3}H]5-HT (5-hydroxy[^{3}H]tryptamine trifluoroacetate, 94 Ci/mmol) was purchased from Amersham (Arlinton, Heights, IL); [^{125}I]RTI-55 (3β-(4-iodophenyl)-tropane-2β-carboxylic acid methylester tartrate, 2200 Ci/mmol) was purchased from Dupont NEN (Boston, MA). Trypsin-EDTA, glutamine, ampicillin/streptomycin were purchased from Life Technologies (Long Island, NY); Modified Eagle Medium (MEM) and Dulbecco's MEM (DMEM) were derived from Invitrogen (Carlsbad, CA) reagents and prepared in the Vanderbilt Media Core. hSERT (Ramamoorthy et al., 1993), hNET (Pacholczyk et al., 1991) and hDAT (Giros et al., 1992) cDNAs have been described. hDAT was a gift from Dr. Caron (Duke University, Durham, NC). RBL-2H3 cells (ATCC, Manassas, VA) were maintained in MEM containing 15% fetal bovine serum (Invitrogen), 1% L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Chinese hamster ovary (CHO) cells (ATCC) were maintained in DMEM containing 10% fetal bovine serum, 1% L-glutamine and penicillin /streptomycin. For comparison of the response of monoamine transporters to cGMP/PDE5 inhibitor, CHO cells were transfected with cDNAs for hSERT, hDAT or hNET (100 ng/well for 24 well plate). Transfections were performed using Mirus reagent (Madison, WI) according to manufacturer's protocol (0.3 μl/well). SERT, DAT or NET cDNAs were pre-incubated with the reagent at ambient temperature for 30 min before adding to plated CHO cells.

[^{3}H]5-HT transport assays were performed as described previously (Ramamoorthy et al., 1998; Zhu et al., 2004). Briefly, RBL-2H3 cells were seeded in 24-well plate (40,000 cells/well) 24 h prior to the 5-HT uptake assay. CHO cells were plated at 20,000/well 16-24 h before transfection and the uptake assay was performed 24 h following the transfection. Medium was removed by aspiration and cells were washed once with Krebs-Ringer's HEPES (KRH) buffer containing 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 g/l glucose, 10 mM HEPES, PH 7.4. Cells were incubated in triplicate at 37 °C in KRH buffer (0.2 ml/well) containing 100 μM pargyline, 100 μM ascorbic acid and 1.0 mM tropolone (Sigma), with or without modifiers. After 10 min incubation with [^{3}H]5-HT (100 nM for RBL-2H3 cells, 20 nM for SERT-transfected CHO cells), [^{3}H]dopamine (50 nM), or [^{3}H]norepinephrine (50 nM) at 37 °C, buffer was aspirated and the cells were washed three times with ice-cold KRH buffer. Cells were solubilized with 0.5 ml Microscint 20 (Packard Bioscience, Meriden, CT) and tritium-labled monoamine accumulation was quantitated using a TopCount plate scintillation counter (Packard Bioscience, Meriden, CT). Specific 5-HT, dopamine and norepinephrine uptake was determined by subtracting the amount of [^{3}H]5-HT, [^{3}H]dopamine and [^{3}H]norepinephrine accumulated in the presence of 10 μM paroxetine, GBR 12935 and desipiramine, respectively.

For studies measuring SERT surface density, we quantitated the binding of the high affinity cocaine analog

[^{125}I]RTI-55 (5 nM) to intact cells at 4 °C for 45 min in PBS/CM buffer (phosphate buffered saline, pH 7.4 with 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$) in the presence or absence of a membrane permeant (10 μM paroxetine) or membrane impermeant (100 μM 5-HT) displacer, defining surface and total specific binding respectively (Zhu et al., 2004). Binding was terminated by two rapid washes with ice-cold PBS/CM. Cells were solubilized with 1% sodium dodecyl sulfate (SDS) and [^{125}I]RTI-55 bound was quantified on a Gamma 4000 counter (Beckman, Irvine, CA). Data from transport and binding assays were analyzed using GraphPad Prism (GraphPad, San Diego, CA).

3. Results

To evaluate the impact of PDE5 inhibition on basal and adenosine receptor-stimulated 5-HT transport, RBL-2H3 cells were pre-incubated for varying times with medium, sildenafil (varying concentrations), or with the adenosine receptor agonist NECA (varying concentrations) plus 4 μM sildenafil, prior to the addition of [^{3}H]-5-HT and measurement of amine accumulation. As shown in Fig. 1A, 4 μM sildenafil alone (points at 0 NECA concentration) evoked a significant stimulation of 5-HT transport activity (118.6±1.8%, $p<0.05$, Student's t-test) relative to vehicle. Additionally, 4 μM sildenafil yielded an additive stimulation of uptake activity when combined with NECA (1 μM 143.3±4.5%, $p<0.01$, Student's t-test). The effects of 4 μM sildenafil were time-dependent, reaching a maximum at 10 min of pre-incubation (Fig. 1B). The sildenafil effect on SERT activity was dose-dependent, reaching a maximum at 4 μM (Fig. 1C), a concentration where maximum potentiation of NECA (1 μM) stimulation was also seen. Saturation kinetic analysis revealed that sildenafil's effects on 5-HT uptake arise from a change in 5-HT Vmax (control: 405±24 fmol/well/min vs. sildenafil: 462±33 fmol/well/min vs. NECA±sildenafil: 545±34 fmol/well/min, n=3) with no significant change in Km (control: 0.84±0.15 μM vs. sildenfil: 0.82±0.18 μM vs. NECA±sildenafil: 0.77±0.15 μM, n=3). As evidence of a cGMP and PKG involvement in sildenafil's actions, the PKG inhibitor H8 completely abolished sildenafil stimulation of 5-HT uptake (Fig. 2A), as well as the effects of sildenafil plus NECA (Zhu et al., 2004). Importantly, at this concentration, H8 has no significant effects on its own. Consistent with these data,

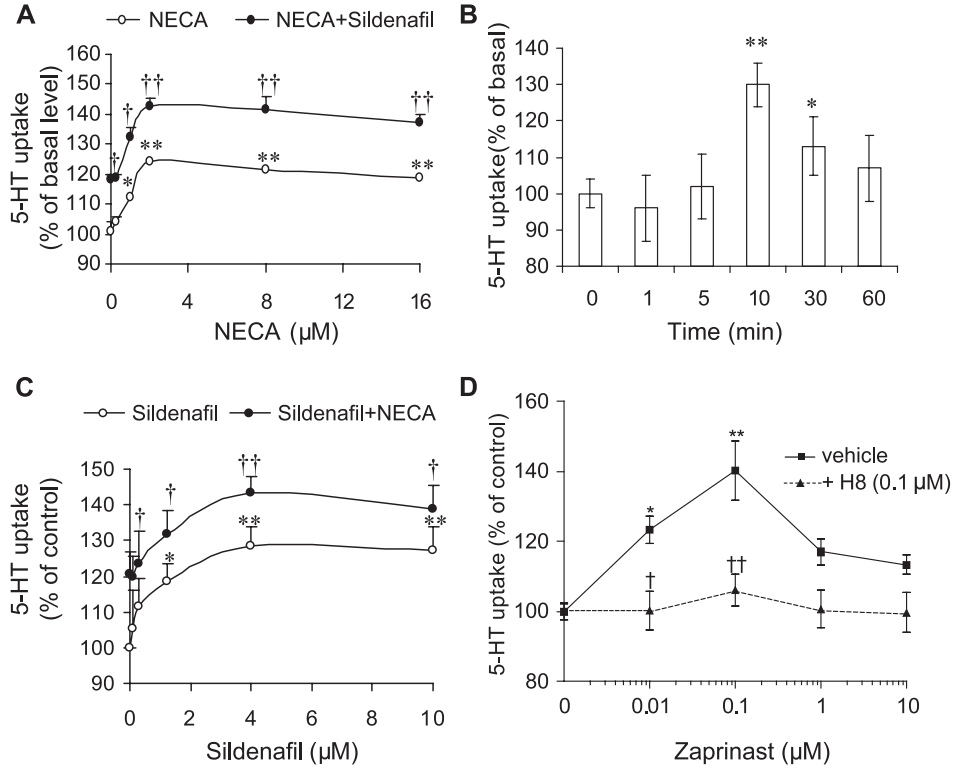

Fig. 1. Sildenafil elevates basal and NECA-enhanced 5-HT uptake in RBL-2H3 cells. (A) Cells (4×10^4/well) were treated with sildenafil at the indicated concentrations for 10 min in the absence/presence of 1 μM NECA prior to transport assay. Sildenafil-induced 5-HT uptake is enhanced by NECA. (B) Influence of pretreatment duration on sildenafil (4 μM) stimulation of 5-HT uptake. (C) Potentiation of NECA effects on SERT by sildenafil. Cells were treated with NECA at the indicated concentrations for 10 min in the absence/presence of 4 μM sildenafil prior to 5-HT transport assays. Co-application of sildenafil and NECA potentiates 5-HT uptake. Values are expressed as the mean of findings from at least three experiments±SEM. *$p<0.05$, **$p<0.01$ (one-way analysis of variance (ANOVA)-Dunnett) vs. basal controls; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. sildenafil (A) or NECA (C) at corresponding dose. (D) Zaprinast stimulates a 5-HT uptake in an H8-sensitive manner. Cells were pre-incubated with vehicle or H8 (0.1 μM) for 15 min followed by the treatment with zaprinast at indicated concentration. *$p<0.05$, **$p<0.01$ (one-way ANOVA-Dunnett) vs. basal controls; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. zaprinast.

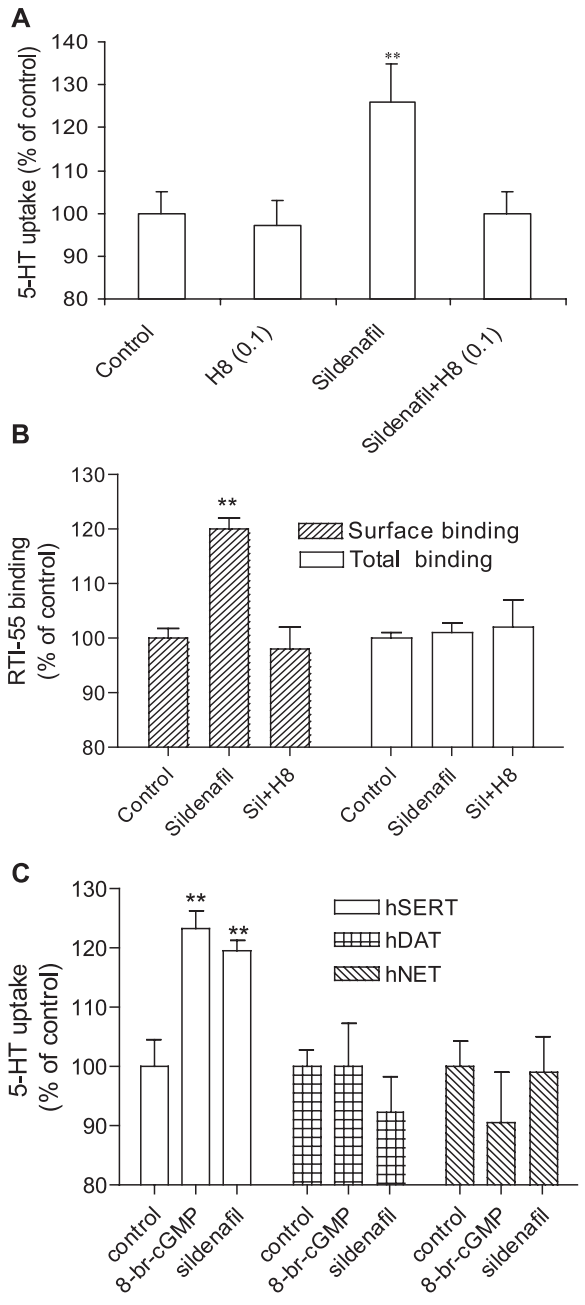

Fig. 2. The PKG inhibitor H8 blocks sildenafil-stimulation of 5-HT uptake and cell surface [^{125}I]RTI-55 binding. (A) PKG dependence of sildenafil-induced 5-HT uptake. RBL-2H3 cells were pretreated for 20 min with H8 (0.1 μM) or vehicle followed by incubation with sildenafil (4 μM) for 10 min followed by 5-HT transport assays. H8 abolishes sildenafil-stimulated 5-HT uptake. (B) PKG dependence of sildenafil-induced surface binding. Treatment with sildenafil induces an increase in SERT surface binding. RBL-2H3 cells were pre-incubated with vehicle or H8 (0.1 μM) for 20 min followed by incubation with sildenafil (4 μM) for 10 min before chilling cells on ice and measurement of 5-HT sensitive [^{125}I]RTI-55 binding. Sildenafil induces a significant increase in surface binding that is sensitive to H8. Total binding is not affected. (C) CHO cells transfected with hSERT, hDAT and hNET cDNAs were treated with (1) 8-bromo-cGMP (10 μM), (2) sildenafil (4 μM) for 10 min prior to uptake assay. Both 8-bromo-cGMP and sildenafil induced an increase of 5-HT uptake, but failed to stimulate dopamine or norepinephrine uptake. Values are expressed as mean values (n=3)±SEM. **p<0.01 vs. control (Student's t-test).

zaprinast, a structurally distinct PDE5 inhibitor, also increases SERT activity in an H8-sensitive manner (Fig. 1D).

Stimulation of 5-HT uptake capacity could arise from an increase in SERT surface density or from the conversion of surface-resident carriers to more active states. In this regard, NET is known to be subject to both trafficking-dependent and -independent modes of regulation (Apparsundaram et al., 2001), and our most recent studies reveal both modes participate in SERT regulation (Zhu et al., 2004). To explore these possibilities, we implemented a whole cell, radioligand binding paradigm using the cocaine analogue [^{125}I]RTI-55. RTI-55 is membrane permeant and can label both surface and intracellular sites. We have developed a paradigm where paroxetine (10 μM) is used as a displacer to monitor total (surface plus intracellular) SERT binding to compare with levels of surface SERT, as defined with the hydrophilic ligand 5-HT (100 μM) (Zhu et al., 2004). Additionally, our assay temperature (4 °C) is chosen to limit 5-HT uptake and prevent access to intracellular SERT sites labeled by the radioligand. By this assay, we find that approximately 30% of SERTs are surface resident in RBL-2H3 cells. Their levels can be reduced by acute phorbol ester treatment, or enhanced by NECA application (Zhu et al., 2004). When cells were treated with sildenafil under conditions that evoke a change in 5-HT transport activity and then chilled for binding assays, we detected no changes in the density of paroxetine-defined, total [^{125}I]RTI-55 binding (Fig. 2B). In contrast, we observed a significant increase in 5-HT-defined [^{125}I]RTI-55 surface binding. The level of increase in surface density (120±3.4% vs. control) matches well the expectation from transport assays (see Fig. 1). Importantly, as with transport assays, the PKG inhibitor H8 completely blocked the elevations in 5-HT sensitive [^{125}I]RTI-55 binding triggered by sildenafil without having effects on its own (Fig. 2B). These findings indicate that the previously ascertained changes in 5-HT transport capacity triggered by sildenafil arise from an increase in surface SERT density.

To assess whether the activation of a cGMP/PKG pathway plays a similar role for regulation of SERT, DAT and NET, CHO cells were transfected separately with human SERT, DAT or NET cDNAs, followed by incubation with 8-bromo-cGMP and sildenafil. Consistent with our previous study (Zhu et al., 2004), both 8-bromo-cGMP and sildenafil stimulated 5-HT uptake. In contrast, neither dopamine nor norepinephrine uptake was significantly affected by these two compounds (Fig. 2C). These findings suggest that among biogenic amine transporters, SERT appears to be a specific target for cGMP-dependent regulation, as modeled in transfected cells.

4. Discussion

Sildenafil is the first effective oral treatment for erectile dysfunction (Goldstein et al., 1998). The drug acts by

inhibiting PDE5, an enzyme found in the corpus cavernosum, allowing cGMP to accumulate and causing relaxation of the smooth muscle with concomitant increased blood flow leading to an erection (Marmor and Kessler, 1999). Recently, central side effects associated with sildenafil, including headache, dizziness, depression, insomnia, abnormal dreams and anxiety, as well as aggressive behavior, have been reported (Milman and Arnold, 2002). Although primary actions are peripheral, sildenafil appears to cross the blood-brain barrier (Milman and Arnold, 2002); thus, it is very likely that some side effects may be caused by actions of sildenafil in the brain, including inhibition of CNS PDE5. Guanylyl cyclase (GC)-mediated production of cGMP is also known to be triggered by nitric oxide synthetase (NOS) activation and nitric oxide production. In the brain, NOS, GC and PDE5 are present at highest activities in hippocampus, cerebral cortex, and basal ganglia (Garthwaite and Boulton, 1995), areas known to receive dense 5-HT projections (Nestler et al., 2001). Importantly, NOS is expressed in serotonergic neurons and projections from the raphe nucleus (Simpson et al., 2003). These findings indicate that stimulation of NOS may increase cGMP levels in serotonergic neurons leading to GC activation. The subsequent increase in cGMP may then act via PKG to regulate SERT activity. Thus the actions of sildenafil we monitor in vitro using RBL-2H3 cells may have parallels in CNS alterations in cGMP level and serotonergic signaling. Sildenafil could lower synaptic 5-HT levels by increasing 5-HT transport, as observed in RBL-2H3 cells. Studies are underway to assess these possibilities. Such actions would be consistent with findings of sildenafil-associated anxiety and depression, effects that may be effectively counteracted by selective serotonin reuptake inhibitors.

Although the sildenafil effect on 5-HT uptake, like the NECA effect (Zhu et al., 2004), arises from changes in transport capacity, the ability of sildenafil to generate additive stimulation when NECA effects are saturated could be interpreted as evidence of a separate pathway other than the cGMP/PKG pathway previously implicated in NECA responses (Miller and Hoffman, 1994; Zhu et al., 2004). Alternatively, NECA effects may be dose limited by recruitment of receptors with opposing actions or by efficient hydrolysis of cGMP produced by NECA stimulation. The latter explanation is supported by the fact that the PKG inhibitor H8 abolished sildenafil stimulation of 5-HT uptake as well as the effects of sildenafil plus NECA (Zhu et al., 2004). These findings indicate that in RBL-2H3 cells a sildenafil-triggered elevation in PKG activity (most likely via elevated cytosolic cGMP levels; Miller and Hoffman, 1994), underlies the stimulation of SERT transport capacity. This conclusion is reinforced by our finding that a separate PDE5 inhibitor, zaprinast, also increases SERT activity in an H8-sensitive manner.

Our findings of sildenafil-modulated SERT activity is made all the more intriguing by recent findings that an obsessive compulsive disorder-associated SERT coding variant (Ozaki et al., 2003), which has been found to exhibit constitutively stimulated SERT activity, is reportedly refractory to nitric oxide-triggered and guanylyl cyclase-dependent SERT activation (Kilic et al., 2003). Possibly, neuronal SERTs may be targeted by PKG to affect alterations in transport capacity, and sildenafil could potentiate basal or receptor-linked stimulation of this pathway, as it does in RBL-2H3 cells. Certainly, we also recognize that multiple neurotransmitter systems may be engaged by sildenafil in the brain in vivo, making interpretations of physiologic roles for these pathways difficult to interpret with pharmacological inhibitors alone. In this regard, we have also found stimulation of SERT by other PDE5 inhibitors including zaprinast. As such, these findings, in conjunction with our previous findings (Zhu et al., 2004), encourage further studies to explore the role of PKG-linked SERT regulation in vivo, and the impact of such modulations on psychiatric disorders and their therapeutics.

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