RIC-3 Exclusively Enhances the Surface Expression of Human Homomeric 5-Hydroxytryptamine Type 3A (5-HT₃A) Receptors Despite Direct Interactions with 5-HT₃A, -C, -D, and -E Subunits* [S]

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Although five 5-hydroxytryptamine type 3 (5-HT₃) subunits (A–E) have been cloned, knowledge on the regulation of their assembly is limited. RIC-3 has been identified as a chaperone for the pentameric ligand-gated nicotinic acetylcholine and 5-HT₃ receptors. Therefore, we examined the impact of RIC-3 on differently composed 5-HT₃ receptors with the focus on 5-HT₃C, -D, and -E subunits. The influence of RIC-3 on these receptor subtypes is supported by the presence of RIC3 mRNA in tissues expressing at least one of the subunits 5-HT₃C, -D, and -E. Furthermore, immunocytochemical studies on transfected mammalian cells revealed co-localization in the endoplasmic reticulum and direct interaction of RIC-3 with 5-HT₃A, -C, -D, and -E. Functional and pharmacological characterization was performed using HEK293 cells expressing 5-HT₃A or 5-HT₃A + 5-HT₃B (or -C, -D, or -E) in the presence or absence of RIC-3. Ca²⁺ influx analyses revealed that RIC-3 does not influence the 5-HT concentration-response relationship on 5-HT₃A receptors but leads to differential increases of 5-HT-induced maximum response (Eₘₐₓ) on cells expressing different subunits. Increases of Eₘₐₓ were due to analogously enhanced Bₘₐₓ values for binding of the 5-HT₃ receptor antagonist [³H]GR65630. The observed enhanced cell surface expression of the tested 5-HT₃ subunit combinations correlated with the increased surface expression of 5-HT₃A as determined by flow cytometry. In conclusion, we showed that RIC-3 can interact with 5-HT₃A, -C, -D, and -E subunits and predominantly enhances the surface expression of homomeric 5-HT₃A receptors in HEK293 cells. These data implicate a possible role of RIC-3 in determining 5-HT₃ receptor composition in vivo.

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Cell surface proteins are translocated to the endoplasmic reticulum (ER)² of eukaryotic cells during synthesis. Thereby, posttranslational covalent modifications such as N-glycosylation and disulfide bond formation occur within the ER during the folding of the polypeptide. The assembly and trafficking of newly translated polypeptides are achieved with the help of chaperones. Many receptor-interacting proteins, which are involved in the trafficking and clustering of ligand-gated ion channels (LGICs), have already been identified (1). The assembly of the subunits in the ER is the rate-limiting step in the biogenesis of oligomeric LGICs (2). The ER-resident chaperones immunoglobulin heavy chain-binding protein (BiP) and calnexin have been shown to participate in subunit assembly of LGICs such as nicotinic acetylcholine (nACh) receptors (3–5) and 5-hydroxytryptamine type 3 (5-HT₃) receptors (6). In addition to these generalized chaperones, much more selective proteins such as stargazin for α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptors (AMPA) (7), 14-3-3 for nACh receptors (8), and RIC-3 for nACh and 5-HT₃ receptors (9) have been found. The latter has been identified in Caenorhabditis elegans as a protein involved in cholinergic signaling (10). It has been shown to enhance the surface expression of various nACh receptor subtypes (11–15) and of homomeric 5-HT₃A receptors (16), probably through an interaction with unassembled subunits in the ER (16, 17), whereas the two other members of the superfamily of Cys-loop LGIC, i.e. γ-aminobutyric acid type A and glycine receptors, seem to be unaffected by RIC-3 (11, 12, 14, 17).

Like all other Cys-loop LGICs, 5-HT₃ receptors are oligomeric complexes composed of five subunits. They exhibit a central role in the bidirectional brain-gut axis, which represents the neuronal connection between the enteric and the central nerv-
ous system via the vagus nerve and mediates the regulation of digestion, emotions, and cognition. Consequently, besides their well established role in chemo-/radiotherapy-induced nausea and vomiting, 5-HT$_3$ receptors are involved in the pathophysiology of neurogastrointestinal and neuropsychiatric disorders (44). To date, five 5-HT3 subunits have been cloned from human: 5-HT3A, -B, -C, -D, and -E (19–23). The 5-HT3A subunit is able to form functional homomeric receptors, whereas the other subunits are only functional when co-expressed with 5-HT3A. Because these subunits are co-expressed in various tissues, e.g. the gastrointestinal tract (23, 24), specific mechanisms must exist that determine receptor composition, which in turn defines the properties of 5-HT$_3$ receptors. Limited information is available regarding these mechanisms and factors involved. However, some chaperone molecules have been shown to be involved in determining subunit composition of LGICs. The protein 14-3-3 has been reported to alter the stoichiometry of α4β2 nACh receptors (25), and there is first evidence that RIC-3 plays a role in determining subunit composition of nACh and 5-HT$_3$ receptors. 1) It has been shown to promote the expression of homomeric 5-HT$_3$A at the expense of heteromeric 5-HT$_3$AB receptors in mammalian cells (17), and 2) it leads to a preferential expression of DEG-3-rich DEG-3/DES-2 nACh receptors in C. elegans (26, 27).

The major aim of this study was to investigate the impact of the specific chaperone RIC-3 on 5-HT$_3$ receptors of various compositions. Given that the interaction of BiP and calnexin with 5-HT$_3$A and 5-HT$_3$AB receptors is known (6), another aim of this study was to determine whether these generalized chaperones also monitor the folding and assembly of 5-HT$_3$ receptors containing the human subunits 5-HT3C, -D, and -E. This may help to elucidate the mechanism of how homomeric, as well as heteromeric, 5-HT$_3$ receptor formation is regulated in vivo and may provide a first step toward the development of more selective compounds for the treatment of neurogastrointestinal and neuropsychiatric disorders.

**MATERIALS AND METHODS**

**Chemicals and Drugs**—Coeleterazine h was from Nanolight (Pinetop, AZ). 5-HT creatinine sulfate (serotonin) was obtained from Sigma. [H]GR65630 (3-(5-[H]methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone; specific activity, 76.4 Ci/mmol) was from PerkinElmer Life Sciences.

**Expression Constructs**—The human 5-HT3A, -B, -C, -D, -E, and -Ea subunit-encoding cDNAs from HTR3A, -B, -C, -D, -E, and -Ea (Table 1) were cloned into the expression vector pcDNA3 (Invitrogen). To enable detection of the encoded proteins in flow cytometry or glycosylation experiments, Myc and/or HA epitope tags were introduced within the extracellularly located N terminus of the subunits (see Ref. 28). The apoaequorin cDNA (GenBank™ accession number L29571), originally derived from cytAEQ/pcDNA1 (Molecular Probes-Invitrogen), was subcloned into HindIII/Xbal-digested pcDNA3.1/zeo(+) (Invitrogen). Oligonucleotide primers based on the human RIC3 sequence (GenBank accession number NM_024557; sense, GACCCAGTGTACGAATCATG; antisense, GAGGAGAGAGGTCACCTTGT) were used to amplify RIC3 cDNA from human liver cDNA. The resulting fragment was subcloned into pCR2.1 (Invitrogen), excised with HindIII/EcoRI, and subcloned into pcDNA3.1(−). The fidelity of the cDNA sequences was verified by sequencing.

**Expression Analysis**—RNAs from 14 different human adult tissues (Clontech) were reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) as designed by the manufacturer. PCR analysis was performed using different gene-specific primers (Table 1). Reaction mixtures of 25 μl contained 10–100 ng of template, 25 pmol of each primer, 200 μM dNTPs (MBI Fermentas, St. Leon-Roth, Germany), 1.5 mM MgCl$_2$, 1× PCR buffer, and 2 units of HotStarTag DNA polymerase (Qiagen, Hilden, Germany). Thermal cycling was performed as follows: initial denaturation at 94 °C for 15 min followed by 35–40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. Final extension was carried out at 72 °C for 5 min.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 and human osteosarcoma U2OS cells (ATCC, Manassas, VA) were grown as monolayers in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO$_2$ at 37 °C. Transient transfection was performed with Polyfect transfection reagent (Qiagen) according to the manufacturer’s instructions. For immunocytochemical experiments and radioligand binding, the following mixtures of cDNAs were used: (a) single subunits (5-HT3A, -B, -C, -D, and -Ea): 20% 5-HT3 subunit cDNA and 80% pcDNA3; and (b) co-expression of 5-HT3A with 5-HT3B (or -C, -D, -E, or -Ea): 20% 5-HT3 subunit cDNA and 80% pcDNA3 (1:4 ratio to promote the formation of heteromeric receptors). For immunofluorescence and flow cytometry experiments, Myc- or HA-tagged subunit constructs were used. For aequorin assays, cDNA amounts used were as follows: (a) homomeric 5-HT$_3$A receptors: 67% aequorin cDNA and 33% 5-HT3A cDNA and pcDNA3 combination (1:4); and (b) co-expression of 5-HT3A with 5-HT3B (or -C, -D, -E, or -Ea): 67% aequorin cDNA and 33%

**TABLE 1**

| Gene/primer | Sequence (5′ → 3′) | Amplicon size | GenBank accession no. |
|-------------|--------------------|---------------|-----------------------|
| HTR3A       | CTTGCTTCCGTAGCAAGACTG | 159 | AJ003079 |
|             | GACGCCTTCCCCAGATCTTCTG | | |
| HTR3C       | TCCCCAGAGAGACTGCAAGA | 418 | AF459285 |
|             | TGACATTCCGATGTGAGTATG | | |
| HTR3D       | CTGGGTACATGGCTTCTGTG | 624 | AI159812 |
|             | TGGAACAGGCTATTACACA | | |
| HTR3E       | ATGTTAAGCTTCACCTTATATCCGGC | 524 | AI159813 (E) |
|             | CTGTCCACCTTGTACGTTT | | DQ644022 (Ea) |
| RIC3        | GACCCACCGTGAAGCTGATG | 1180 | NM_024557 |
|             | GAGAGAGAGGTCACCTTGT | | |
| ARF         | GCCAGGTTCCTCTCACCTCCTG | 336 | NM_00102227.1 |
|             | GCTGTTCCGACGCACTTGGT | | |

* a for, forward; rev, reverse; ARF, ADP-ribosylation factor.

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**Kim J, Lee K, Park H, et al. Modulation of 5-HT$_3$ Receptor Expression by RIC-3. J Biol Chem 2010;285:26957–67.**

**Abstract**—The specific chaperone RIC-3 plays a role in determining subunit composition of 5-HT$_3$ receptors. This study investigated the impact of RIC-3 on 5-HT$_3$ receptors of various compositions, and monitored the folding and assembly of 5-HT$_3$ receptors containing the human subunits 5-HT3C,-D, and -E. The fidelity of the cDNA sequences was verified by sequencing. Expression analysis revealed that RIC-3 modulates 5-HT$_3$ receptor expression in HEK 293 and U2OS cells. Immunocytochemical experiments showed that RIC-3 binds to and promotes the formation of heteromeric receptors. Radioligand binding assays confirmed that RIC-3 modulates 5-HT$_3$ receptor expression.

**Conclusion**—RIC-3 is a specific chaperone that modulates 5-HT$_3$ receptor expression.

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5-HT3A and 5-HT3B (or -C, -D, -E, or -Ea) cDNA combination (1:4). For expression of RIC-3, ⅓ of RIC-3 cDNA (related to the total 5-HT3 subunit cDNA amount) was included unless otherwise indicated.

**Immunofluorescence Experiments**—HEK293 and U2OS cells, seeded on poly-l-lysine-coated coverslips in 12-well plates, were transfected using a total amount of 1 μg of DNA/well. Cells were analyzed 24 h after transfection. Briefly, cells were washed twice using 1× phosphate-buffered saline (PBS) and fixed by incubation in 3.75% paraformaldehyde for 30 min. Afterward, they were washed 3 × 5 min in 1× PBS at room temperature and then permeabilized in 0.1% Triton X-100, PBS. The primary antibodies mouse anti-Myc (Cell Signaling Technology, 9B11), mouse anti-HA (Sigma, HA-7), and sheep anti-RIC-3, diluted in 1× PBS, were applied for 1 h at room temperature. Cells were washed 3 × 5 min in 1× PBS and incubated with the fluorochrome-labeled secondary antibodies (anti-mouse/sheep Alexa Fluor 488 (Invitrogen)) in 1× PBS for 1 h. From this point on, every step was carried out light-protected. Cells were washed 3 × 5 min in 1× PBS. A nuclear counterstain with 4′,6-diamidino-2-phenylindole (1:10,000) was carried out. Then cells were washed twice in 1× PBS at room temperature and mounted in Mowiol (Calbiochem, Merck). The slides were stored at 4 °C until microscopic examination. Microscopy was performed with a Zeiss Axiophot system, and images were taken and analyzed using the Leica FW4000 application (Leica, Nussloch, Germany).

**Co-immunoprecipitation**—HEK293 cells, seeded in 6-cm cell culture dishes coated with poly-l-lysine, were transfected with a total amount of 10 μg of DNA/dish. Twenty-four hours following transfection cells were 1-methionine-starved for 30 min before being labeled with [35S]methionine/cysteine (0.2 mCi/dish; Hartmann Analytic, Braunschweig, Germany) for 24 h. Cells were lysed in a 10 mM sodium phosphate-based lysis buffer (6) for 1 h on ice. After centrifugation (16,000 × g for 10 min), the supernatant was incubated with 10 μl of UltraLink Immobilized Protein A/G resin (Thermo Fisher Scientific Inc., Rockwell, IL) for 16 h at 4 °C to remove nonspecific binding. After centrifugation (16,000 × g for 5 min), the supernatant was split and incubated with either 5 μl of the respective 5-HT3 subunit- or chaperone-specific antibody for 24 h at 4 °C. After 6 h, 5 μl of the UltraLink resin were added. The resin was washed three times with a 10 mM sodium phosphate-based buffer containing 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 50 mM NaCl, and 1 mM sodium orthovanadate. Analysis was performed using SDS-polyacrylamide gel electrophoresis with NuPAGE 4–12% Bis-Tris gels (Invitrogen) followed by vacuum drying and autoradiography on BioMax films (Eastman Kodak Co.) at −80 °C.

**Site-directed Mutagenesis for Glycosylation Studies**—According to the technique of gene splicing by overlap extension (29, 30), mutated 5-HT3Ea constructs with specific nucleotide exchanges affecting the predicted N-glycosylation sites were created by two-step polymerase chain reaction with flanking primers and internal primers carrying the desired mutation. Mutagenesis of 5-HT3C was performed using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used are listed in [supplemental Table 1](#).

**Tunicamycin Treatment of HEK293 Cells**—Twenty-four hours posttransfection, HEK293 cells were incubated with 5 μg/ml tunicamycin for 24 h to block N-linked glycosylation.

**Western Blotting**—Transfected cells were harvested 48 h posttransfection and incubated in 300 μl of lysis buffer (6) for 1 h on ice. Total protein was determined using the BCA Protein Assay kit (Pierce), and 10 μg of protein were loaded on 4–12% Bis-Tris NuPAGE gels (Invitrogen). Gels were blotted onto polyvinylidene difluoride membranes using the XCell system (Invitrogen). Detection was accomplished following the “Odyssey Western Blot Analysis” protocol (Li-Cor Biosciences, Lincoln, NE). The primary antibodies anti-5-HT3C and anti-5-HT3D/E ([supplemental Table 2](#)) were diluted 1:500; secondary antibody (donkey anti-rabbit IRDye 680, Li-Cor Biosciences) was diluted 1:10,000.

**Radioligand Binding Assay**—HEK293 cells, seeded in 75-cm$^2$ culture flasks, were transfected with a total amount of 15 μg of cDNA/flask. Preparation of membranes and [3H]GR65630 binding was carried out 48 h later as described previously (28, 30). For saturation experiments, 4 μg of membranes were incubated in duplicates with five increasing concentrations (0.02–1.5 nM) of [3H]GR65630 for 1 h. Nonspecific binding was determined on mock-transfected cells. Incubation mixtures were filtered through GF/B filters, presoaked with 0.5% polyethyleneimine, using a Brandel cell harvester followed by three washes with ice-cold buffer. Radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

**Aequorin Luminescence-based Ca$^{2+}$ Influx Assay**—HEK293 cells, seeded in 25- or 75-cm$^2$ culture flasks, were transfected with a total amount of 5 or 15 μg of cDNA/flask, respectively. The aequorin assay was performed as described previously (31). Harvested cells were loaded with 5 μM coelenterazine h for 2.5 h at room temperature. Suspensions of cells in assay buffer were used for luminometric determination of intracellular Ca$^{2+}$ transients in 96-well plates in a Centro LB 960 luminometer (Berthold, Bad Wildbad, Germany). Luminescence was recorded 5 s prior and 15–60 s upon autoinjection of 5-HT at a sampling rate of 2 Hz. At the end of the experiments in which 5-HT maximum responses were recorded, cells were lysed by autoinjection of 0.1% Triton X-100 (v/v), 50 mM CaCl$_2$, and remaining aequorin luminescence was recorded to obtain the maximum possible Ca$^{2+}$ response.

**Flow Cytometry**—HEK293 cells, seeded in 12-well plates, were transfected with a total amount of 0.75 μg of cDNA/well. Forty-eight hours following transfection, cells were harvested after treatment with Accutase (PAA Laboratories, Pasching, Austria) and washed with PBS. Cells expressing HA- and Myc-tagged 5-HT3 subunits were incubated with mouse anti-HA or mouse anti-Myc antibody ([supplemental Table 2](#)) for 1 h on ice. After washing the cells with PBS, they were incubated with the secondary Alexa Fluor 488 anti-mouse antibody ([supplemental Table 2](#)) for 20 min on ice. Following a washing step with PBS, cells were resuspended in 150 μl of FACS buffer (BD Perm/Wash, BD Biosciences). Samples were run on a FACSCalibur system (BD Biosciences). For each staining condition, 30,000
cells were analyzed using CellQuest Pro 4.0.2 software (BD Biosciences). Cells with a mean fluorescence intensity (MFI) >99% of mock-transfected cells were defined as positive. Data are presented as “fluorescence indices” (FIs) as has been previously done for γ-aminobutyric acid type A receptors: FI = percentage of positive cells × MFI (32).

Data Analysis—Relative light units (RLU) for increases of the intracellular Ca\(^{2+}\) concentration, measured as aequorin luminescence, were obtained by subtraction of baseline luminescence from the 5-HT-induced peak luminescence. In 5-HT maximum response experiments, the peak luminescence (RLU\(_{\text{peak}}\)) was normalized against total aequorin luminescence (RLU\(_{\text{max}}\)) after cell lysis to control for differences in transfection efficiency and cell number (RLU\(_{\text{peak}}\)/(RLU\(_{\text{peak}}\) + RLU\(_{\text{max}}\))). The concentration-response curves and saturation binding curves as well as the pEC\(_{50}\) values, Hill slopes, and binding constants maximum binding capacity (B\(_{\text{max}}\)) and K\(_d\) were calculated by means of GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Data are given as means ± S.E. Statistical analysis was performed with unpaired Student’s t test or one-way analysis of variance followed by Dunnett’s or Tukey’s post hoc test. Differences were considered significant at \(p < 0.05\).

RESULTS

Co-expression of RIC-3 and 5-HT3C, -D, and -E

We performed comparative expression analysis by reverse transcription-PCR, amplifying parts of the coding regions of the genes HTR3A, -C, -D, and -E and the complete coding region of RIC3 using 14 cDNAs prepared from human adult tissues (Fig. 1). HTR3C and HTR3D presented with different splice forms corresponding to isoforms that were described recently (33). In line with previous data, transcription of HTR3A and HTR3C was ubiquitous, and for HTR3D, highest expression was detectable in colon and lung (23). However, HTR3D could not be amplified from kidney, and weak bands were visible in brain tissue from caudate nucleus and hippocampus and in the periphery in liver, heart, and stomach. Primers for HTR3E were also suitable to amplify HTR3Ea. Consistent with earlier studies, expression of HTR3E was restricted to colon and intestine (23, 34). The mRNA for canonical RIC3 (RIC-3a; band size, 1.18 kb) was expressed in all tissues tested except kidney. Thus, it was present in those tissues expressing at least one of the 5-HT3 subunits A, C, D, or E. Three additional products with sizes of approximately 1.0, 0.8, and 0.6 kb could be amplified, presenting with a tissue-specific expression pattern. These might presumably correspond to splice isoforms of RIC3, which have been described previously (12, 35).

Co-localization of 5-HT3 subunits and Calnexin or RIC-3

Immunofluorescence experiments of two different mammalian cell lines (HEK293 and U2OS) transiently expressing 5-HT3C, -D, or -Ea subunits revealed a co-staining of these subunits with the ER-resident chaperone calnexin and thereby confirmed the localization of the 5-HT3 subunits to the ER (supplemental Fig. 1). To obtain insight into the possible interaction of the 5-HT3 subunits and the more specific chaperone RIC-3, further immunofluorescence experiments on the same cell lines transiently expressing 5-HT3C, -D, or -Ea subunits and RIC-3 were carried out.
These experiments revealed a co-localization of all tested subunits and RIC-3 in the ER (Fig. 2).

**Direct Interaction of 5-HT3C, -D, and -Ea with BiP/Calnexin**

To further analyze the putative interaction between the subunits 5-HT3A, -C, -D, and -E and the globally acting ER-resident chaperones BiP and calnexin, immunoprecipitation experiments of transfected and metabolically labeled HEK293 cells were performed. A direct protein interaction between 5-HT3A, -C, and -Ea and the two chaperones was confirmed because all subunits co-precipitated with BiP or calnexin. In the case of 5-HT3D, a direct BiP or calnexin interaction remained questionable in this experimental setup because the expected band was hardly visible (supplemental Fig. 2A). However, additional immunoprecipitation experiments followed by Western blot confirmed an interaction of 5-HT3D with BiP or calnexin (supplemental Fig. 2B).

**Direct Interaction of 5-HT3A, -C, -D, and -Ea with RIC-3 and N-Glycosylation of 5-HT3C and -Ea**

To additionally determine the existence of direct protein interactions between the subunits 5-HT3A, -C, -D, and -E and RIC-3, the same technique as that used for calnexin and BiP was applied. For that purpose, specific antibodies raised against the 5-HT3 subunits A, C, D, and E and RIC-3 were used (supplemental Table 2) (16).3 The interaction of RIC-3 with the 5-HT3B subunit could not be studied because no suitable antibody was available. Previous experiments revealed that the 5-HT3D/E antibody works better for the isoform 5-HT3Ea than for 5-HT3E. Thus, our analysis was restricted to only 5-HT3Ea. Immunoactive bands of expected sizes were detectable for 5-HT3A, -C, and -Ea (approximately 55 kDa) and for 5-HT3D (approximately 35 kDa) (Fig. 3). In lysates of cells co-transfected with RIC-3 and 5-HT3 subunit cDNAs, the corresponding immunoactive bands for the 5-HT3 subunits were detected by anti-RIC-3, indicating that RIC-3 co-precipitated with 5-HT3A, -C, -D, and -Ea subunits.

The co-immunoprecipitation of RIC-3 and 5-HT3A revealed an immunoactive band of a size below that of the completely glycosylated 5-HT3A subunit. This finding indicates that RIC-3 might exclusively interact with only partially glycosylated and thus immature 5-HT3A subunits. In contrast, RIC-3 co-precipitated with 5-HT3C, -D, and -Ea bands of all sizes, representing differentially glycosylated subunits in the case of 5-HT3C and 5-HT3Ea that would fit to the expected size increase of approximately 3 kDa per N-glycosylation site (37).

In Western blot experiments on transfected cells pretreated with tunicamycin, block of N-linked glycosylation led to a loss of higher molecular mass bands for 5-HT3C and -Ea. In contrast, the band for 5-HT3D remained unchanged (Fig. 4A). This suggests that N-glycosylation does occur on 5-HT3C and -Ea, whereas 5-HT3D is not glycosylated as has been predicted previously (23). All predicted N-glycosylation sites were verified by Western blot analysis of cells expressing 5-HT3C or -Ea after knock-out of the respective sites (23) using site-directed mutagenesis (Fig. 4B).

**Influence of RIC-3 on 5-HT-induced Ca2+ Influx through 5-HT3 Receptors**

**Homomeric 5-HT3A Receptors**—5-HT led to concentration-dependent aequorin luminescence reflecting intracellular Ca2+ elevation when applied to coelenterazine h-loaded HEK293 cells transiently expressing apoaequorin, RIC-3, and 5-HT3A. The resulting concentration-response curve was characterized by a pEC50 value of 5.77 ± 0.02 and a Hill coefficient of 3.12 ± 0.22. These functional parameters did not differ from those

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5-HT3A + 5-HT3E did not significantly differ from that determined on cells expressing the homomeric 5-HT3A receptor (274.1 ± 30.2 and 262.4 ± 27.4%, respectively). In contrast, RIC-3 led to a significantly smaller increase of $E_{\text{max}}$ on 5-HT3AB receptors of 179.0 ± 14.6% compared with that on 5-HT3A receptors. The most prominent influence of RIC-3 was detected on cells expressing 5-HT3A + 5-HT3C or 5-HT3A + 5-HT3Ea. The $E_{\text{max}}$ values were increased to 426.5 ± 32.1 and 513.4 ± 37.0%, respectively, in the presence of RIC-3 (Fig. 6).

**Influence of RIC-3 on Cell Surface Expression of 5-HT3 Receptors of Diverse Composition**

[$^3$H]GR65630 Binding Studies—The enhancement of human 5-HT3A receptor cell surface expression by human RIC-3 in mammalian cells has been shown before (16). Thus, we hypothesized that the increased 5-HT $E_{\text{max}}$ values for Ca$^{2+}$ influx reflect the promotion of cell surface expression by RIC-3. To clarify this issue, saturation binding studies with the 5-HT3 antagonist [$^3$H]GR65630 on membranes from transfected HEK293 cells were carried out. Experiments were performed for homomeric 5-HT3A receptors and specific 5-HT3 subunit combinations, which determined on 5-HT3A receptors in the absence of RIC-3 ($pEC_{\text{app}}$ 5.76 ± 0.03; Hill coefficient, 3.19 ± 0.26) (Fig. 5A). By contrast, RIC-3 increased the 5-HT-induced $E_{\text{max}}$. The RIC-3-mediated elevation of $E_{\text{max}}$ depended on the RIC-3 cDNA amount (Fig. 5B). Significantly increased Ca$^{2+}$ responses were detected for 5-HT3A:RIC-3 cDNA ratios between 1:0.01 and 1:1. The maximum RIC-3 effect was observed with a 1:0.1 cDNA ratio (279.9 ± 16.9% of control in the absence of RIC-3). For this reason, the 5-HT3 subunit:RIC-3 cDNA ratio 1:0.1 was used for further experiments.

**Co-expression of 5-HT3A and 5-HT3B (or -C, -D, -E, or -Ea)**—To determine the impact of RIC-3 on different 5-HT3 subunit combinations, cells expressing 5-HT3A (homomeric 5-HT3A receptor as control) or 5-HT3A + 5-HT3B (or -C, -D, -E, or -Ea) in the presence or absence of RIC-3 were analyzed for 5-HT-induced maximum Ca$^{2+}$ responses. In analogy to the results described above, 5-HT induced a significantly higher maximum response of 298.5 ± 21.9% in cells expressing 5-HT3A and RIC-3 compared with the response in the absence of RIC-3 (Student’s $t$ test, $p < 0.001$). The RIC-3-mediated increases of $E_{\text{max}}$ on cells expressing 5-HT3A + 5-HT3D or exhibited a significantly different influence of RIC-3 on the 5-HT-induced $E_{\text{max}}$ compared with the effect of RIC-3 on the 5-HT3A receptor, i.e. 5-HT3A + 5-HT3B (or -C or -Ea) (see above). Co-expression of RIC-3 and 5-HT3A led to an increase of the $B_{\text{max}}$ of [$^3$H]GR65630 to 232.7 ± 21.0% compared with the $B_{\text{max}}$ in the absence of RIC-3 (Student’s $t$ test, $p < 0.001$). This is related to an increase of the cell surface expression of the receptor (Table 2). RIC-3 also led to enhanced $B_{\text{max}}$ values for the examined 5-HT3 subunit combinations, however, to different extents as compared with increases in 5-HT-induced $E_{\text{max}}$ (Table 2). Co-expression of RIC-3 together with 5-HT3A + 5-HT3C and 5-HT3A + 5-HT3Ea resulted in $B_{\text{max}}$ increases of 319.0 ± 17.9 and 317.5 ± 22.2%, respectively. These values are significantly higher than the RIC-3-mediated $B_{\text{max}}$ increase of the homomeric 5-HT3A receptor. On the other hand, the $B_{\text{max}}$ determined on cells transfected with 5-HT3A + 5-HT3B cDNAs was less elevated in the presence of RIC-3 (184.1 ± 12.6%) than that of homomeric 5-HT3A receptors; however, the significance level was not reached in this case (Fig. 6 and Table 2). As expected, the dissociation constants ($K_d$) for [$^3$H]GR65630 on the examined receptors were not different in the presence or absence of RIC-3 (Table 2).
**Modulation of 5-HT_3 Receptor Expression by RIC-3**

**Flow Cytometry Experiments**—Because the radioligand [³H]GR65630 does not allow discrimination between heteromeric and homomeric 5-HT_3 receptors, an immunocytochemical approach was additionally applied. Therefore, FACS analyses with Myc-/HA-tagged subunits to enable the cell surface detection of particular subunits in cells expressing 5-HT3A + 5-HT3B (or -C, -D, -E, or -Ea) were carried out. In one set of experiments, cells expressing the HA-tagged 5-HT3A subunit plus one of the Myc-tagged 5-HT3B, -E, and -Ea subunits or the empty plasmid vector (HA-5-HT3A as control) in the presence or absence of RIC-3 were measured. In another set, cells expressing the Myc-tagged 5-HT3A subunit plus one of the HA-tagged 5-HT3C and -D subunits or the empty plasmid vector (Myc-5-HT3A as control) were analyzed.

Detection of HA-5-HT3A and Myc-5-HT3A in cells exclusively expressing 5-HT3A subunits revealed significantly enhanced FIs, reflecting increases of surface expression of homomeric 5-HT_3A receptors to 181.2 ± 17.2 and 161.0 ± 10.43% of control, respectively, in the presence of RIC-3 (Fig. 7). RIC-3 also mediated significant elevation of cell surface expression of 5-HT3A subunits when co-expressed with 5-HT3E or 5-HT3D subunits to 201.7 ± 10.13 and 146.6 ± 8.4% of control, respectively. These increases were not significantly different from those measured for the corresponding homomeric 5-HT_3A receptor. In contrast, co-expression of 5-HT3Ea or 5-HT3C subunits led to significantly higher RIC-3-mediated increases of the cell surface expression of 5-HT3A subunits compared with that for the 5-HT_3A receptor expressed alone (252.3 ± 22.72 and 195.0 ± 11.04%, respectively). Unexpectedly, the 5-HT3C, -D, -E, and -Ea subunits seemed to be expressed at very low frequencies on the surface of HEK293 cells. An average number of about 3% positive cells could be measured for these subunits, whereas for 5-HT3A the number of positive cells ranged from about 19 to 40% in the absence of RIC-3 (data not shown). Co-expression of RIC-3 did not alter the expression of 5-HT3C, -D, -E, and -Ea subunits on the cell surface (data not shown). In contrast, Myc-5-HT3B was well detectable on the cell surface, and RIC-3 led to an increased surface expression of HA-5-HT3A after co-expression of Myc-5-HT3B to 150.9 ± 15.0% of control. However, the expression of Myc-5-HT3B itself was not altered by RIC-3 (110.8 ± 14.7% of control).

**DISCUSSION**

Only a limited amount of information exists on the regulation of 5-HT_3 receptor expression and composition in different tissues. Therefore, we investigated the impact of the chaperone RIC-3 on the surface expression and function of diverse 5-HT_3 receptors, focusing on the 5-HT3C, -D, and -E subunits. Comparative mRNA expression analysis showed expression of alternative RIC3 transcripts in human tissues expressing at least one of the subunits 5-HT3C, -D, and -E subunits. Comparative mRNA expression analysis showed expression of alternative RIC3 transcripts in human tissues expressing at least one of the subunits 5-HT3C, -D, and -E. This is in line with the ubiquitous expression of RIC3 mRNA in central and peripheral tissues that has been shown in previous studies (12, 35). Thus, RIC-3 is likely to be co-expressed in cells expressing 5-HT_3 receptors, enabling interactions between the chaperone and 5-HT3 subunits in vivo. Furthermore, we showed co-localization of RIC-3 and 5-HT3C, -D, and -E subunits in the ER of two...
mammalian cell lines. Direct interaction of RIC-3 and the 5-HT3A subunit has been shown previously by co-immunoprecipitation (16). Our co-immunoprecipitation studies with HEK293 cells expressing 5-HT3 subunits and RIC-3 revealed that RIC-3 does not only interact with 5-HT3A but also with 5-HT3C, -D, and -Ea. Because the splice isoform 5-HT3Ea only differs from 5-HT3E in its signal sequence at the very N-terminal end (28), RIC-3 interaction with 5-HT3E is very likely. However, comparative expression analysis suggests that the RIC-3-mediated regulation of 5-HT3 receptor expression might be even more complex because, in the case of HTR3C and HTR3D, splice variants were detectable. The existence of additional HTR3 splice variants was described recently (33). Consequently, their role in receptor assembly and trafficking requires further investigation.

Analogous co-immunoprecipitation experiments with BiP and calnexin also revealed a direct interaction of these generalized chaperones with 5-HT3A, -C, -D, and -Ea subunits that previously has only been shown for 5-HT3AB receptors (6). Interaction with these chaperones in vivo is very likely because they are co-localized with 5-HT3 subunits in the ER, which was shown for calnexin by immunofluorescence. Additional findings regarding posttranslational modification of the subunits 5-HT3C, -D, -E, and -Ea came from N-glycosylation studies of transfected HEK293 cells. So far, N-glycosylation has only been shown for the subunits 5-HT3A and -B (6, 37). Our experiments revealed that all tested subunits except 5-HT3D are N-glycosylated and confirmed the previously predicted glycosylation sites (23). This points to an additional layer of complexity in the 5-HT3 receptor system based on posttranslational modification, which might play a role in receptor maturation and surface expression as has been shown for γ-aminobutyric acid type A and 5-HT\textsubscript{3}A receptors (37, 38).

Recent studies revealed a RIC-3-mediated increase of cell surface expression of functional nACh and 5-HT\textsubscript{3}A receptors. The ability of RIC-3 to act as a chaperone seems to be influenced by the host cell environment. Consistent with this, human RIC-3 enhanced the cell surface expression of recombinant human 5-HT\textsubscript{3}A receptors in mammalian cells (9, 13, 16), whereas it led to inhibition of recombinant murine 5-HT\textsubscript{3}A receptors in Xenopus oocytes (12, 39). In consideration of these species- and cell-dependent effects, we studied the impact of human RIC-3 on human 5-HT3 subunits heterologously expressed in HEK293 cells. As previously reported, RIC-3 exhibited no influence on the potency of the agonist 5-HT on homomeric 5-HT\textsubscript{3}A receptors (16). Furthermore, the affinities of the radioligand \textsuperscript{3}HGR65630 to all tested 5-HT3 subunit combinations were not altered after RIC-3 co-expression. Thus, RIC-3 does not influence the conformation of the 5-HT\textsubscript{3}A receptor ligand-binding site. These results suggest that RIC-3 does not affect the function of 5-HT\textsubscript{3} receptors. As expected from its action as a chaperone, radioligand binding and functional Ca\textsuperscript{2+} influx studies revealed that RIC-3 enhances the cell surface expression of functional 5-HT\textsubscript{3} receptors in particular for all tested 5-HT3 subunit combinations, i.e. 5-HT3A and 5-HT3A + 5-HT3B (or -C, -D, -E, or -Ea).

As determined by measuring maximum 5-HT\textsubscript{3}A-induced Ca\textsuperscript{2+} influx through homomeric 5-HT\textsubscript{3}A receptors, the RIC-3-mediated increase of receptor expression was strongly dependent on the RIC-3 levels. RIC-3 enhanced maximum Ca\textsuperscript{2+} responses in cells with 5-HT3A:RIC-3 cDNA ratios of 1:0.01

**TABLE 2**

| Subunits | $B_{max}$ (pmol/mg protein) | $K_d$ (nM) |
|---------|-----------------|----------|
| 5-HT3A  | 25.01 ± 2.26    | 57.80 ± 4.55 |
| 5-HT3A + 5-HT3B | 5.01 ± 0.35 | 9.22 ± 0.90 |
| 5-HT3C  | 7.89 ± 1.28    | 24.47 ± 3.43 |
| 5-HT3Ea | 4.11 ± 0.42    | 12.73 ± 1.01 |

*p < 0.001.

*p < 0.01.

**FIGURE 7.** Flow cytometry analysis of HEK293 cells transiently expressing either 5-HT3A or 5-HT3A + 5-HT3B (or -C, -D, -E, or -Ea) in the presence or absence of RIC-3. Cells were transfected with Myc- or HA-tagged subunit cDNAs to enable the cell surface detection with mouse anti-HA (clone HA-7) or mouse anti-Myc antibody (clone 9B11). The secondary antibody was Alexa Fluor 488 donkey anti-mouse IgG. Fls are expressed as percentages of the respective Fl values in the absence of RIC-3 (means ± S.E. of six to nine independent transfections). Shown is the cell surface expression of the 5-HT3A subunit detected by anti-HA (A) or anti-Myc antibody (B). For co-transfections of 5-HT3A and 5-HT3B cDNA, expression of both subunits is shown (A). Significant differences compared with control without RIC-3 (**, p < 0.01; ***, p < 0.001) or compared with the RIC-3 effect on the homomeric 5-HT3A receptor (+, p < 0.05) are indicated.
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to 1:1. Further increase of the RIC-3 cDNA amount abolished the RIC-3-mediated elevation of the maximum Ca2+ signal. This might be due to the formation of RIC-3 aggregates, which have been observed after RIC-3 overexpression (17, 40, 41) and shown to reduce the amount of nACh receptor subunits for the formation of functional receptors (40). This is in line with a recent study showing that co-expression of RIC-3 and the nAChα7 subunit in tsA201 cells only led to a measurable cell surface expression of the homomeric nAChα7 receptor when small amounts of RIC-3 cDNA were used (42). This concentration dependence of the chaperone activity of RIC-3 might be an essential mechanism of the expression regulation of nACh and 5-HT3 receptors in different cell types or at different times. Cheng et al. (16) also observed an enhanced surface expression of 5-HT3A subunits depending on the RIC-3 amount. However, they found an increase of surface expression of 5-HT3A up to a 5-HT3A:RIC-3 cDNA ratio of 1:4. These discrepancies might be due to application of different expression constructs or to different incubation times after transfection because the interaction with RIC-3 was shown to be transient (16).

The extent of the RIC-3-mediated increase of cell surface expression varied between different 5-HT3 subunit combinations. This was shown by three approaches: 5-HT-induced Ca2+ influx, radioligand saturation binding, and FACS studies. FACS analyses revealed that on cells co-expressing 5-HT3A and 5-HT3B subunits RIC-3 exclusively enhanced cell surface expression of 5-HT3A, whereas the amount of 5-HT3B remained constant. Hence, a predominant formation of homomeric 5-HT3A receptors at the expense of heteromeric 5-HT3AB receptors is promoted. This result confirms data of recent studies (17, 30). The net effect was therefore a less pronounced RIC-3-mediated increase in 5-HT3 receptor surface expression on cells expressing 5-HT3A + 5-HT3B compared with cells exclusively expressing 5-HT3A. This was seen in both Ca2+ influx and radioligand binding studies and is further supported by data of Cheng et al. (17), who also measured a slightly smaller RIC-3-induced increase of $B_{\text{max}}$ for [3H]GR65630 binding on cells expressing 5-HT3A + 5-HT3B compared with those exclusively expressing 5-HT3A.

Unexpectedly, FACS experiments on cells co-expressing 5-HT3A and 5-HT3C (or -D, -E, or -Ea) subunits revealed that 5-HT3C, -D, -E, and -Ea are expressed on the cell surface at very low levels, which were not increased by RIC-3. The lack of an RIC-3-mediated increase of surface expression of 5-HT3C, -D, -E, and -Ea would be in line with its interaction with mature subunits. Thus, comparable with its reported action on the 5-HT3B subunit, RIC-3 appears to have an inhibitory effect on these subunits as well. Their marginal surface expression levels, measured in FACS experiments, seem to contradict results from previous immunofluorescence and biotinylation studies showing co-expression of 5-HT3A and 5-HT3C (or -D, -E, or -Ea) on the cell surface (28). However, immunofluorescence images showed a lower fluorescence intensity for the subunits 5-HT3C, -D, -E, and -Ea compared with 5-HT3A on the cell surface (see Fig. 1 in Ref. 28). Low levels of surface expression restricted to a small number of cells, visible in immunofluorescence, are likely to be missed in flow cytometry, which is less sensitive. We conclude that only a low amount of the subunits 5-HT3C, -D, -E, and -Ea is part of mature receptors on the cell surface of HEK293 cells. This might be one reason why no differences in the pharmacological properties of cells expressing 5-HT3A + 5-HT3C (or -D, -E, or -Ea) and those expressing homomeric 5-HT3A receptors could be detected until now (28). Furthermore, an explanation for the lacking surface expression of these subunits could be the requirement of yet unidentified chaperones for incorporation into functional 5-HT3 receptors, which are not present in HEK293 cells. This would be comparable with the situation regarding nAChα7 receptors some years ago. Efficient surface expression of these receptors in cell lines like HEK293 or Chinese hamster ovary was not achieved until the discovery of RIC-3 as an nACh receptor chaperone (15, 43).

RIC-3 mediated the most prominent increase of 5-HT3 receptor cell surface expression on cells expressing 5-HT3A + 5-HT3Ea (or -C) measured by radioligand binding and 5-HT-induced Ca2+ influx. This was confirmed by FACS analyses showing that the RIC-3-mediated increase of surface expression of the 5-HT3A subunit was highest on these cells. Cells expressing the latter subunit combinations have been shown previously to exhibit significantly lower 5-HT3 receptor surface expression levels compared with cells expressing homomeric 5-HT3A receptors (28, 30, 33). Thus, we hypothesize that the inhibitory effect of 5-HT3Ea and -C subunits on 5-HT3 receptor expression might be abolished by RIC-3, leading to a facilitated cell surface expression of mature homomeric 5-HT3A receptors. On the other hand, the RIC-3-mediated increases of 5-HT3 receptor cell surface expression on cells expressing 5-HT3A + 5-HT3D (or -E) were not different from that on cells expressing homomeric 5-HT3A receptors. This is in agreement with the fact that these subunit combinations did not alter 5-HT3 receptor surface expression compared with cells expressing homomeric 5-HT3A receptors (28). However, the question whether this kind of regulation would be the same in native cells, which express the required chaperones, still remains. Thus, the function of the subunits 5-HT3C, -D, and -E is still elusive, and the discovery of yet unidentified essential chaperone molecules will enable further studies regarding 5-HT3 receptor composition.

In summary, we showed interaction of the two general chaperones BiP and calnexin and the specific chaperone RIC-3 with 5-HT3A, -C, -D, and -E subunits. Combined with previous findings (17), our results support the hypothesis that RIC-3 serves to ensure the production of homomeric 5-HT3A receptors at the expense of heteromeric receptors incorporating 5-HT3B–E subunits. Therefore, RIC-3 seems to play an important role in determining 5-HT3 receptor composition in vivo. Analogous results have been reported for nACh receptors of C. elegans; RIC-3 led to a preferential expression of DEG-3-rich DEG-3/DES-2 receptors (26, 27). Interestingly, RIC3 mRNA levels have been shown to be elevated in the post-mortem brains of individuals with bipolar disorder and schizophrenia (18), and accumulating evidence exists that 5-HT3 receptors are likely to be involved in both diseases (36). This underlines the putative role of 5-HT3 receptor modulation by RIC-3, which might be disturbed in neuropsychiatric diseases. Therefore, the investigation of 5-HT3 receptor maturation and regulation will
help to gain insight into the pathomechanism and to generate novel approaches for the therapy of these diseases.

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REFERENCES

1. Collingridge, G. L., Isaac, J. T., and Wang, Y. T. (2004) Nat. Rev. Neurosci. 5, 952–962
2. Green, W. N., and Millar, N. S. (1995) Trends Neurosci. 18, 280–287
3. Blount, P., and Merlie, J. P. (1991) J. Cell Biol. 113, 1125–1132
4. Gelman, M. S., Chang, W., Thomas, D. Y., Bergeron, J. J., and Prives, J. M. (1995) J. Biol. Chem. 270, 15085–15092
5. Wanamaker, C. P., and Green, W. N. (2007) J. Biol. Chem. 282, 31113–31123
6. Boyd, G. W., Low, P., Dunlop, J. I., Robertson, L. A., Vardy, A., Lambert, J. J., Peters, J. A., and Connolly, C. N. (2002) Mol. Cell. Neurosci. 21, 38–50
7. Vandenberghe, W., Nicoll, R. A., and Bredt, D. S. (2005) J. Neurosci. 25, 1095–1102
8. Jeanclos, E. M., Lin, L., Treuell, M. W., Rao, J., DeCoster, M. A., and Anand, R. (2001) J. Biol. Chem. 276, 28281–28290
9. Millar, N. S. (2008) Br. J. Pharmacol. 153, Suppl. 1, S177–S183
10. Nguyen, M., Alfonso, A., Johnson, C. D., and Rand, J. B. (1995) Genetics 140, 527–535
11. Halevi, S., McKay, J., Palfreyman, M., Yassin, L., Eshel, M., Jorgensen, E., L E., Cohen, C. J., and Aiyar, J. (2005) Mol. Pharmacol. 68, 26158–26166
12. Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M., and Treinin, M. (2003) J. Neurosci. 23, 1573–1581
13. Landsdell, S. J., Collins, T., Yabe, A., Gee, V. J., Gibb, A. J., and Millar, N. S. (2008) J. Neurochem. 105, 1573–1581
14. Landsdell, S. J., Gee, V. J., Harkness, P. C., Doward, A. I., Baker, E. R., Gibb, A. J., and Millar, N. S. (2005) Mol. Pharmacol. 68, 1431–1438
15. Williams, M. E., Burton, B., Urrutia, A., Shcherbatko, A., Chavez-Noriega, L. E., Cohen, C. J., and Aiyar, J. (2005) J. Biol. Chem. 280, 1257–1263
16. Cheng, A., McDonald, N. A., and Connolly, C. N. (2005) J. Biol. Chem. 280, 22502–22507
17. Cheng, A., Bollan, K. A., Greenwood, S. M., Irving, A. J., and Connolly, C. N. (2007) J. Biol. Chem. 282, 26158–26166
18. Severance, E. G., and Yolken, R. H. (2007) Neuroscience 148, 454–460
19. Belelli, D., Balcarek, J. M., Hope, A. G., Peters, J. A., Lambert, J. J., and Blackburn, T. P. (1995) Mol. Pharmacol. 48, 1054–1062
20. Davies, P. A., Pitsis, M., Hanna, M. C., Peters, J. A., Lambert, J. J., Hales, T. G., and Kirkness, E. F. (1999) Nature 397, 359–363
21. Dubin, A. E., Huvar, R., D’Andrea, M. R., Pyati, J., Zhu, J. Y., Joy, K. C., Wilson, S. J., Galindo, J. E., Glass, C. A., Luo, L., Jackson, M. R., Lovenberg, T. W., and Erlander, M. G. (1999) J. Biol. Chem. 274, 30799–30810
22. Miyake, A., Mochizuki, S., Takemoto, Y., and Akuzawa, S. (1995) Mol. Pharmacol. 48, 407–416
23. Niesler, B., Frank, B., Kapeller, J., and Rappold, G. A. (2003) Gene 310, 101–111
24. Chetty, N., Coupar, I. M., Tan, Y. Y., Desmond, P. V., and Irving, H. R. (2009) Neurogastroenterol. Motil. 21, 551–558, e14–e15
25. Exley, R., Moroni, M., Sasdelli, F., Houldian, L. M., Lukas, R. J., Sher, E., Zwart, R., and Bermudez, I. (2006) J. Neurochem. 98, 876–885
26. Ben-Ami, H. C., Yassin, L., Farah, H., Michaeli, A., Eshel, M., and Treinin, M. (2005) J. Biol. Chem. 280, 28053–28060
27. Cohen Ben-Ami, H., Biala, Y., Farah, H., Elishvitz, E., Battat, E., and Treinin, M. (2009) Biochemistry 48, 12329–12336
28. Niesler, B., Walsteb, J., Combrink, S., Möller, D., Kapeller, J., Rietdorf, J., Bönisch, H., Göttерт, M., Rappold, G., and Brüss, M. (2007) Mol. Pharmacol. 72, 8–17
29. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 61–68
30. Walsteb, J., Hammer, C., Bönisch, H., Rappold, G., and Niesler, B. (2008) Pharmacogenet. Genomics 18, 793–802
31. Walsteb, J., Combrink, S., Brüss, M., Göttерт, M., Niesler, B., and Bönisch, H. (2007) Anal. Biochem. 368, 185–192
32. Lo, W. Y., Botzolakis, E. J., Tang, X., and Macdonald, R. L. (2008) J. Biol. Chem. 283, 29740–29752
33. Holbrook, J. D., Gill, C. H., Zebda, N., Spencer, J. P., Leyland, R., Rance, K. H., Trinh, H., Balmer, G., Kelly, F. M., Yusaf, S. S., Courtenay, N., Luck, J., Rhodes, A., Modha, S., Moore, S. E., Sanger, G. J., and Gunthorpe, M. J. (2009) J. Neurochem. 108, 384–396
34. Karnovsky, A. M., Gotow, L. F., McKinley, D. D., Pichnan, J. L., Ruble, C. L., Mills, C. J., Schellin, K. A., Slightom, J. L., Fitzgerald, L. R., Benjamin, C. W., and Roberds, S. L. (2003) Gene 319, 137–148
35. Seredenina, T., Ferraro, T., Terstappen, G. C., Caricasole, A., and Ronca-rati, R. (2008) Biosci. Rep. 28, 299–306
36. Thompson, A. J., and Lunnis, S. C. (2007) Expert Opin. Ther. Targets 11, 527–540
37. Monk, S. A., Williams, J. M., Hope, A. G., and Barnes, N. M. (2004) Biochem. Pharmacol. 68, 1787–1796
38. Connolly, C. N., Krishek, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1996) J. Biol. Chem. 271, 89–96
39. Castillo, M., Mulet, J., Gutiérrez, L. M., Ortiz, I. A., Castelan, F., Gerber, S., Sala, S., Sala, F., and Criado, M. (2005) J. Biol. Chem. 280, 27062–27068
40. Shteingauz, A., Cohen, E., Biala, Y., and Treinin, M. (2009) J. Cell Sci. 122, 807–812
41. Wang, Y., Yao, Y., Tang, X. Q., and Wang, Z. Z. (2009) J. Neurosci. 29, 12625–12635
42. Alexander, J. K., Jefford, G., Criado, M., Sagher, D., and Green, W. N. (2007) in Annual Meeting of the Society for Neuroscience, San Diego, November 3–7, 2007, 575.3/K3, Society for Neuroscience, Washington, D. C.
43. Cooper, S. T., and Millar, N. S. (1997) J. Neurochem. 68, 2140–2151
44. Walsteb, J., Rappold, G., and Niesler, B. (2010) Pharmacol. Ther., in press