Risperidone Irreversibly Binds to and Inactivates the h5-HT₇ Serotonin Receptor

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ABSTRACT

Risperidone displays a novel mechanism of antagonism of the h5-HT$_7$ receptor. Pretreatment of the cells with 5 or 20 nM risperidone, followed by removal of the drug from the media, renders the 5-HT$_7$ receptors unresponsive to 10 μM 5-HT for at least 24 h. Thus, risperidone seems to be producing a rapid, long-lasting inactivation of the h5-HT$_7$ receptor. Whole-cell radioligand binding studies indicate that risperidone interacts in an irreversible or pseudo-irreversible manner with the h5-HT$_7$ receptor, thus producing the inactivation. Internalization of the h5-HT$_7$ receptor was not detected by monitoring green fluorescent protein-labeled fluorescent forms of the h5-HT$_7$ receptor exposed to 20 nM risperidone. Ten other antagonists were tested for h5-HT$_7$-inactivating properties, and only 9-OH-ris-peridone and methiothepin were found to demonstrate the same anomalous properties as risperidone. These results indicate that the h5-HT$_7$ receptor may possess unique structural features that allow certain drugs to induce a conformation resulting in an irreversible interaction in the intact membrane environment. This may indicate that the h5-HT$_7$ receptor is part of a subfamily of G-protein-coupled receptors (GPCRs) possessing this property or that many GPCRs have the potential to be irreversibly blocked, but only select drugs can induce this effect. At the very least, the possibility that highly prescribed drugs, such as risperidone, are irreversibly antagonizing GPCR function in vivo is noteworthy.

G-protein-coupled receptors (GPCRs) are the largest single family of receptors expressed in the mammalian genome (Lefkowitz, 2004). Approximately 600 genes express GPCRs, which serve to detect the levels of a multitude of extracellular and intracellular substances and induce intracellular changes designed to adapt to the changes in the extracellular environment. Synthetic agonists mimic endogenous substances in stimulating GPCRs, and competitive antagonists act by binding to the receptor but causing no effect, thereby preventing agonist stimulation of the receptor. Competitive antagonists display two hallmark characteristics: 1) antagonist inhibition can be overcome by increasing agonist levels while maintaining constant competitive antagonist levels and 2) agonist concentration-response curves are shifted to the right in the presence of a constant level of competitive antagonist (Egan et al., 2000). Additions and exceptions to these rules have been observed, including receptor reserve, which predicts maximal receptor functionality at agonist fractional occupancies less than 100%, and constitutive activity, which is the degree of receptor stimulation observed in the absence of any agonist (Teitler et al., 2002; Kenakin, 2004; Purohit et al., 2005).

Risperidone was one of the first of a new generation of antipsychotic drugs developed based on potency as a 5-HT$_{2A}$ and D$_2$ dopamine receptor antagonist (Leysen et al., 1988; Meltzer et al., 1989). These "atypical" antipsychotic drugs have been found to be superior to the original "typical" antipsychotic drugs in that they produce fewer extrapyramidal side-effects and are beneficial in treating the negative symptoms of psychosis (Meltzer et al., 1989). As part of an ongoing investigation into the neurochemical effects of atypical antipsychotic drugs, we produced a cell line expressing a constitutively active mutant form of the h5-HT$_7$ receptor (Purohit et al., 2004, 2005). We noted that some antipsychotic drugs, including risperidone, displayed inverse agonist properties with potencies similar to their affinities for the receptor, whereas other drugs displayed far lower potencies than anticipated based on their affinities for the receptor (Roth et al., 1994; Purchit et al., 2005). It was hypothesized that an allosteric mechanism might explain some of the apparent discrepancies in the potencies of the inverse agonists, and this allosteric mechanism might be functional in the native

ABBREVIATIONS: GPCR, G-protein-coupled receptor; PCR, polymerase chain reaction; GFP, green fluorescent protein; ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; Ro-20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone.

h5-HT$_7$ receptor. Therefore, the present study was performed to investigate, in greater detail, the mechanism of action of risperidone at the h5-HT$_7$ receptor expressed in HEK-293 cells. Risperidone was a potent inhibitor of cAMP production induced by 10 μM 5-HT, with an IC$_{50}$ approximately equal to its K_I value of 2 nM. Competitive antagonism predicts that risperidone's potency should have been dramatically reduced in the presence of 5-HT at a concentration ~200-fold over its EC$_{50}$ at the h5-HT$_7$ receptor. Therefore, we conducted a series of experiments to determine the paradoxical high potency of risperidone in inhibiting 5-HT stimulation of the h5-HT$_7$ receptor. Our results indicate that risperidone interacts in an irreversible or pseudo-irreversible manner, producing an inhibition of h5-HT$_7$ receptor activity that seems to be rapid, potent, and essentially complete. This mechanism of action seems to be observed only with the h5-HT$_7$ receptor, in that risperidone has been demonstrated to be a classic competitive antagonist at other serotonin and dopamine receptors.

GPCR desensitization has been studied extensively in vitro (Lefkowitz and Williams, 1978; Sibley and Lefkowitz, 1985; Freedman and Lefkowitz, 1996; Gainetdinov et al., 2004; Lefkowitz et al., 1983; Lefkowitz, 2004). As a rule, receptor desensitization occurs because of a prolonged interaction of a high concentration of an agonist with the receptor. The time course of the desensitization involves hours and often results in a partial loss of activity (usually between 30 and 50%). This process has been shown to be due to the phosphorylation of agonist-occupied GPCRs followed by the binding of arrestin proteins that uncouple the receptor from GTP-binding proteins. A GPCR inactivation process occurring through the interaction of an antagonist with the receptor, at low concentrations, complete within 30 min (see *Results*), is a novel observation. It is noteworthy that methiothepin and risperidone were reported to produce an insurmountable antagonism of 5-HT$_7$-mediated contraction of dog basilar arteries, whereas six other antagonists produced a competitive antagonism (Terron and Falcon-Neri, 1999). Thus, the results and interpretations described herein involving recombinant cell function in vitro are likely to be occurring in vivo.

Materials and Methods

Radioligand Binding. Radioligand binding studies in membrane homogenates were performed as described previously (Purohit et al., 2005). HEK cells stably expressing h5-HT$_7$ receptors (100 mm dish; ~100% confluent) were scraped and collected in 50 mM Tris-HCl, 0.5 mM EDTA, and 10 mM MgSO$_4$, pH 7.6 (at 23°C); centrifuged at 10,000g for 30 min; homogenized using a Polytron homogenizer (Kinematica, Basel, Switzerland); and centrifuged again at 10,000g for 30 min. The membranes were resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM MgSO$_4$, and 0.1% ascorbic acid, pH 7.6 (at 23°C). Assays were performed in triplicate 1.0-ml volumes containing 10 μg of membrane protein (which was added last). Assays containing 2 nM [^{3}H]5-HT (24 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), 1 nM [^{3}H]mesulergine (82 Ci/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK), or 1 nM [^{3}H]risperidone (43 Ci/mmol; Janssen Pharmaceutica, New Brunswick, NJ) (Gommeren et al., 1997) were performed in the absence and presence of 10 μM clozapine to detect the level of available h5-HT$_7$ receptors. Specific binding was generally ~85–95% of total binding. Tubes were incubated for 30 min at 37°C and filtered, and the filters were washed with 10 ml of Tris buffer. Radioactivity was measured using a liquid scintillation counter at an efficiency of 40%. Experimental results were analyzed using Prism

Software (GraphPad Software, San Diego, CA). Protein content of the samples was determined with the use of a BCA protein assay kit (Pierce Chemical, Rockford, IL).

Whole-cell radioligand binding studies were performed as described previously with minor modifications (Shenoy et al., 2006). Radioligands were incubated with the cells for 30 min in the presence and/or absence of nonradioactive drugs, the media was removed, cells were rinsed with PBS, and cells were lysed with 3% trichloroacetic acid. The dissociation rate experiments involved a 30-min incubation with radioligand, addition of 10 μM mesulergine, and addition of 3% TCA at the appropriate time points. The released radioactivity was added to scintillation vials and counted on a Beckman scintillation counter (efficiency 40%).

cAMP Assay. A modification of the procedure of Salomon et al. (1974) was used. Cells were plated in six-well plates (2×10^6 cells/well) for 24 h and then incubated with [^{3}H]adenine (4 μCi/well) for 24 h. Ro-20-1724 (0.25 mM), a phosphodiesterase inhibitor, was included in all subsequent media treatments. Typical drug treatment experiments involved a 30-min pretreatment with media or drug followed by exposure to 5-HT for 15 min. Where applicable, media was aspirated and replaced three times to remove drugs, followed by exposure to 5-HT for 15 min. Control experiments demonstrated that this procedure produced no effect on the cells' responsiveness to 5-HT (see *Results*), and [^{3}H]risperidone, [^{3}H]5-HT, and [^{3}H]mesulergine tracer experiments demonstrated that three washes eliminated >99% of the drug from the original media. Cells were then lysed, and total cAMP was isolated and eluted using Dowex column chromatography. [^{3}H]cAMP levels were assessed using liquid scintillation counting in Ultima Gold XR. For radioligand binding studies, subsequent to the final aspiration, radioligand binding buffer was added, and cells were treated as described above (see *Radioligand Binding*).

Creation of GFP Fusion Proteins. h5-HT$_7$-GFP fusion proteins were created using the pEGFP-N1 vector from Clontech (Mountain View, CA). This vector contains multiple cloning sites upstream and in frame with the GFP, enabling the creation of a fusion protein with the GFP attached to the C terminus of the receptor. PCR was performed to amplify the entire open reading frame of the h5-HT$_7$ with primers designed to remove the stop codon, while adding SstI and BamHI restriction sites at the 5' and 3' ends, respectively. SstI/BamHI digests of the PCR products were ligated into the pEGFP vector in frame with the GFP DNA sequence. DNA sequencing was performed (Center for Comparative Functional Genomics facility) to confirm that no additional mutations were created during the PCR step. DH5α *Escherichia coli* were transformed with the vector/receptor cDNA and grown overnight in Luria-Bertani broth with selection antibiotic, and DNA were extracted using a miniprep kit from QIAGEN (Valencia, CA).

Confocal Microscopy in Living Cells. HEK-293 cells were transiently transfected with h5-HT$_7$–GFP fusion protein in 100-mm dishes. Twenty-four hours after transfection, cells were seeded in serum-free medium on polylysine-coated glass coverslips and incubated in serum-free medium overnight before microscopy. The cells were imaged live in PBS on a confocal imaging system (LSM-510 META; Zeiss, Thornwood, NY) using a 63$\times$ 1.4 numerical aperture oil immersion objective at room temperature. The GFP was excited with the 488 nm line from an argon laser and emission collected using a band pass filter at 500 to 550 nm. The pinhole was set at 1.32 Airy units that results in a z-resolution of 2 μm. Several images of GFP expressing cells were collected to establish a baseline and then either 300 nM clozapine or 20 nM risperidone was added to the cells, and incubation was continued for an additional 30 min. Images were collected at a rate of 1 image/min for 30 min. Cells expressing the GFP fusion protein were also imaged in phosphate-buffered saline for only 30 min to establish that receptors did not internalize in the absence of added drug (data not shown). Images were collected using the Aim software (Zeiss) and the figures were assembled using Adobe Photoshop.

Results

Contrary to classic competitive antagonism, risperidone was found to inhibit 50 nM and 10 μM 5-HT-stimulated cAMP with identical IC_{50} values (K_I = 2.6 nM) (data not shown). To further investigate the mechanism producing this unexpected effect of risperidone, the effects of risperidone and clozapine on increasing concentrations of 5-HT was investigated (Fig. 1). The inhibition by 20 nM risperidone of 5-HT-mediated stimulation of cAMP accumulation in HEK-293 cells stably expressing h5-HT$_7$ receptors was clearly insurmountable at 5-HT concentrations as high as 10 μM. The EC_{50} of 5-HT at the h5-HT$_7$ receptor was 64 nM (Fig. 1), indicating that 10 μM 5-HT was 156-fold in excess of its EC_{50}. Risperidone at 20 nM is 10-fold in excess of its K_i value for the h5-HT$_7$ receptor (Purohit et al., 2005). Likewise, clozapine was included at a concentration of 300 nM, which is ~10-fold over its K_I for the h5-HT$_7$ receptor (Purohit et al., 2005). Unlike risperidone, clozapine's inhibition of 10uM 5-HT was surmountable, consistent with a classic competitive antagonism mechanism of action. Figure 1B demonstrates classic competitive antagonism by risperidone of 5-HT-stimulated inositol phosphate accumulation at r5-HT$_{2A}$ receptors. The rightward shift of the 5-HT concentration-response curve and no significant decrease in maximal stimulation are hallmarks of classic competitive antagonism.
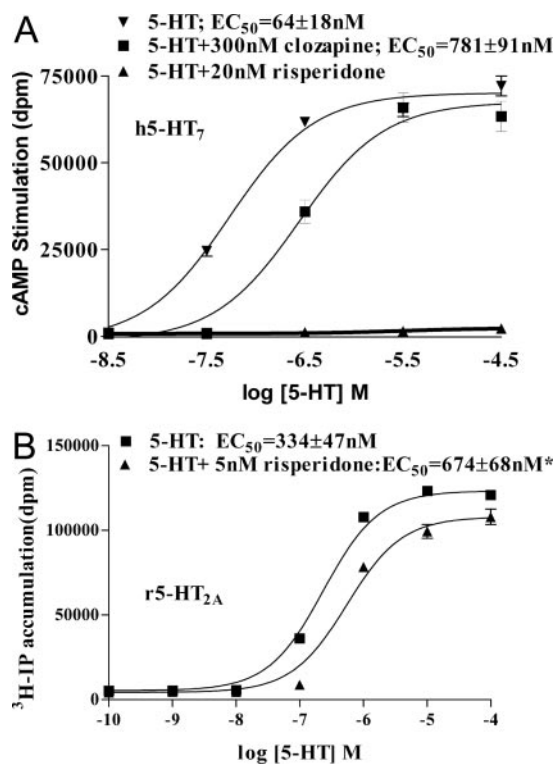
The results in Fig. 1B, compared with the effect of risperidone demonstrated in Fig. 1A (insurmountable antagonism), clearly demonstrate the unusual nature of risperidone's actions at the h5-HT$_7$ receptor, while demonstrating that risperidone did act as a classic competitive antagonist at the r5-HT$_{2A}$ receptor. The data in Fig. 1 indicate that the mechanism of action of risperidone in inhibiting 5-HT-mediated stimulation of cAMP accumulation was different from that of clozapine and was not due to classic competitive antagonism.

Figure 2 demonstrates the unusual effect of 20 nM risperidone, 100 nM 9-OH-risperidone, and 30 nM methiothepin in producing a dramatic inactivation of the h5-HT$_7$ receptor after removing the drug (washout). Cells were treated with no drug (A), 20 nM risperidone (B), 100 nM 9-OH-risperidone (C), 30 nM methiothepin (D), or 300 nM clozapine (E) for 30 min. The media was aspirated and replaced with fresh media three times, and 10 μM 5-HT was then added to the wells. After 15 min, the level of cAMP accumulation was measured. Although clozapine was present at saturating concentrations, there was no inhibition of 10 μM 5-HT, which was 588-fold over its EC_{50} (Fig. 1). However, risperidone, 9-OH-risperidone, and methiothepin produced a profound loss of 10 μM 5-HT-induced cAMP stimulation. These results are consistent with the unusual effects observed in Fig. 1 for risperidone. Mesulergine, penfluridol, amperozide, and cinanserin were found to behave in a classic competitive manner (Fig. 2). Zotepine, fluperlapine, ziprasidone, fluphenazine, chlorpromazine, perphenazine, and thioridazine were also found to produce no inhibition of 10 μM 5-HT stimulation (data not shown).

A possible explanation for the effects of risperidone observed in Figs. 1 and 2 is an irreversible or pseudo-irreversible interaction of risperidone with the h5-HT$_7$ receptor. To determine whether this was occurring, radioligand binding experiments were performed on intact cells stably expressing h5-HT$_7$ receptors. Figure 3 displays the effect of a 30-min
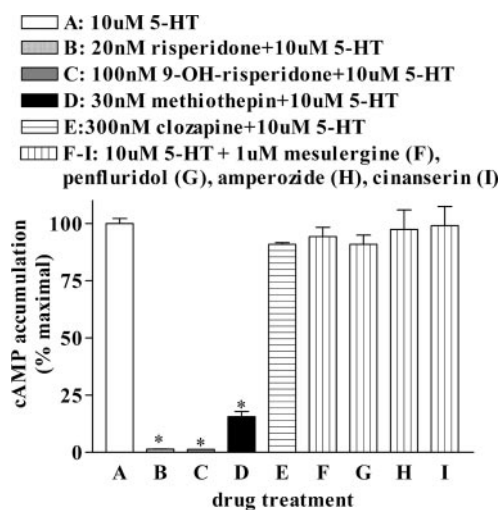

Fig. 1. A, insurmountable antagonism of 5-HT-stimulated cAMP production by 20 nM risperidone, and competitive antagonism by 300 nM clozapine. The EC_{50} values for 5-HT in the absence and presence of 300 nM clozapine were 64 and 781 nM, respectively, consistent with a competitive antagonist mechanism. The lack of 5-HT-stimulated cAMP production in the presence of 20 nM risperidone is not consistent with a competitive mechanism. B, surmountable antagonism of 5-HT-stimulated r5-HT$_{2A}$ receptors by 5 nM risperidone. These results indicate that risperidone is a competitive antagonist at the r5-HT$_{2A}$ but not at the h5-HT$_7$ receptor. The results are the means and S.E.M. of four independent experiments performed in triplicate. *, $p < 0.01$ versus 5-HT EC_{50} (Student's t test).

Fig. 2. Inactivation of the h5-HT$_7$ receptor. After a 30-min incubation with media containing no drug (A), 20 nM risperidone (B), 100 nM 9-OH-risperidone (C), 30 nM methiothepin (D), 300 nM clozapine (E), 1 μM mesulergine (F), 1 μM penfluridol (G), 1 μM amperozide (H), or 1 μM cinanserin (I), the cells were thoroughly washed and stimulated with 10 μM 5-HT for 15 min. Results are the mean ± S.E.M. of three independent experiments. In addition (F–I), 1 μM mesulergine, penfluridol, amperozide, and cinanserin produced no residual effects on 10 μM 5-HT stimulation. *, $p < 0.001$ versus A or E.

preincubation with risperidone, 9-OH-risperidone, methiothepin, or clozapine on [^{3}H]5-HT binding. The drugs were removed by repeated aspiration and addition of fresh media, and [^{3}H]5-HT radiolabeling studies were performed on the intact cells (see *Materials and Methods*). In parallel with the cAMP production studies (Fig. 2), risperidone, 9-OH-risperidone, and methiothepin pretreatment significantly inhibited subsequent radiolabeling of the h5-HT$_7$ receptors, whereas pretreatment with clozapine produced no effect on the level of [^{3}H]5-HT specific binding observed. These results indicate that risperidone, 9-OH-risperidone, and methiothepin seem to be interacting irreversibly with the h5-HT$_7$ receptor binding site, which would explain the results observed in Fig. 2. To more directly demonstrate this irreversible interaction, [^{3}H]risperidone specific binding to intact cells was investigated (Fig. 4A). In this experiment, h5-HT$_7$ receptors were labeled with 1 nM [^{3}H]risperidone or 1 nM [^{3}H]mesulergine for 30 min. The media was removed and cells washed three times, and the amount of radioligand bound to the receptor was determined. The presence of [^{3}H]risperidone-specific binding after repeated washing of the cells was a direct demonstration of the irreversible interaction of risperidone with the h5-HT$_7$ receptor. Figure 4B displays the lack of dissociation of specific [^{3}H]risperidone binding after equilibration with h5-HT$_7$ receptor–expressing cells and the addition of 10 μM mesulergine. It is noteworthy that this same pretreatment with risperidone did not produce an irreversible effect when the cells were homogenized and membrane homogenates were pretreated with risperidone and then radiolabeled (data not shown). This might indicate that a soluble factor or a factor loosely associated with the plasma membrane was involved in the irreversible interaction observed in the whole-cell preparation. Figure 5 displays the time course of the rapid loss of h5-HT$_7$ receptors exposed to 5 nM risperidone. These results demonstrate a far more rapid inactivation process than the classical desensitization phenomena associated with GPCRs (see *Discussion*).

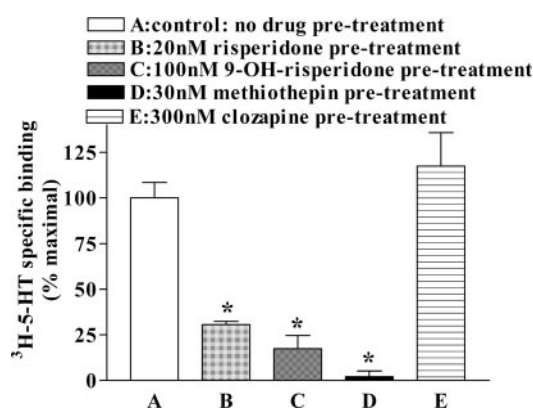To determine the time course of the recovery of function after removal of drugs, cAMP accumulation assays and

whole-cell radioligand binding assays were performed 24 h subsequent to application and removal of the antagonists (Fig. 6). At the 24-h time point, there was only a slight recovery of both function and radioligand binding. These results indicate that the inactivation was an extremely long-lasting effect, and the functional effect and the radioligand binding effect were further evidence that risperidone, 9-OH-risperidone, and methiothepin were binding irreversibly to the h5-HT$_7$ receptor.

To determine the concentration-dependent effect of risperidone on the 5-HT concentration-dependent stimulation of h5-HT$_7$ receptors, cells were exposed to 1, 3, and 5 nM risperidone, washed, and exposed to varying concentrations of 5-HT (Fig. 7). The results demonstrate a concentration-dependent effect on the maximal 5-HT stimulation with no significant effect on EC$_{50}$ values (69, 87, and 44 nM for control, 1 and 3 nM risperidone pretreatments, respectively).
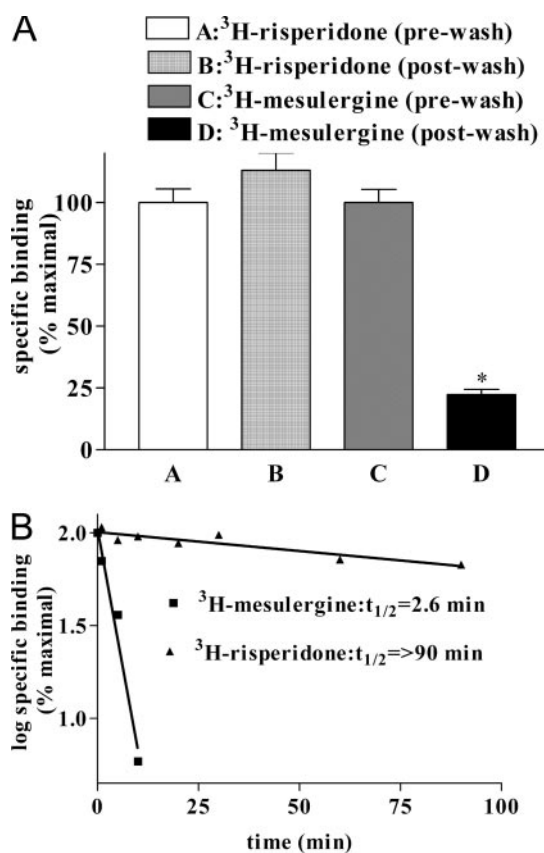

Fig. 4. A, irreversibility of specific [^{3}H]risperidone binding to h5-HT$_7$ receptors on intact cells. The cells were first exposed to 1 nM [^{3}H]risperidone or 1 nM [^{3}H]mesulergine ($\pm$10 μM clozapine) for 30 min, subjected to intact cell radioligand binding immediately, or washed three times with media then subjected to intact cell radioligand binding. The stability of the specific binding of [^{3}H]risperidone to thorough washing (A versus B) indicates an irreversible interaction. The loss of specific [^{3}H]mesulergine binding indicates a reversible interaction (C versus D). Results are the means $\pm$ S.E.M. of three experiments performed in triplicate [*, $p <$ 0.01 for C versus D (ANOVA)]. B, h5-HT$_7$ dissociation time course for [^{3}H]risperidone and [^{3}H]mesulergine. After a 30-min equilibration with 1 nM [^{3}H]risperidone and 1 nM [^{3}H]mesulergine in the presence and absence of 10 μM clozapine, dissociation was initiated with 10 μM mesulergine (see *Materials and Methods*). [^{3}H]Risperidone specific binding to the h5-HT$_7$ receptor does not dissociate over a 90-min period, whereas [^{3}H]mesulergine readily dissociates from the receptor, consistent with an irreversible or pseudo-irreversible interaction of risperidone with the h5-HT$_7$ receptor and a classic reversible interaction of mesulergine with the h5-HT$_7$ receptor.

Fig. 3. Irreversible antagonism of specific [^{3}H]5-HT binding to h5-HT$_7$ receptors by risperidone, 9-OH-risperidone, and methiothepin. A, 30-min preincubation with no-drug media, followed by three washes (washout), followed by whole-cell [^{3}H]5-HT (2 nM) radioligand binding assay. B, 30-min preincubation with 20 nM risperidone, followed by washout, followed by binding assay. C, as in B but using 100 nM 9-OH risperidone. D, as in B but using 30 nM methiothepin. E, as in B but using 300 nM clozapine. The drug concentrations were 10-fold in excess of their K_I values. Mesulergine (1 μM) was used to determine nonspecific binding, which averaged 2200 dpm and was 90% specific. Results are the means $\pm$ S.E.M. of three independent experiments performed in triplicate.
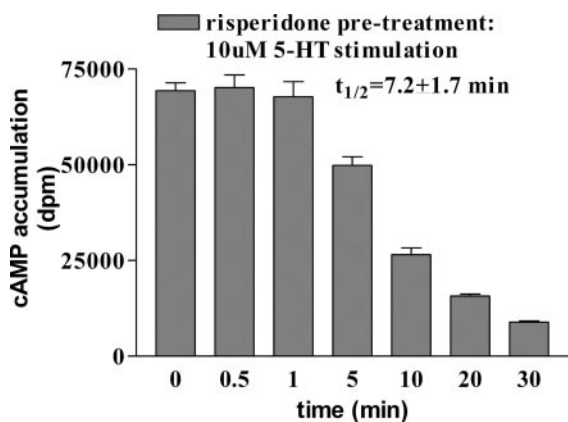
Fig. 5. Time course of risperidone-induced inactivation of h5-HT$_7$ receptors. Cells were preincubated with 5 nM risperidone for the indicated times, followed by treatment with 10 μM 5-HT for 15 min. The $t_{1/2}$ is the mean $\pm$ S.E.M. of three independent experiments.
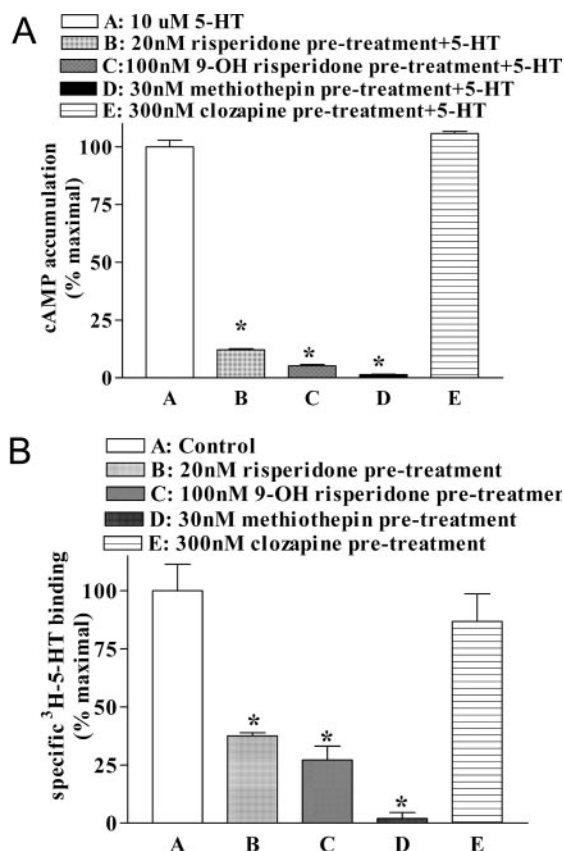

Fig. 6. A, irreversible inactivation of h5-HT$_7$ receptors. Cells were treated with the indicated drugs for 30 min, the drugs were removed, and 24 h later, 10 μM 5-HT-stimulated cAMP production was measured. A, control (no drug pretreatment); B, 20 nM risperidone pretreatment; C, 100 nM 9-OH-risperidone pretreatment; D, 30 nM methiothepin pretreatment; and E, 300 nM clozapine pretreatment. Maximal stimulation ranged from 50,000–80,0000 dpm. Results are the means $\pm$ S.E.M. of three experiments performed in triplicate [∗, $P <$ 0.01 B–D versus A (ANOVA)]. B, irreversible inhibition of [^{3}H]5-HT binding to h5-HT$_7$ receptors, 24 h after exposure and removal of drugs. Cells were treated with the indicated drugs for 30 min, the drugs were removed, and 24 h later, 2 nM [^{3}H]5-HT specific binding to h5-HT$_7$ receptors was measured. A, control (no drug pretreatment); B, 20 nM risperidone pretreatment; C, 100 nM 9-OH risperidone pretreatment; D, 30 nM methiothepin pretreatment; E, 300 nM clozapine pretreatment. Mesulergine (1 μM) was used to determine nonspecific binding, which averaged 2200 dpm and was 90% specific. The results are the means $\pm$ S.E.M. of three independent experiments [∗, $P <$ 0.01 B–D versus A (ANOVA)].

These results are consistent with a concentration-dependent inactivation of h5-HT$_7$ receptors as a result of the increasing levels of risperidone interacting with the receptors during the 30-min pretreatment.

GPCR internalization has received a great deal of attention as a factor in desensitization mechanisms (Shenoy et al., 2006). To determine whether internalization is a key factor in the rapid inactivation of the h5-HT$_7$ receptor, GFP-tagged h5-HT$_7$ receptors were expressed in HEK cells, and the effects of 5-HT, risperidone, and clozapine on internalization of the receptors was monitored with confocal microscopy (Fig. 8). No obvious internalization was produced by risperidone or clozapine under conditions that produce inactivation of the GFP-tagged h5-HT$_7$ receptors. The h5-HT$_7$-GFP fusion protein was found to demonstrate the same inactivating response to risperidone and 9-OH-risperidone as the native h5-HT$_7$ receptor (data not shown). These observations indicate that a robust, rapid internalization mechanism is apparently not involved in the inactivation of the h5-HT$_7$ receptor observed in our studies.
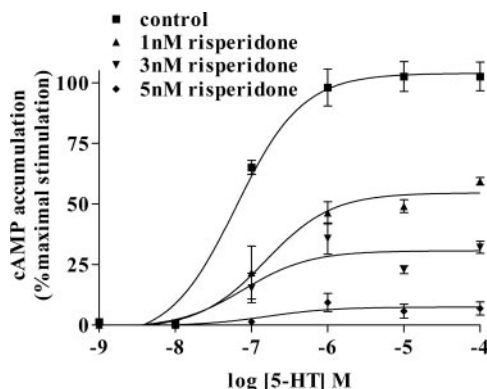

Fig. 7. Concentration-dependent inactivation of h5-HT$_7$ receptor function by risperidone. Cells were pre-exposed to 0, 1, 3, and 5 nM risperidone for 30 min, washed, and stimulated with 10 μM 5-HT. Maximal 5-HT stimulation ranged from 60,000 to 80,000 dpm. Results are the means $\pm$ S.E.M. of three independent experiments performed in triplicate. There were no significant differences in the EC$_{50}$ values for 5-HT (63, 69, 87, and 44 nM for control, 1, 3, and 5 nM risperidone, respectively). There was a significant difference in the maximal (10^{-4} M 5-HT) stimulation for control, 1, 3, and 5 nM risperidone treatments ($p <$ 0.01; ANOVA).
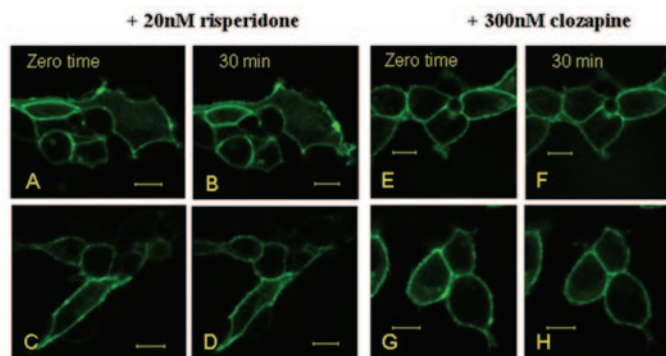

Fig. 8. Confocal imaging of GFP-tagged h5-HT$_7$ receptors: lack of acute effects of risperidone and clozapine on receptor internalization. HEK-293 cells expressing GFP-tagged h5-HT$_7$ receptors were imaged live in the absence (time 0, A and C) and 30 min after the addition of 20 nM risperidone (B and D). Likewise, h5-HT$_7$-GFP–expressing cells were imaged in the absence (zero time, E and G) and 30 min after the addition of 300 nM clozapine (F and H). Scale bar, 10 μm.

Discussion

The data presented strongly indicate that risperidone, 9-OH-risperidone, and methiothepin produce an irreversible or pseudo-irreversible interaction with the h5-HT₇ receptor. This irreversible interaction results in a rapid inactivation of the h5-HT₇ receptor after exposure to any of these three drugs. The irreversible interaction of risperidone, 9-OH-risperidone, and methiothepin seems to be a somewhat unusual effect, because the majority of antagonists produce classic competitive antagonism.

The biological and pharmacological implications of this work are not trivial. A study of dog cerebral artery 5HT₇-mediated relaxation produced results corresponding to our results (Terron and Falcon-Neri, 1999). In that study, methiothepin and risperidone were found to produce an insurmountable antagonism of 5-HT₇-mediated relaxation of an in vivo prostaglandin-induced cerebral arterial constriction. Clozapine, mesulergine, and spiperone were found to produce surmountable antagonism in that study, corresponding to the surmountable antagonism of the h5-HT₇ receptor produced by the drugs in our studies. The inactivation of cerebral arterial 5-HT₇ receptors by risperidone would be predicted to produce an elevated, long-lasting basal constriction of the cerebral artery, because the 5-HT₇ receptor seems to contribute a vasodilatory response. This effect, in turn, might be expected to produce a propensity to cerebrovasculature constriction and stroke in situations in which the cerebral artery is stimulated to constrict. It is noteworthy that risperidone has been reported to produce an elevated likelihood of stroke in clinical trials in patients suffering from agitation due to dementia and/or Alzheimer's disease (Wooltorton, 2002; Schneider et al., 2006). The higher incidence of stroke in the patients treated with risperidone may have nothing to do with the h5-HT₇ receptor inactivation noted in our studies. However, the inactivating effect noted herein does provide a possible pharmacological mechanism for the higher incidence of stroke in the patients treated with risperidone. Given the enormous population of patients that have been and are being prescribed risperidone, any unusual interaction or effect of this drug on a significant pharmacological target, such as brain and/or cardiovascular h5-HT₇ receptors, is worthy of note and further investigation.

The unusual effect of risperidone, 9-OH-risperidone, and methiothepin on the h5-HT₇ receptor raises the possibility that this is not just an idiosyncratic effect of these drugs on this particular GPCR. An irreversible antagonist interaction with the M_5 muscarinic receptor has been reported (Grant and El Fakahany, 2005). It is possible that some of the drugs commonly used and categorized as competitive antagonists may be producing irreversible interactions not observed with the currently accepted methods of receptor antagonism testing. Concentration-dependent inhibition of agonist stimulation provides an IC_{50} value for an antagonist but does not provide a mechanism. Although most studies of antagonists do include experiments that reveal mechanisms (i.e., Schild analyses) many published studies do not. For instance, the classic paper on the potent antagonist properties of risperidone at the 5-HT₂ receptor includes an inhibition curve but not a Schild analysis (Leysen et al., 1988). Our own studies using recombinant cells demonstrate the classic competitive antagonist properties of risperidone at the r5-HT₂ₐ receptor

(Fig. 1B). It is clear that a majority of drugs being developed as therapeutics undergo detailed mechanistic studies that would reveal irreversible or pseudo-irreversible effects. However, the studies described herein emphasize the importance of not assuming a competitive mechanism for a drug at one receptor based on a competitive mechanism demonstrated at another receptor.

Finally, the lack of irreversibility observed in the interaction of risperidone with the h5-HT₇ receptor on membrane preparations that have been subjected to repeated homogenization and resuspension (data not shown) may be a key observation. This result implies that a factor present in the intact cells (and in the isolated dog cerebral artery preparation) may be key to the irreversible interaction and is lost during the membrane preparation. This factor may be common to GPCRs or may be associated with a subset of GPCRs. Given the importance of GPCRs as cellular components and as drug targets, any novel factor that modulates drug-receptor interaction with GPCRs is a notable contribution. Studies to determine the reason for the difference in the risperidone-h5-HT₇ receptor interaction between the intact cell preparations and the homogenate preparations should reveal important novel mechanistic insights into GPCR function.

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