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Heterocomplex formation of 5-HT_{2A}-$mGlu_2$ and its relevance for cellular signaling cascades

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ABSTRACT

Dopamine, serotonin and glutamate play a role in the pathophysiology of schizophrenia. In the brain a functional crosstalk between the serotonin receptor 5-HT_{2A} and the metabotropic glutamate receptor $mGlu_2$ has been demonstrated. Such a crosstalk may be mediated indirectly through neuronal networks or directly by receptor oligomerization. A direct link of the 5-HT_{2A}-$mGlu_2$ heterocomplex formation to receptor function, i.e. to intracellular signaling, has not been fully demonstrated yet. Here we confirm the formation of 5-HT_{2A}-$mGlu_2$ heterocomplexes using quantitative Snap/Clip-tag based HTRF methods. Additionally, $mGlu_2$ formed complexes with 5-HT_{2B} and $mGlu_5$ but not 5-HT_{2C} indicating that complex formation is not specific to the 5-HT_{2A}-$mGlu_2$ pair. We studied the functional consequences of the 5-HT_{2A}-$mGlu_2$ heterocomplex addressing cellular signaling pathways. Co-expression of receptors in HEK-293 cells had no relevant effects on signaling mediated by the individual receptors when $mGlu_2$ agonists, antagonists and PAMs, or 5-HT_{2A} hallucinogenic and non-hallucinogenic agonists and antagonists were used. Hallucinogenic 5-HT_{2A} agonists induced signaling through $G_{q/11}$, but not G_i and thus did not lead to modulation of intracellular cAMP levels. In membranes of the medial prefrontal cortex [^{3}H]-LY341495 binding competition of $mGlu_{2/3}$ agonist LY354740 was not influenced by 2,5-dimethoxy-4-iodoamphetamine (DOI). Taken together, the formation of GPCR heterocomplexes does not necessarily translate into second messenger effects. These results do not put into question the well-documented functional cross-talk of the two receptors in the brain, but do challenge the biological relevance of the 5-HT_{2A}-$mGlu_2$ heterocomplex.

1. Introduction

Schizophrenia is a devastating mental disorder that affects about 1% of the population over the lifetime (Freedman, 2003; Sawa and Snyder, 2003; Tamminga and Holcomb, 2005). While the etiology of the disease is not completely understood, three neurotransmitter systems seem to be particularly relevant: dopamine, serotonin and glutamate. Currently marketed antipsychotic drugs block the dopamine D_2 receptor at therapeutic exposures (Carlsson, 1978; Talbot and Laruelle, 2002). Several studies demonstrated psychotomimetic effects of hallucinogenic compounds, like LSD, mescaline and DOI, due to 5-HT_{2A} receptor agonism (Geyer and Vollenweider, 2008; Gonzalez-Maeso et al., 2007; Schreiber et al., 1994), which were attenuated by selective 5-HT_{2A} receptor antagonists (Vollenweider et al., 1998), and by atypical antipsychotics (Meltzer et al., 1989). Preclinical and clinical studies found that channel blocking *N*-methyl-D-aspartate (NMDA) receptor antagonists (e.g., ketamine and phencyclidine) induce a syndrome in healthy volunteers resembling positive and negative symptoms of schizophrenia, suggesting that modulation of the glutamate transmitter system could be a valid approach to treat schizophrenia. Indeed, a metabotropic glutamate receptor ($mGlu_{2/3}$) agonist was found to be efficacious during a phase II clinical trial in schizophrenia (Patil et al., 2007). In this study LY2140023, a prodrug of the $mGlu_{2/3}$ receptor orthosteric agonist LY404039, attenuated both positive and negative symptoms to a degree comparable to the atypical antipsychotic olanzapine. Together with results from subsequent clinical trials (Kinon et al., 2011; Stauffer et al., 2011)

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT_{2A}, serotonin receptor 2A; DOI, 2,5-dimethoxy-4-iodoamphetamine; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; HTRF, homogenous time-resolved FRET; mGlu, metabotropic glutamate receptor; mPFC, medial prefrontal cortex; PAM, positive allosteric modulator; Tet, tetracycline.

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these data indicate the relevance of $mGlu_{2/3}$ receptor agonists as a novel antipsychotic principle, which had been suggested based on preclinical cytochemical, neurochemical and behavioral studies (Mezler et al., 2010; Moghaddam and Adams, 1998).

Besides direct activation of $mGlu_{2/3}$ receptors, such orthosteric agonists also modulate the activity of the 5-HT_{2A} serotonin receptor subtype. A reciprocal functional inhibition of 5-HT_{2A} agonism and $mGlu_{2/3}$ agonism has been described in the prefrontal cortex of rat (Aghajanian and Marek, 1999; Marek et al., 2000). In these studies, 5-HT_{2A} receptor activation induced excitatory postsynaptic currents (EPSCs) in the medial prefrontal cortex (mPFC) (Aghajanian and Marek, 1999), and a $mGlu_{2/3}$ antagonist further enhanced the frequency and amplitude of EPSCs (Marek et al., 2000). By contrast $mGlu_{2/3}$ agonists or 5-HT_{2A} receptor antagonists suppressed EPSCs and attenuated behavioral effects of serotonergic hallucinogens (*e.g.* LSD) (Gewirtz and Marek, 2000) and dissociative anesthetics (*e.g.* PCP) (Moghaddam and Adams, 1998). These findings are consistent with a proposed inhibitory autoreceptor function of $mGlu_{2/3}$ and suggest that presynaptic glutamate release is positively regulated by 5-HT_{2A} receptor activation. In particular these results demonstrate that the effects of $mGlu_2$ and 5-HT_{2A} receptor modulation are functionally antagonistic in the intact brain.

While the efficacy of the known $mGlu_{2/3}$ cross-reactive orthosteric agonists can not unequivocally be attributed to either of the two receptor types, novel $mGlu_2$-selective positive allosteric modulators (PAMs) like BINA generated similar effects (Benneyworth et al., 2007). Together with studies in $mGlu_2$ and $mGlu_3$ knockout mice (Spooren et al., 2000; Woolley et al., 2008), these data support the idea that $mGlu_2$, but not $mGlu_3$ is responsible for the functional 5-HT_{2A} antagonism.

Since functional antagonism between 5-HT_{2A} and $mGlu_2$ receptors is well established, it was of particular interest to investigate a possible direct molecular interaction between these two receptors. 5-HT_{2A} and $mGlu_2$ receptors are both localized in brain cortex (Marek et al., 2000), but direct demonstration of the existence of 5-HT_{2A}-$mGlu_2$ heterocomplexes was made only recently by Gonzalez-Maeso et al. (2008). This study found that $mGlu_2$ and 5-HT_{2A} directly interact in recombinant cell lines and are present in the same neuronal cells in culture. Additionally, these findings implicate functional consequences related to the pharmacology of antipsychotics due to interactions at the 5-HT_{2A}-$mGlu_2$ complex. It was shown that the formation of the 5-HT_{2A}-$mGlu_2$ complex enhances $G_{\alpha i}$ activation of hallucinogenic 5-HT_{2A} agonists, which is described to be involved in hallucinogen-specific signaling (Gonzalez-Maeso et al., 2008, 2007). Activation of the $mGlu_2$ component suppresses the neuropsychological effects of hallucinogens. A further study reported that a 5-HT_{2A}-$mGlu_2$ heterocomplex serves as integration point balancing $G_{\alpha i}$- and $G_{\alpha q}$-dependent signaling (Fribourg et al., 2011). This indicates a direct crosstalk between the two receptors and remodels the idea of a synaptic mechanism of 5-HT_{2A}-induced glutamatergic transmission.

To further substantiate these findings, we co-expressed $mGlu_2$ in an inducible manner within a constitutive 5-HT_{2A} background in HEK-293 cells. We determined the reciprocal influence of the two receptor types on receptor expression. Also, we evaluated the pharmacological responses of 5-HT_{2A} and $mGlu_2$ by measuring intracellular calcium and cAMP levels upon stimulation with agonists, antagonists, hallucinogens and PAMs. We confirm the 5-HT_{2A}-$mGlu_2$ heterocomplex formation, and demonstrate that $mGlu_2$ additionally interacts with 5-HT_{2B} and $mGlu_5$, but not with 5-HT_{2C}. The heterocomplex formation of 5-HT_{2A}-$mGlu_2$ alone did not result in a functional crosstalk. Furthermore, an effect of hallucinogenic 5-HT_{2A} agonists on $G_{\alpha i}$-mediated signaling could not be observed. While our data do not question the functional crosstalk of these neurotransmitter systems in the brain, these results argue that the functional interaction of $mGlu_2$ and 5-HT_{2A} may not be mediated by interaction in form of heteromers.

2. Materials and methods

2.1. Materials

LANCE cAMP 384 kit was purchased from PerkinElmer (Rodgau, Germany), FLIPR calcium 4 assay kit from Molecular Devices (Ismaning, Germany), and TagLite HTRF reagents were purchased from Cisbio Bioassays (Codolet, France). Compounds LY341495, LY354740, LY379268, LY404039, LY487379, BINA, 5-HT, 2-methylserotonin, R-(+)lisuride, Ketanserin, Mianserin, DOI (2,5-dimethoxy-4-iodoamphetamine), Mescaline, and [^{3}H]LY341495 can be obtained commercially (Tocris, Bristol, UK; Merck, Darmstadt, Germany; Sigma–Aldrich, Munich, Germany; and Selleck Chemicals, Houston, TX, USA). MGS0039 was synthesized at Abbott. All other reagents were purchased from Sigma–Aldrich.

2.2. Generation of cDNA constructs and cell lines

Plasmids encoding 5-HT_2 receptor subtypes A-C or $mGlu_5$ N-terminally fused to the Snap tag (pSnap-HTR2A, pSnap-HTR2B, pSnap-HTR2C, and pSnap-hGRM5) and $mGlu_2$ N-terminally fused to the Clip tag (pClip-hGRM2) were purchased from Cisbio Bioassays.

T-REx-293 cells (Invitrogen, Darmstadt, Germany) were cultured in DMEM (Invitrogen) with 5 μg/mL blasticidin S HCl (Invitrogen) and selected with 800 μg/mL geneticin (Invitrogen) for stable 5-HT_{2A} (pcDNA3.1; Invitrogen) expression and with 150 μg/mL hygromycin B (Roche, Mannheim, Germany) for tetracycline-inducible $mGlu_2$ (pcDNA5/TO) expression (293-H_{2A}-iG_2). Additionally, cell lines expressing solely inducible $mGlu_2$ (293-iG_2) or 5-HT_{2A} (293-H_{2A}) were generated. Maximal $mGlu_2$ expression was induced by 48 h stimulation with 1 μM tetracycline. For protein interaction assays, HEK 293T/17 cells (ATCC #CRL-11268) were transiently transfected using Lipofectamine (Invitrogen). 293T/17 cells showed better transfection rates and higher expression levels than regular HEK-293 cells and were thus used for all transient expression experiments. All cells were cultured at 37 °C under 5% CO_2 atmosphere in DMEM high glucose GlutaMAX-I (Invitrogen), 10% charcoal/dextran treated FBS (Thermo Scientific HyClone, Bonn, Germany), gentamycin (Invitrogen), and appropriate selection antibiotics.

2.3. HTRF assay

To measure HTR-FRET signals, 293T/17 cells were co-transfected with Snap-tagged 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, or $mGlu_5$ receptor and Clip-tagged $mGlu_2$ receptor 24 or 48 h prior to assay performance. Cells were labeled with Tag-lite Clip-Lumi4-Tb, Snap-Lumi4-Tb, and/or Clip-Red according to the manufacturer's protocol (Cisbio Bioassays) and HTR-FRET signal (665 nm) and Lumi4-Tb donor signal (620 nm) were measured using a PheraStar Plus (BMG Labtech, Ortenberg, Germany). HTRF ratio (665 nm/620 nm $\times$ 10^4) was calculated to eliminate quenching and dispensing errors. Co-transfections and substrate labeling were carefully titrated to obtain expression and labeling ratios of 1.

2.4. cAMP assay

Determination of intracellular cAMP through time resolved fluorescence energy transfer (TR-FRET) was studied with the LANCE cAMP detection kit according to the manufacturer's instructions using 96-well half area microplates (Costar, Corning, Amsterdam, The Netherlands). Cells were serum-starved for five hours and stimulation with compounds was done for 30 min. For $mGlu_2$ receptor antagonist and PAM testing, cells were simultaneously stimulated with EC_{80} or EC_{20} concentrations of glutamate, respectively. Pre-stimulation of $mGlu_2$ with LY404039 (EC_{80}) or LY341495 (EC_{100}) was done for 5 h prior to 5-HT_{2A} receptor stimulation with DOI.

2.5. Ca^{2+} mobilization assay

Calcium mobilization was measured by FLIPR (Fluorometric Imaging Plate Reader; Molecular Devices) with the calcium-specific indicator assay calcium 4 according to manufacturer's protocol. Stable T-REx-293 cell lines were seeded in 96-well (4×10^4 cells/well) clear-bottomed poly-D-lysine coated black microplates (Greiner Bio-One, Frickenhausen, Germany) two days prior to assay performance and incubated in glutamate and serum free medium for 5 h before the measurements.

2.6. Radioligand binding assay

Preparation of membranes and binding assay were performed as described by Wright et al. (2001). Assay volume of 500 μl consisted of 1 nM [^{3}H]LY341495, 14 μg protein of rat cerebral cortex membranes (Wistar Wistar) in 10 mM potassium phosphate and 100 mM potassium bromide, pH 7.6. Non-specific binding was