Antidepressants and Antipsychotic Drugs Colocalize with 5-HT₃ Receptors in Raft-Like Domains

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Despite different chemical structure and pharmacodynamic signaling pathways, a variety of antidepressants and antipsychotics inhibit ion fluxes through 5-HT₃ receptors in a noncompetitive manner with the exception of the known competitive antagonists mirtazapine and clozapine. To further investigate the mechanisms underlying the noncompetitive inhibition of the serotonin-evoked cation current, we quantified the concentrations of different types of antidepressants and antipsychotics in fractions of sucrose flotation gradients isolated from HEK293 (human embryonic kidney 293) cells stably transfected with the 5-HT_{3A} receptor and of N1E-115 neuroblastoma cells in relation to the localization of the 5-HT₃ receptor protein within the cell membrane. Western blots revealed a localization of the 5-HT₃ receptor protein exclusively in the low buoyant density (LBD) fractions compatible with a localization within raft-like domains. Also, the antidepressants desipramine, fluoxetine, and reboxetine and the antipsychotics fluphenazine, haloperidol, and clozapine were markedly enriched in LBD fractions, whereas no accumulation occurs for mirtazapine, carbamazepine, moclobemide, and risperidone. The concentrations of psychopharmacological drugs within LBD fractions was strongly associated with their inhibitory potency against serotonin-induced cation currents. The noncompetitive antagonism of antidepressants at the 5-HT₃ receptor was not conferred by an enhancement of receptor internalization as shown by immunofluorescence studies, assessment of receptor density in clathrin-coated vesicles, and electrophysiological recordings after coexpression of a dominant-negative mutant of dynamin I, which inhibits receptor internalization. In conclusion, enrichment of antidepressants and antipsychotics in raft-like domains within the cell membrane appears to be crucial for their antagonistic effects at ligand-gated ion channels such as 5-HT₃ receptors.

Key words: 5-HT₃ receptor; antidepressants; antipsychotics; lipid rafts; receptor internalization; ligand-gated ion channel

Introduction

In addition to the classical mechanisms of action of antidepressants and antipsychotics (Kinon and Lieberman, 1996; Blakely, 2001; Pacher et al., 2001), a variety of these psychopharmacological drugs may modulate neuronal excitability through interference with ligand-gated ion channels. Both an indirect modulation of ligand-gated ion channels, e.g., GABA_A receptors, via endogenous neuroactive steroids (Uzunov et al., 1996; Romeo et al., 1998; Marx et al., 2003) and a direct modulation of members of this receptor family, e.g., the GABA_A receptor (Korpi et al., 1995; Mozrzymas et al., 1999; Malatynska et al., 2000; Robinson et al., 2003) and the 5-HT₃ receptor (Fan, 1994; Breiting et al., 2001; Eisensamer et al., 2003; Rammes et al., 2004), have been

described. This inhibitory action at 5-HT₃ receptors has been observed for both antidepressants (Eisensamer et al., 2003) and antipsychotics (Rammes et al., 2004) independently of their chemical structure and pharmacodynamic properties. With the exception of the known competitive antagonists mirtazapine and clozapine, all of the other antidepressants (Eisensamer et al., 2003) and antipsychotics (Rammes et al., 2004) investigated thus far inhibit the serotonin-induced ion flux through 5-HT₃ receptors in a noncompetitive manner. However, the molecular mechanisms underlying this noncompetitive antagonism have not yet been elucidated. Although the localization of neurotransmitter receptors within the cell membrane is of major importance for receptor function, only few data are available concerning the lipid raft association of neurotransmitter receptors. In contrast to the metabotropic glutamate receptor type-1, the GABA_B receptor (Becher et al., 2001), the α7 subunit of the nicotinic acetylcholine receptor (Bruses et al., 2001), and the AMPA receptor (Suzuki et al., 2001) have been found in lipid rafts.

Lipid rafts are membrane microdomains enriched with glycosphingolipids and cholesterol that compartmentalize membrane proteins. Lipid rafts are thought to participate in a variety of signaling pathways, membrane trafficking, and other cellular functions (Brown and London, 1998; Tsui-Pierchala et al., 2002).

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Table 1. Extraction and HPLC conditions for quantification of psychopharmacological drugs

Drug	Extraction		HPLC		Detection (nm)	
	Base	Isoamyl–alcohol	Internal standard	Gradient (% B)	UV	Fluorescence
Carbamazepine	NaOH	0%	protriptyline	28–35	214	
Clozapine	NaOH	1.5%	protriptyline	28–35	214	
Desipramine	NaHCO ₃	1.5%	trimipramine	28–35	214	
Fluoxetine	NaHCO ₃	1.5%	protriptyline	28–35	214	220/305
Fluphenazine	NaOH	1.5%	protriptyline	28–50	214	
Haloperidol	NaOH	1.5%	protriptyline	28–35	214	
Mirtazapine	NaHCO ₃	1.5%	protriptyline	5–35	214	295/370
Moclobemide	NaOH	28.6%	protriptyline	5–35	214	
Reboxetine	NaHCO ₃	1.5%	protriptyline	5–35	214	
Risperidone	NaHCO ₃	1.5%	protriptyline	5–35	214	

B, Acetonitrile.

Biochemically, lipid rafts are characterized as detergent-resistant membranes (DRMs) that float at a light buoyant density (LBD) on sucrose gradients. Moreover, detergent-free methods have also been successfully used in isolating membrane fractions with similar biochemical characteristics (Smart et al., 1995; Song et al., 1996; Luria et al., 2002), so-called raft-like domains.

Thus far, it is not known whether the 5-HT₃ receptor is located in such raft-like domains. Moreover, no data exist on the real concentrations of antidepressants and antipsychotics within such membrane microdomains and whether these psychopharmacological drugs and the receptor protein are indeed colocalized within the cell membrane. We therefore quantified the concentrations of different types of antidepressants and antipsychotics in cell fractions of human embryonic kidney 293 (HEK 293) cells stably transfected with the 5-HT_{3A} receptor and of N1E-115 neuroblastoma cells in relation to the membrane localization of the 5-HT₃ receptor protein. Because receptor internalization is a major principle of the downregulation of the GABA_A receptor (Tehrani and Barnes, 1997; Connolly et al., 1999) that shares structural features with the 5-HT₃ receptor, we investigated whether the antagonistic effects of antidepressants at the 5-HT₃ receptor are also conferred via an enhancement of receptor internalization.

Materials and Methods

Chemicals and drugs. The following compounds were used: desipramine (DMI), carbamazepine, clozapine, haloperidol, risperidone, fluphenazine (RBI, Köln, Germany), fluoxetine (Tocris Bioscience, Köln, Germany), reboxetine (generous gift from Pfizer, New York, NY), mirtazapine (a generous gift from NV Organon, Oss, The Netherlands), and moclobemide (a generous gift from Hoffman-LaRoche, Grenzach, Germany). For stock solutions (10 mM), desipramine, fluoxetine, reboxetine, and fluphenazine were dissolved in H₂O; for all other compounds, stock solutions (10 mM) were prepared in pure ethanol. Thus, the maximal ethanol concentration in experiments using drug concentrations of 10 μ M was 0.1%.

DNA constructs. The human 5-HT_{3A} receptor subunit cDNA was cloned into the mammalian expression vector pCDM8 (Davies et al., 1999). The tagged 5-HT_{3A} receptor subunit [5-HT_{3A}-hemagglutinin (HA)] was obtained by tagging the 5-HT_{3A} receptor with the HA epitope (YPYDVPDYA) between amino acid 5 and 6 (T29-tag-T30) using site-directed mutagenesis (Boyd et al., 2002). The mutated cDNA was cloned into the expression vector pRK5. The dominant-negative acting mutant of dynamin I bearing a replacement of Lys(44) with alanine (K44A) was also cloned into the expression vector pRK5. The K44A mutant dynamin was generously provided by Dr. Christopher N. Connolly (Department of Pharmacology and Neuroscience, Ninewells Medical School, University of Dundee).

Cell cultures and transfection. HEK 293 cells purchased from the European collection of cell cultures [European Collection of Cell Cultures

(ECACC), Salisbury, UK] and HEK 293 cells stably expressing the human 5-HT_{3A} receptor (HEK-5-HT_{3A} cells) (Lankiewicz et al., 1998) were cultured in minimum essential medium (MEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Invitrogen), 1% glutamine, nonessential amino acids and antibiotics (Invitrogen). Mouse N1E-115 neuroblastoma cells were purchased from the ECACC and nourished with MEM (Eagle; Invitrogen) supplemented with 15% FCS (Invitrogen). The medium of N1E-115 cells was exchanged completely daily. Once every 3 d, cells were reseeded onto fresh Petri dishes after treatment with trypsin-EDTA (1% in PBS), resuspension in MEM, and centrifugation at 200 $\times$ g for 4 min. Cells were incubated at 37°C, 5% CO₂, and 95% humidity.

Exponentially growing HEK 293 cells (2×10^6 cells) were transfected with 10 μ g of DNA (pCDM8, pRK5) by electroporation (Electro Cell Manipulator 600 Electroporation System; BTX, San Diego, CA). Cells were harvested 18–24 h after transfection.

Lipid extraction and analytical methods. Samples were extracted with chloroform–methanol according to the slightly modified method of Bligh and Dyer (1959). Briefly, a 150 μ l fraction of the density gradient and 560 μ l of chloroform–methanol (1:2, v/v) were vigorously mixed and centrifuged for 10 min at 14,000 $\times$ g. The supernatant was mixed with 190 μ l of chloroform and 190 μ l of water and centrifuged for 30 min at 14,000 $\times$ g. The bottom lipid phase was evaporated and then dissolved in isopropanol. The recovery of cholesterol was >90% as determined by a cholesterol standard.

Cholesterol was assayed spectrophotometrically using a commercially available kit [Roche Diagnostics (Mannheim, Germany) Diagnostic Kit number 139050].

Protein was determined by the Bradford assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA).

Extraction procedure for HPLC. Four hundred microliters of sucrose gradient fraction, 600 μ l of water, and 0.1 ml of 2 μ g/ml internal standard solution (Table 1) and 1 ml of 2 M sodium–hydrogen–carbonate buffer, adjusted to pH 10.5 with NaOH, or 0.2 ml of 6 M NaOH (Table 1) were vortexed. Five milliliters of n-hexane with various isoamyl–alcohol concentrations (Table 1) were added, and the samples were mixed for 20 min at room temperature. After centrifugation at room temperature for 15 min at 4000 $\times$ g, the organic layer was transferred to a tube containing 0.25 ml of 0.18 M phosphoric acid, mixed for 20 min, and centrifuged at 4000 $\times$ g for 10 min. The organic layer was then discarded, and an aliquot (20 μ l) of the aqueous phase was injected for chromatographic separation. The extraction recoveries were >80%.

HPLC. A Beckman Coulter (Palo Alto, CA) 166 variable-wavelength UV detector, a Merck (Darmstadt, Germany) L-7480 fluorescence detector, and a Beckman Coulter gradient pump 126 Solvent Module equipped with a Beckman Coulter autoinjector 508 autosampler were used for the HPLC analysis. Separations were made on a Luna 5 μ m C18(2) 250 $\times$ 4.6 mm column (Phenomenex, Torrance, CA). The mobile phases [A, 0.22% orthophosphoric acid, adjusted to pH 3.5 with NaOH (6 M); B, acetonitrile] were degassed for 15 min in an ultrasonic bath immediately before use. The column temperature was 60°C, and the flow of the mobile phase was 1.0 ml/min. A mobile phase gradient was

used for the chromatographic analysis (Table 1). The substances and their metabolites were determined by UV absorption or fluorescence at the described wavelength (Table 1). The coefficient of variance was <15% for the different methods used.

Quantification of psychopharmacological drugs. Samples were calibrated by using spiked samples at different concentrations. The concentrations were in the measurement range of the respective substances. Quantification was performed by calculating the internal-standard peak/area ratio of the analyte, and a regression model was fitted to the peak/area ratio of each compound to internal standard versus concentration.

Sucrose gradient centrifugation and Western blotting. HEK-5-HT_{3A} cells or N1E-115 cells of 10 or 17 confluent plates (diameter, 10 cm) were washed with PBS and incubated on plates with 10 μ M concentrations of the respective drug diluted in PBS for 10 min at room temperature. Raft-like domains were prepared using a detergent-free method (Gimpl and Fahrenholz, 2000). All procedures were performed at 4°C. Cells were washed twice with ice-cold PBS and resuspended in 2 ml of high-salt HEPES buffer (20 mM HEPES, 5 mM EDTA, pH 7.4, 1 M NaCl). The suspension was thoroughly homogenized and subsequently brought to 40% sucrose by 1:1 (v/v) dilution with an 80% (w/v) sucrose stock solution in high-salt HEPES buffer. This solution was applied to the bottom of a 14 $\times$ 89 mm centrifuge tube (Beckman Coulter) and overlaid with 3 ml of 35% (w/v) sucrose and subsequently with 3 ml of 5% (w/v) sucrose in 20 mM HEPES, 5 mM EDTA, pH 7.4, 0.5 M NaCl. Samples were ultracentrifuged for 20 h at 200,000 $\times$ g (SW-41 rotor), 4°C and fractionated (12 fractions with 800 μ l each) from the top to the bottom of the gradient. For some experiments, we used 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as detergent, diluted in 20 mM HEPES, 5 mM EDTA, pH 7.4, 150 mM NaCl. This buffer was also used for the sucrose gradient; all other procedures were as described above. For Western blot analysis, aliquots of each fraction were adjusted by reducing sample buffer (10% SDS, 20% glycerol, 125 mM Tris, 1.0 mM EDTA, 0.002% bromophenol blue, and 10% β -mercaptoethanol), denatured by heating at 95°C for 5 min, loaded onto SDS-PAGE, and transferred to nitrocellulose transfer membrane (Protein; Schleicher Schuell Bioscience, Dassel, Germany) or polyvinylidene fluoride transfer membrane (Immobilon-P; Neolab, Heidelberg, Germany). Caveolin (rabbit anti-caveolin polyclonal antibody; BD Biosciences Clontech, Heidelberg, Germany), caveolin-1 (mouse anti-caveolin-1 monoclonal antibody; BD Biosciences, Lexington, KY), caveolin-2 (mouse anti-caveolin-2 monoclonal antibody; BD Biosciences Clontech), flotillin-1 (goat anti-flotillin-1 polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA), and 5-HT₃ receptor subunits (rabbit anti-5-HT₃ receptor polyclonal antibody; a generous gift from Merck Sharp and Dohme, Essex, UK) on the blots were detected by specific antibodies, as indicated and visualized by an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) according to the protocols of the manufacturer.

Immunofluorescence. HEK-5-HT_{3A}-HA cells cultured on poly-L-lysine (10 μ g/ml)-coated coverslips were incubated for 20 min in PBS containing 0.5% BSA and 10% FCS as a blocking solution. Subsequent antibody dilutions were performed in blocking solution; all other solutions were prepared with PBS. After incubation with primary mouse anti-HA antibody (1:100; Santa Cruz Biotechnology) for 1 h on ice, cells were incubated with 100 μ M desipramine or fluoxetine for 15 min at room temperature and fixed in 3% paraformaldehyde for 15 min. Cells were quenched in 50 mM NH₄Cl for 10 min, blocked for 20 min, and incubated with secondary goat anti-mouse Alexa Fluor 488 (30 min, 10 μ g/ml; Invitrogen, Paisley, UK) for detection of surface HA-5-HT_{3A} receptors. Cells were permeabilized with 0.5% NP-40 during subsequent quenching and blocking, processed as described above, and incubated with secondary goat anti-mouse Alexa Fluor 568 (30 min, 10 μ g/ml; Invitrogen) to detect internalized receptors. Coverslips were examined using a confocal microscope (LSM 510 META NLO; Zeiss, Oberkochen, Germany).

Preparation of clathrin coated vesicles. HEK-5-HT_{3A} cells of 40 confluent plates (diameter, 10 cm) were harvested, washed twice with PBS, and homogenized in 3 vol of isolation buffer (10 mM morpholinoethane sulfonic acid, pH 6.5, containing 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl₂). After centrifugation at 17,000 $\times$ g for 20 min, the supernatant

was recentrifuged at 100,000 $\times$ g for 1 h. The resulting pellet was resuspended in 2 ml of isolation buffer and after mixing with 2 ml of 12.5% (w/v) Ficoll and 2 ml of 12.5% (w/v) sucrose in isolation buffer, the sample was centrifuged at 42,000 $\times$ g for 40 min. The supernatant was mixed with 3 vol of 0.125% Triton X-100 in isolation buffer and incubated for 30 min on ice. After centrifugation (100,000 $\times$ g, 90 min), the pellet was resuspended in 50 mM Tris, 1 mM EDTA, pH 7.5. All procedures were performed at 4°C.

Binding assay. For ligand-binding experiments, clathrin-coated vesicle (CCV) samples were incubated in microtiter plates in a total volume of 250 μ l at 37°C for 1 h with the indicated concentrations of [³H]GR65630 (75 Ci/mmol; PerkinElmer, Boston, MA). Bound ligand was separated from free ligand by washing with ice-cold assay buffer and rapid filtration through Whatman GF/B filters with a Titertek cell harvester (Nunc, Wiesbaden, Germany). Radioactivity was determined by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 10 μ M MDL 72222 (tropanyl 3,5-dichlorobenzoate). Specific binding represented ~90% of the total binding. Binding data were analyzed with the EBDA and LIGAND programs (Munson and Rodbard, 1980), which provide a nonlinear, least-square regression analysis.

Electrophysiological recordings. 5-HT-induced inward Na⁺ currents were recorded from lifted HEK cells transiently transfected with the 5-HT_{3A} receptor and the dynamin K44A mutant in the whole-cell voltage-clamp configuration under visual control using an inverted microscope (Zeiss) as described previously (Wetzel et al., 1998). Cells were kept in a bath solution containing (in mM) 140 NaCl, 2.8 KCl, and 10 HEPES, pH 7.2. Patch electrodes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a horizontal pipette puller (Zeitz-Instruments, Augsburg, Germany) to yield pipettes with a resistance of 3–6 M Ω . Pipettes were filled with a solution containing (in mM) 130 CsCl, 2 MgCl₂, 2 CaCl₂, 2 ATP, 0.2 Tris-GTP, 10 glucose, 10 HEPES, and 10 EGTA, pH 7.2. After the whole-cell configuration was established, the cells were lifted from the glass substrate and 10 μ M 5-HT and/or DMI (1 μ M) was applied using a fast superfusion device. We applied these concentrations because 10 μ M serotonin were used for the determination of the IC₅₀ value for the inhibition of the serotonin response by DMI in our previous study (Eisensamer et al., 2003), which was in the low micromolar range.

For control experiments, a piezo translator-driven double-barreled application pipette was used to expose the lifted cell to either a 5-HT-free or 5-HT-containing solution. A 2 s 5-HT pulse was delivered every 60 s. Current signals were recorded at a holding potential of –50 mV with an EPC-9 amplifier (HEKA, Lambrecht, Germany) and were analyzed using the HEKA PulseFit 8.5 and Igor Pro version 4.5 (WaveMetrics, Lake Oswego, OR) software on a Power Macintosh G3 computer (Apple, Cupertino, CA).

Results

LBD fractions were isolated from recombinant HEK cells stably expressing the 5-HT_{3A} receptor (HEK-5-HT_{3A} cells) and from N1E-115 neuroblastoma cells. LBD fractions at the interface between the 5 and 35% sucrose layers (fractions 3–6) contained 4–8% of the total cellular proteins, whereas the bulk of proteins remained in the 40% sucrose layer at the bottom of the ultracentrifuge tube (Fig. 1*a,b*). In contrast, cholesterol was enriched in the LBD fractions with a specific concentration (micrograms of cholesterol per milligram protein) up to 900-fold higher than that of the high buoyant density (HBD) fractions (fractions 8–12) (Fig. 1*a*), indicating that LBD fractions are highly enriched in raft-like domains. LBD fractions of HEK-5-HT_{3A} cells indeed represent raft-like domains, because caveolin-2 and flotillin-1 are preferentially located within the respective LBD fractions (Fig. 1*d*). To test whether LBD fractions contain caveolae, a subpopulation of raft-like domains, which are characterized by their invaginated morphology and the presence of the scaffolding protein caveolin-1 (Rothberg et al. 1992; Fra et al., 1995; Murata et al., 1995), we probed the gradient fractions with a monoclonal anti-

Figure 1. 5-HT₃ receptors were present in LBD fractions both of HEK-5-HT_{3A} cells and N1E-115 neuroblastoma cells. Cells were solubilized by sonication in high-salt buffer, and extracts were fractionated by discontinuous density-gradient ultracentrifugation. Fractions (0.8 ml) were harvested from the top of the gradient (fraction 1, top of gradient; fraction 12, base of gradient). **a**, Fractions of sucrose gradients obtained from HEK-5-HT_{3A} cells (12 fractions with 0.8 ml each) were assayed for protein concentration (milligrams of protein per milliliter fraction) and cholesterol concentration (micrograms of cholesterol per milligram of protein). **b**, Proteins obtained from HEK-5-HT_{3A} cells were analyzed by reduced SDS-PAGE (10% gel, 25 μ l aliquots per lane), followed by Ponceau staining; the position of molecular weight marker proteins is shown on the left. **c**, Replicate gels were immunoblotted with a polyclonal antibody against 5-HT₃ receptors. Native HEK 293 cells were used as negative control for 5-HT₃ receptor immunoreactivity. The position of molecular weight marker proteins is shown on the left. **d**, Replicate gels were immunoblotted with a polyclonal antibody against caveolin, a monoclonal antibody against caveolin-2, and a polyclonal antibody against flotillin-1. The position of molecular weight marker proteins is shown on the left.

body against caveolin-1. Neither HEK-5-HT_{3A} cells nor N1E-115 neuroblastoma cells showed caveolin-1 immunoreactivity in our Western blot procedure (data not shown). Thus, both cell types apparently express only flat lipid rafts.

When the gradient fractions of HEK-5-HT_{3A} and N1E-115 cells were probed with a polyclonal antibody against 5-HT₃ receptors, we found a strong immunoreactivity exclusively in the LBD fractions of whole-cell homogenates and also of plasma membranes as starting material (Fig. 1c). The same results were seen when 1% CHAPS was used as detergent (data not shown). These results suggest that 5-HT₃ receptors are associated with raft-like domains. The 5-HT₃ receptors of drug-treated cells were also exclusively localized in LBD fractions (data not shown). Moreover, drug incubation did not affect the concentration of cholesterol in LBD fractions (data not shown).

If 5-HT₃ receptors indeed are lipid raft associated, functional noncompetitive 5-HT₃ receptor antagonists such as antidepressants and antipsychotics might interact with the receptor within these membrane microdomains. To investigate whether antidepressant and antipsychotic drugs colocalize with the 5-HT₃ receptor in raft-like domains, we quantified the respective psychopharmacological drugs in relation to the localization of the 5-HT₃ receptor protein within the cell membrane. The drug concentration in each fraction was determined by HPLC. We found rather low specific concentrations (9–28 μ mol/mg protein) (Fig. 2, Table 2) of DMI, fluoxetine, reboxetine, mirtazapine, fluphenazine, haloperidol, and risperidone in HBD fractions of HEK-5-HT₃ cells. Only clozapine was substantially enriched in HBD fractions (Table 2). However, this was attributable to an accumulation exclusively in fraction 8 (Fig. 2). Fluoxetine and fluphenazine were mainly

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tration (milligrams of protein per milliliter fraction) and cholesterol concentration (micrograms of cholesterol per milligram of protein). **b**, Proteins obtained from HEK-5-HT_{3A} cells were analyzed by reduced SDS-PAGE (10% gel, 25 μ l aliquots per lane), followed by Ponceau staining; the position of molecular weight marker proteins is shown on the left. **c**, Replicate gels were immunoblotted with a polyclonal antibody against 5-HT₃ receptors. Native HEK 293 cells were used as negative control for 5-HT₃ receptor immunoreactivity. The position of molecular weight marker proteins is shown on the left. **d**, Replicate gels were immunoblotted with a polyclonal antibody against caveolin, a monoclonal antibody against caveolin-2, and a polyclonal antibody against flotillin-1. The position of molecular weight marker proteins is shown on the left.

enriched in LBD fractions, with their concentrations in LBD fractions being ~10 times higher than those in HBD fractions (Fig. 2, Table 2). Also, DMI, haloperidol, reboxetine, and clozapine were concentrated twofold to fourfold higher in LBD fractions than in HBD fractions. In contrast, mirtazapine and risperidone were not accumulated in LBD fractions (~10 $\mu\text{mol}/\text{mg}$ protein). Carbamazepine and moclobemide were not found in neither LBD nor HBD fractions (<0.5 $\mu\text{mol}/\text{mg}$ protein) (Fig. 2, Table 2). The concentrations of DMI, fluoxetine, reboxetine, fluphenazine, haloperidol, carbamazepine, moclobemide, and risperidone in the LBD fractions showed a highly significant association (Spearman correlation coefficient, -0.878 ; $r = 0.004$) with their antagonistic potencies expressed as IC_{50} values for their inhibition of the 5-HT-evoked Na^+ current through 5-HT₃ receptors (Eisensamer et al., 2003). However, we found no such association with drug concentrations in the HBD fractions. These results indicate that the antagonistic effects of noncompetitive compounds, such as antidepressants or antipsychotics, at the 5-HT₃ receptor depend on the drug concentration within the LBD fractions; i.e., higher concentrations exert more pronounced functional antagonistic effects at the 5-HT₃ receptor.

Similar results were obtained with N1E-115 cells incubated with DMI, fluoxetine, carbamazepine, or moclobemide (data not shown).

To investigate putative mechanisms underlying the antagonistic action of antidepressants and antipsychotics at 5-HT₃ receptors, we questioned whether these psychopharmacological drugs might induce receptor internalization.

First, we measured the effect of DMI at 5-HT-induced Na^+ currents through HEK-5-HT₃ cells coexpressing K44A dynamin, a dominant-negative acting mutant of dynamin I that blocks internalization. Our patch-clamp recordings showed that DMI markedly reduced the amplitude of the 5-HT-induced Na^+ current, even in the presence of K44A dynamin (Fig. 3a), indicating that the antagonistic effect of DMI is not dependent on receptor internalization. Second, we performed immunofluorescence experiments with HA epitope-tagged recombinant 5-HT₃ receptors expressed in HEK 293 cells (HEK-5-HT₃-HA cells). The epitope tag added between amino acids 5 and 6 of the mature polypeptide appears to be silent (Boyd et al., 2002). The immunofluorescence patterns of DMI- or fluoxetine-treated cells

Figure 2. Concentrations of antidepressant and antipsychotic drugs in fractions of sucrose-flotation gradients isolated from HEK-5-HT_{3A} cells. Drug concentrations were determined by HPLC. Results are expressed as specific concentrations (micromoles per milligram of protein) and represent the mean $\pm$ SD of four independent experiments. Fractions were prepared as described above. Error bars represent SD.

Table 2. Concentration of antidepressant and antipsychotic drugs in LBD and HBD fractions of HEK-5-HT_{3A} cells

Fractions	DMI	Fluoxetine	Reboxetine	Mirtazapine	Carbamazepine	Moclobemide	Fluphenazine	Haloperidol	Clozapine	Risperidone
LBD	68.63 ± 18.79	218.35 ± 33.45	29.17 ± 12.96	8.97 ± 2.29	0.28 ± 0.18	0.46 ± 0.43	205.41 ± 34.03	61.99 ± 7.71	110.07 ± 18.64	11.46 ± 3.08
HBD	16.05 ± 3.35	23.46 ± 2.87	9.41 ± 1.11	12.19 ± 1.96	0.08 ± 0.06	0.20 ± 0.16	15.53 ± 8.26	28.14 ± 9.58	47.45 ± 8.87	9.80 ± 3.96

Sucrose density fractions were prepared as described in Materials and Methods. Drug concentrations were determined by HPLC. Results are expressed as specific concentrations (micromoles per milligram of protein) and represent the mean ± SD of four independent experiments. Fractions 3–6 represent LBD fractions; fractions 8–12 represent HBD fractions.

Figure 3. The antagonistic effect of antidepressant drugs at 5-HT₃ receptors is independent of receptor internalization. **a**, Effect of DMI on the 5-HT-induced Na⁺ currents through HEK-5-HT₃ cells coexpressing K44A dynamin. Na⁺ currents of HEK-5-HT₃ cells expressing K44A dynamin were recorded in the whole-cell voltage-clamp configuration. Representative original recordings before and after application of DMI and subsequent washout are shown. The top bar indicates the application of 10 μM 5-HT, and the bottom bar indicates the presence of 1 μM DMI. **b**, Effect of DMI and fluoxetine on the subcellular localization of 5-HT₃ receptors. Living HEK 293 cells transiently transfected with HA-tagged 5-HT₃ receptors were incubated with anti-HA antibody for 1 h and subsequently incubated with 100 μM DMI, 100 μM fluoxetine, or PBS (PBS control) for 15 min before fixation. Staining control was incubated in the absence of anti-HA antibody and subsequently incubated with PBS before fixation. Surface-bound receptors were detected before permeabilization using a fluorescein-conjugated secondary antibody. Internalized receptors were subsequently detected after permeabilization using a rhodamine-conjugated secondary antibody.

were indistinguishable from those of controls (Fig. 3b). Thus, in accordance with the electrophysiological recordings, these data indicate that noncompetitive 5-HT₃ receptor antagonists do not induce receptor internalization. Because certain types of raft-associated receptors can get internalized via CCVs (Abrami et al., 2003), we isolated CCVs of HEK-5-HT₃ cells preincubated with 10 μM fluoxetine or PBS (control) for 10 or 30 min and determined receptor density by ligand binding assay. We found comparable receptor densities in CCVs of both drug-treated and control cells (data not shown). Thus, our data do not support the

hypothesis that antidepressants and antipsychotics antagonize 5-HT₃ receptor-mediated currents via an enhancement of receptor internalization.

Discussion

In the present study, we investigated putative molecular mechanisms underlying the noncompetitive antagonistic effects of antidepressants and antipsychotics at the 5-HT₃ receptor.

One possibility might be that these antagonistic effects are mediated through an enhancement of receptor internalization. Receptor internalization is of major importance for receptor recycling and is an important determinant for the availability of functionally active membrane-bound receptors (Calkin and Barnes, 1994; Barnes, 1996; Miranda and Barnes, 1997). For example, chronic administration of benzodiazepines induces internalization of the GABA_A receptor (Tehrani and Barnes, 1997). Moreover, antidepressants (Koshikawa et al., 1987; Burgi et al., 2003; Riad et al., 2004) and antipsychotic drugs (Willins et al., 1999) may enhance receptor internalization/downregulation of 5-HT_{1A} receptors, 5-HT₂ receptors, or β-adrenoceptors (β₁AR), which all are G-protein-coupled receptors. However, only chronic treatment with DMI, but not acute doses of DMI, may cause downregulation of β₁AR *in vivo* (Koshikawa et al., 1987; Burgi et al., 2003). In our study, the noncompetitive antagonism of antidepressants at the 5-HT₃ receptor could be observed after short time exposure to the respective drugs and obviously was not conferred by an enhancement of receptor internalization as shown by immunofluorescence studies, assessment of receptor density in clathrin-coated vesicles, and electrophysiological recordings after coexpression of dynamin K44A, a dominant-negative mutant of dynamin I, which inhibits receptor internalization. Thus, an enhancement of internalization does not explain the functional antagonistic effects of antidepressants at the 5-HT₃ receptor.

To investigate the concentrations of the respective psychopharmacological drugs in relation to the localization of the 5-HT₃ receptor within the cell membrane, we quantified the concentrations of different types of antidepressants and antipsychotics in fractions of sucrose-floating gradients isolated from HEK 293 cells stably transfected with the 5-HT_{3A} receptor and of N1E-115 neuroblastoma cells in relation to the localization of the 5-HT₃ receptor protein. Western blots revealed a localization of the 5-HT₃ receptor protein exclusively in the LBD fractions, which also contain caveolin-2 and flotillin-1. Thus, the LBD fractions indeed represent raft-like domains. This raft association of the 5-HT₃ receptor is consistent with the cluster distribution of the 5-HT₃ receptor observed in the plasma membrane of live HEK cells (Ilegems et al., 2004).

Initially, lipid rafts have been characterized as DRMs with low buoyant density (Brown and Rose, 1992; Sargiacomo et al., 1993; Song et al., 1996). However, questions have been raised about to what extent DRMs reflect raft size and composition *in vivo* (for review, see Edidin, 2003; Munro, 2003). To avoid artifacts by the partitioning of detergent molecules into the lipid bilayer, detergent-free protocols have been developed and have subse-

quently been used extensively to define the molecular composition of raft-like domains (Smart et al., 1995; Song et al., 1996). LBD fractions isolated in a detergent-free environment have been shown to exhibit many characteristics normally associated with DRMs (Luria et al., 2002), although contamination by non-raft membrane fragments cannot be completely excluded (Schnitzer et al., 1995; Stan et al., 1997; Waugh et al., 1999). We also used a detergent-free protocol, because detergents might impair the inclusion and accumulation of the drugs into the cell membrane and might interfere further with the HPLC analysis. The contamination by non-raft membrane fragments in the LBD fraction was investigated by using plasma membranes as starting material. LBD fractions isolated from whole-cell homogenates and plasma membranes showed corresponding results. Thus, contamination by non-raft membrane fragments does not confound our results. Similar to the 5-HT₃ receptor, a variety of ion channels have been shown to be located in raft-like domains (for review, see Martens et al., 2004). Rafts are thought to have unique biophysical properties that directly affect channel function (Tillman and Cascio, 2003). Within the family of ligand-gated ion channels, GABA_A receptors (Dalskov et al., 2005), the $\alpha 7$ subunit of the nicotinic acetylcholine receptor (Bruses et al., 2001), NMDA (Besshoh et al., 2005), and AMPA receptors (Suzuki et al., 2001; Hering et al., 2003) have been detected in lipid rafts. Thus, the enrichment of antidepressants and antipsychotics within these membrane microdomains might also be important for the modulation of other ligand-gated ion channels in addition to the 5-HT₃ receptor.

Moreover, rafts could serve as a platform to cluster distinct signaling molecules with ion channels (Anderson, 1998; Okamoto et al., 1998; Hooper, 1999; Kurzchalia and Parton, 1999) and may play a role in the polarized sorting and/or trafficking of ion channels (Ledesma et al., 1998). However, the raft association of proteins can be highly variable (Smart et al., 1996; Mineo et al., 1999) (for review, see Pike, 2003). For example, treatment of cells with progesterone shifted the caveolae-associated folate receptor to the bulk membrane fractions. Progesterone blocked cholesterol movement from the endoplasmic reticulum to caveolae, which are impaired by cholesterol depletion (Smart et al., 1996). In our study, we did not find any changes in the cholesterol concentration in the LBD fractions or in the raft association of the 5-HT₃ receptor after short-term treatment of the cells with antidepressant or antipsychotic drugs. Interestingly, also only chronic, but not acute, treatment of cells with antidepressants has been shown to result in a reduced amount of the G-protein G_{sα} in caveolae-enriched membrane domains (Toki et al., 1999) and a redistribution of G_{sα} in the cell (Donati et al., 2001).

Similar to the 5-HT₃ receptor, also the antidepressants desipramine, fluoxetine, and reboxetine and the antipsychotics fluphenazine, haloperidol, and clozapine were markedly enriched in LBD fractions, whereas no such accumulation could be shown for mirtazapine, carbamazepine, moclobemide, and risperidone. Only compounds that reach substantial concentrations within these membrane microdomains are capable of acting as noncompetitive functional antagonists at the 5-HT₃ receptor, whereas carbamazepine, moclobemide (Eisensamer et al., 2003), and risperidone (Rammes et al., 2004), which are not accumulated in these membrane fractions, are devoid of antagonistic properties at this ligand-gated ion channel. When considering the competitive antagonists mirtazapine and clozapine, only clozapine is enriched in LBD fraction, whereas mirtazapine is not. This would be compatible with the idea that mirtazapine acts solely as a competitive antagonist at the 5-HT₃ receptor, whereas clozapine, in view of its relatively low binding affinity (Hermann et al., 1996;

Rammes et al., 2004), might act as a simultaneous competitive and noncompetitive antagonist. In addition, the concentrations of psychopharmacological drugs within LBD fractions were strongly associated with their inhibitory potency against serotonin-induced cation currents. In summary, our data indicate that an accumulation of antidepressants and antipsychotics might be important for the functional antagonistic effects of these drugs at the 5-HT₃ receptor.

An enrichment of antidepressants and antipsychotics within LBD fractions of cell membranes has not been demonstrated thus far. Only clozapine has been shown to accumulate in the “very low-density lipoprotein” fraction of plasma samples with highly elevated lipoprotein levels, but no such accumulation occurred in standard plasma (Procysyn et al., 2001). Generally, basic lipophilic compound such as antidepressants, e.g., fluoxetine and imipramine, may bind nonspecifically to membrane phospholipids (Bickel and Steele, 1974; Di Francesco and Bickel, 1977; Romer and Bickel, 1979). Thus, an accumulation of such drugs in phospholipid-rich membrane microdomains such as lipid rafts is highly probable. Because the respective concentrations are reached both in animal studies (Uhr et al., 2000; Weigmann et al., 2000) and in neuroimaging studies in patients treated with fluoxetine (Bolo et al., 2000; Henry et al., 2000), the accumulation of these psychopharmacological drugs in raft-like domains may occur also *in vivo* under therapeutical conditions.

In conclusion, enrichment of antidepressants and antipsychotics in raft-like domains within the cell membrane appears to be crucial for their antagonistic effects at ligand-gated ion channels, such as 5-HT₃ receptors, and may contribute to the understanding of essentially unknown mechanisms of action of these psychopharmacological drugs.

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