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Activation of mGlu2/3 Metabotropic Glutamate Receptors Negatively Regulates the Stimulation of Inositol Phospholipid Hydrolysis Mediated by 5-Hydroxytryptamine$_{2A}$ Serotonin Receptors in the Frontal Cortex of Living Mice

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ABSTRACT

The interaction between 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) serotonin receptors and metabotropic glutamate (mGlu) 2/3 receptors underlies the antipsychotic activity of mGlu2/3 receptor agonists in experimental animals and humans. The molecular nature of this interaction is only partially known. We here report for the first time that pharmacological activation of mGlu2/3 receptors attenuates the stimulation of polyphosphoinositide (PI) hydrolysis mediated by 5-HT$_{2A}$ receptors in the frontal cortex of living mice. Mice were injected intracerebroventricularly with [*myo*-^{3}H]inositol and treated with drugs 1 h after a pretreatment with lithium, which blocks the conversion of inositol monophosphate into free inositol. Systemic injection of the mGlu2/3 receptor agonist (−)-2-oxa-4-aminocyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) inhibited the stimulation of PI hydrolysis induced by the hallucinogenic 5-HT$_{2A}$ receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) without affecting the stimulation by mGlu1/5 or muscarinic receptors. The action of LY379268 was prevented by the preferential mGlu2/3 receptor antagonist (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2′-carboxycyclopropyl)glycine (LY341495). *N*-(4′-cyano-biphenyl-3-yl)-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride (LY566332), a selective mGlu2 receptor enhancer, also reduced DOI-stimulated PI hydrolysis when combined with subthreshold doses of LY379268. Systemic LY379268 inhibited DOI-stimulated PI hydrolysis in mice lacking either mGlu2 or mGlu3 receptors but was inactive in double mGlu2/mGlu3 receptor knockout mice, suggesting that both mGlu2 and mGlu3 receptors interact with 5-HT$_{2A}$ receptors. Surprisingly, contrasting results were obtained in cortical slice preparations, where LY379268 amplified both DOI- and 3,5-dihydroxyphenylglycine-stimulated PI hydrolysis. Amplification was abrogated by the mGlu5 receptor antagonist 2-methyl-6-(phenylethynyl)pyridine, suggesting that experiments in brain slices are biased by an additional component of receptor-stimulated PI hydrolysis. This highlights the importance of in vivo models for the study of the interaction between 5-HT$_{2A}$ and mGlu2/3 receptors.

The evidence that the metabotropic glutamate (mGlu) 2/3 receptor agonist 4-amino-2-thiabicyclo(3.1.0)hexane-4,6-dicarboxylic acid (LY404039, given in the form of a prodrug) relieves schizophrenic symptoms without increasing body weight in a phase IIa clinical study (Patil et al., 2007) is a major breakthrough in the mGlu receptor field and holds great promise for the treatment of psychosis, particularly under conditions in which weight gain limits the use of atypical antipsychotics (e.g., in patients with psychosis associated with Alzheimer's disease or in patients with diabetes). The development of mGlu2/3 receptor agonists as antipsychotic drugs moved from a series of pioneer studies on the interaction between 5-HT$_{2A}$ serotonin receptors and mGlu2/3 receptors in the cerebral cortex. 5-HT$_{2A}$ receptors are activated by hallucinogens such as lysergic acid diethylamide, psilo-

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ABBREVIATIONS: mGlu, metabotropic glutamate; 5-HT, 5-hydroxytryptamine; PI, polyphosphoinositide; LY379268, (−)-2-oxa-4-aminocyclo[3.1.0]hexane-4,6-dicarboxylic acid; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DHPG, 3,5-dihydroxyphenylglycine; LY341495, (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2′-carboxycyclopropyl)glycine; LY566332, *N*-(4′-cyano-biphenyl-3-yl)-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride; MPEP, 2-methyl-6-(phenylethynyl)pyridine; BAY367620, (3a*S*,6a*S*)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[*c*]furan-1-on; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PLC, phospholipase C; InsP, inositol monophosphate; PBS, phosphate-buffered saline; ANOVA, analysis of variance; PLSD, protected least significant difference; LY354740, (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo(3.1.0)hexane-2,6-dicarboxylic acid monohydrate.

cin, and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Vollenweider et al., 1998) and are antagonized by atypical antipsychotics, such as clozapine, olanzapine, risperidone, and quetiapine (Kroeze and Roth, 1998; Marek and Aghajanian, 1998). It is noteworthy that 5-HT_{2A} receptors are up-regulated and mGlu2 receptors down-regulated in the frontal cortex of patients with schizophrenia (González-Maeso et al., 2008), suggesting that a functional unbalance between these two receptors contributes to the development of schizophrenic symptoms. The first evidence for a functional interaction between mGlu2/3 receptors and 5-HT_{2A} receptors was provided by Marek et al. (2000), who showed that the potent and selective mGlu2/3 receptor agonist LY354740 attenuated serotonin-induced excitatory postsynaptic currents recorded in layer V pyramidal cells of the medial prefrontal cortex. These findings suggested that mGlu2/3 receptors negatively regulate the ability of 5-HT_{2A} receptors to stimulate release from thalamic fibers afferent to the prefrontal cortex (Marek et al., 2001). Pharmacological activation of mGlu2/3 receptors has been shown to inhibit the behavioral and electrophysiological effects of hallucinogens (Gewirtz and Marek, 2000; Kłodzinska et al., 2002; Zhai et al., 2003; Winter et al., 2004; Benneyworth et al., 2007) and to prevent the down-regulation of 5-HT_{2A} receptors induced by repeated administrations of DOI in the prefrontal cortex (Marek et al., 2006). Recent evidence sheds light onto the molecular nature of the interaction between 5-HT_{2A} and mGlu2/3 receptors. González-Maeso et al. (2008) have shown that 5-HT_{2A} and mGlu2 receptors form a heterodimeric complex that involves the fourth and fifth transmembrane domains of mGlu2 receptors. Within this complex, activation of mGlu2 receptors specifically inhibits one of the two major signaling pathways activated by 5-HT_{2A} receptors in response to hallucinogens in cultured cortical neurons and in the mouse somatosensory cortex: the pathway mediated by a pertussis toxin-sensitive G_i/G_o protein that ultimately leads to the induction of the immediate early gene egr-2. At least under the conditions described by González-Maeso et al. (2008), activation of mGlu2 receptors fails to affect the canonical signaling pathway activated by 5-HT_{2A} receptors [i.e., the G_q-dependent activation of polyphosphoinositide (PI) hydrolysis], which leads to an increased formation of inositol-1,4,5-trisphosphate and diacylglycerol and ultimately to c-Fos induction. These findings, however, are not in agreement with the evidence that the mGlu2/3 receptor agonist LY379268 or the selective mGlu2 receptor enhancer biphenyl-indanone A prevents the induction of c-fos by hallucinogens in the medial prefrontal cortex (Zhai et al., 2003; Benneyworth et al., 2007). Thus, the nature of the interaction between mGlu2/3 and 5-HT_{2A} is complex, and the detection of immediate early genes as downstream readout systems of receptor activation may be misleading, although it may be advantageous for in vivo studies. We decided to apply the technique of in vivo measurements of receptor-activated PI hydrolysis for the study of the interaction between 5-HT_{2A} and mGlu2/3 receptors using a battery of pharmacological tools, as well as knockout mice lacking mGlu2 receptors [mGlu2(−/−)], mGlu3 [mGlu3(−/−)] receptors, or both [mGlu2(−/−)/mGlu3(−/−)] receptor subtypes.

Materials and Methods

Materials. [*myo*-^{3}H]inositol (18 Ci/mmol) was purchased from GE Healthcare (Milano, Italy); (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and pilocarpine were obtained from Sigma (Milano, Italy); 3,5-dihydroxyphenylglycine (DHPG), LY341495, 2-methyl-6-(phenylethynyl)pyridine (MPEP), and BAY367620 were purchased from Tocris Bioscience Ltd. (Avonmouth, UK). LY379268 and LY566332 were kindly provided by Eli Lilly and Company (Indianapolis, IN). mGlu2(−/−) mice, mGlu3(−/−) mice, double mGlu2(−/−)/mGlu3(−/−) mice, and their wild-type littermates were kindly provided by Eli Lilly and Company. For a detailed description of these mice, see Linden et al. (2005).

Assessment of PI Hydrolysis in Vivo. Male CD1 mice (30–35 g, Charles River Italia, Calco, Italy) or wild-type, mGlu2(−/−), mGlu3(−/−), and mGlu2(−/−)/mGlu3(−/−) mice (30–35 g) were anesthetized with ketamine (100 mg/kg) plus xylazine (10 mg/kg) and injected with [*myo*-^{3}H]inositol (2 μCi/5 μl/2 min, i.c.v.). Twenty-four hours later, mice were treated with lithium ions (administered as LiCl, 10 mmol/kg s.c.) to inhibit the conversion of inositol monophosphate (InsP) into free inositol. DOI, DHPG, or pilocarpine was injected intraperitoneally (DOI, pilocarpine) or intracerebroventricularly (DHPG) 1 h after LiCl injection. DHPG was dissolved in saline containing 50% dimethyl sulfoxide for intracerebroventricular injections. Control mice were injected with the vehicle alone. mGlu2/3 receptor ligands were injected intraperitoneally 5 min before DOI, pilocarpine, or DHPG. Mice were killed 1 h after treatment with DOI, DHPG, or pilocarpine. The frontal cortices and hippocampi were quickly removed and stored at −80°C. On the day of the assay, brain tissue was sonicated in 2 ml (frontal cortex) or 1.25 ml (hippocampus) of water containing 10 mM LiCl. After centrifugation at 10,000*g* for 20 min, the [^{3}H]InsP present in the supernatant was separated by anion exchange chromatography in 10-ml columns containing 1.5 ml of Dowex 1-X-8 resin (formate form, 100–200 mesh; Bio-Rad, Milan, Italy). Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP (Nicoletti et al., 1986). Total radioactivity in the brain regions was determined by counting a 100-μl aliquot of the whole homogenate. Values are expressed as the ratio between the radioactivity measured in the [^{3}H]InsP fraction and the radioactivity measured in the whole homogenate times 100. Independent aliquots of the whole homogenates were added to chloroform and methanol (final water/methanol/chloroform ratio, 1:1:1) for measurements of the radioactivity incorporated into the chloroform phase containing inositol phospholipids.

Intracerebral Infusions in Mice. Male CD1 mice (30–35 g, Charles River) were injected with [*myo*-^{3}H]inositol (2 μCi/5 μl/2 min i.c.v) and implanted with intracerebral cannulas secured with cement, under ketamine (100 mg/kg i.p.) plus xylazine (10 mg/kg i.p.) anesthesia in a Kopf stereotaxic frame. The site of cannula implantation was the left frontal cortex [coordinates: 1.5 mm anterior to the bregma, 1.8 mm lateral to the midline, and 1.6 mm ventral from the surface of skull, according to the atlas of Franklin and Paxinos (1997)]. After surgery, mice were housed in separate cages in a temperature-controlled environment on a 12-h light/dark cycle with ad libitum access to water and food. Twenty-four h later, mice were treated with lithium ions (administered as LiCl, 10 mmol/kg s.c.). One hour later, mice were injected with DOI 2 mg/kg i.p., and 5 min before DOI, mice were injected by the intracerebral cannula with PBS, 3 μl/2 min, or LY379268, 10 nmol/3 μl/2 min. Mice were killed 1 h later, and both frontal cortices were dissected out and used to measure PI hydrolysis as described above.

Measurement of Polyphosphoinositide Hydrolysis in Cortical Slices. Receptor agonist-stimulated PI hydrolysis was also measured in cortical slices, as described by Nicoletti et al. (1986). In brief, male CD1 neonate (7–9 postnatal days) or adult mice were

killed by decapitation, and fresh frontal cortices were sliced (350 × 350 μm) using a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, UK). Slices were incubated at 37°C under constant oxygenation for 30 to 45 min in Krebs-Hensleit buffer equilibrated with 95% O_2/5% CO_2 to pH 7.4. Forty microliters of gravity-packed slices were then incubated for 60 min in 250 μl of buffer containing 1 μCi of [*myo*-^{3}H]inositol. Slices were incubated with LiCl (10 mM for 10 min) followed by the indicated concentrations of DOI and mGlu receptor ligands. One hour later, the incubation was stopped by the addition of 900 μl of methanol/chloroform (2:1), after washing the slices with ice-cold buffer. After further addition of 300 μl of chloroform and 600 μl of water, the samples were centrifuged at low speed to facilitate phase separation, and the upper aqueous phase was loaded into Dowex 1-X-8 columns (Dow Chemical Company, Midland, MI) for the separation of [^{3}H]InsP, as indicated above.

Assessment of Mitogen-Activated Protein Kinase Activation in the Frontal Cortex in Vivo and in Cortical Slices. For in vivo experiments, male CD1 mice were treated with DOI and LY379268 alone or in combination (LY379268 was administered 5 min before DOI) and then killed 15 min later. For in vitro experiments, cortical slices prepared as described above were oxygenated at 37°C for 30 min and then challenged with DOI (100 μM) and LY379268 (1 μM), alone or in combination, for an additional 15 min. For the assessment of mitogen-activated protein kinase (MAPK) activation, cortical tissue was lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 0.01% Nonidet P-40, 100 mM NaCl, , 5 mM EDTA, 50 mM NaF, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin) for 15 min at 4°C. Equal amounts of proteins (100 μg) were separated by 8% SDS-polyacrylamide gel. After separation, proteins were transferred onto nitrocellulose membranes. Membranes were incubated with an antibody against phosphorylated extracellular signal-regulated kinase ERK1/2 (phospho-p44/42 MAPK monoclonal antibody, 1:500; Cell Signaling, Danvers, MA) overnight at 4°C. Blots were then incubated for 1 h with the secondary antibody (1:5000, peroxidase-coupled anti-mouse, or 1:7000, peroxidase-coupled anti-rabbit; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Immunostaining was revealed by the enhanced chemiluminescence Western blotting analysis system (GE Healthcare). Data were normalized for the expression of unphosphorylated ERK1/2 (p44/42 MAPK polyclonal antibody, 1:2000; Cell Signaling).

Results

Activation of mGlu2/3 Receptors Inhibits DOI-Stimulated PI Hydrolysis in Vivo. For measurements of receptor-activated PI hydrolysis in vivo, we adopted a protocol originally developed by Patel and Freedman (1994). Mice were infused with 2 μCi of [*myo*-^{3}H]inositol i.c.v. and were treated 24 h later with lithium ions (10 mmol/kg s.c. as LiCl) to block the conversion of [^{3}H]InsP into free [^{3}H]inositol. Drugs were injected either intracerebroventricularly, intraperitoneally, or subcutaneously 1 h after lithium treatment. The lipid fractions extracted from the frontal cortex and hippocampus of control mice incorporated 4% of the total radioactivity. Basal [^{3}H]InsP formation was 1 to 3% of total radioactivity in the frontal cortex, as reported previously (Johnson et al., 1999). Injection of the mGlu1/5 receptor agonist DHPG (0.6 μmol/3 μl i.c.v.) increased [^{3}H]InsP formation by approximately 50% in the frontal cortex (Fig. 1A). We also measured the PI response to DHPG in the hippocampus, which was selected as a reference brain region (Johnson et al., 1999). The extent of stimulation was approximately 2-fold, a value similar to that reported in rats injected with equimolar doses of DHPG (Johnson et al., 1999). Stimulation of PI hydrolysis by DHPG in the mouse frontal cortex was abolished by a 5-min pretreatment with the selective mGlu5

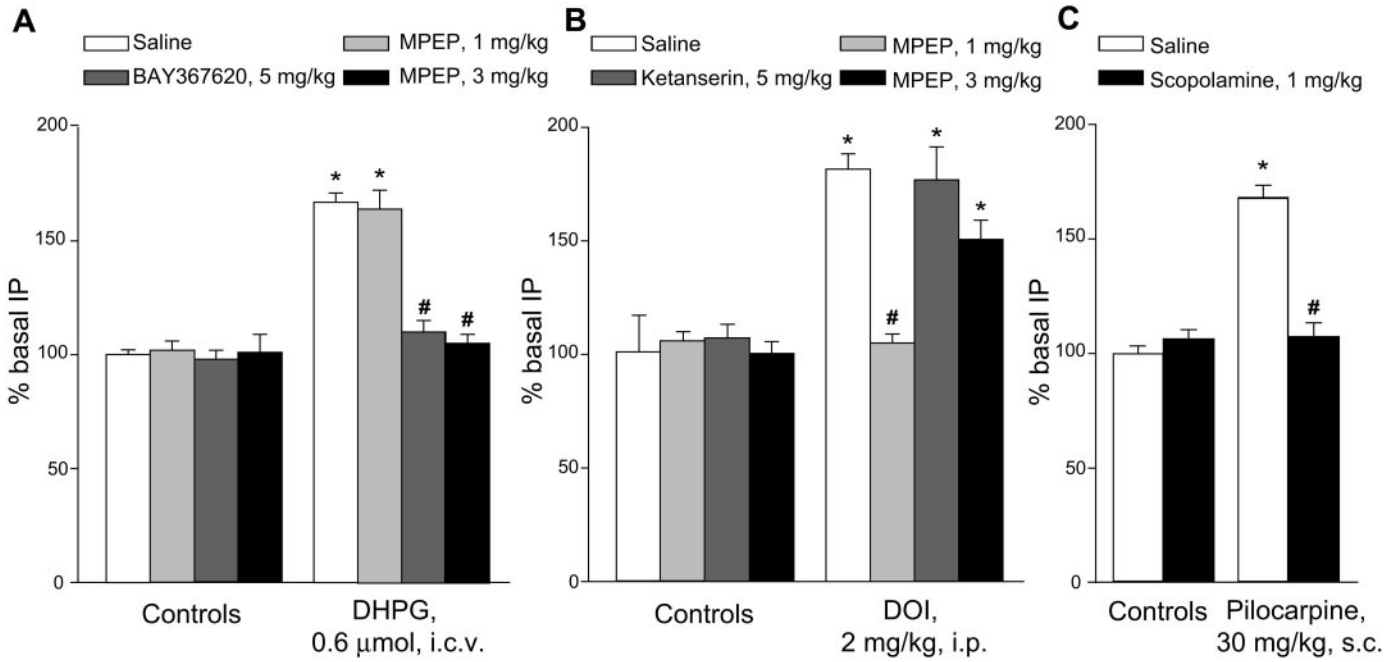

Fig. 1. In vivo stimulation of PI hydrolysis by DHPG (A), DOI (B), or pilocarpine (C) in the mouse frontal cortex. Mice were infused with 2 μCi of [*myo*-^{3}H]inositol i.c.v. and treated 24 h later with lithium ions (10 mmol/kg s.c. as LiCl). DHPG (0.6 μmol i.c.v.), DOI (2 mg/kg i.p.), or pilocarpine (30 mg/kg s.c.) was administered 1 h after lithium treatment. Mice were pretreated intraperitoneally with saline, BAY367620 (5 mg/kg), MPEP (1 or 3 mg/kg), or scopolamine (1 mg/kg) 5 min before the administration of DHPG, DOI, or pilocarpine. Data (mean ± S.E.M.; n = 8–10) are expressed as the percentage of controls pretreated with saline; $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective control mice (∗) or the respective groups of mice pretreated with saline (#).

receptor antagonist MPEP (1 or 3 mg/kg i.p.) but was unaffected by the mGlu1 receptor antagonist BAY367620 (5 mg/kg i.p.) (Fig. 1A). The hallucinogen DOI (2 mg/kg i.p.) was as effective as DHPG in stimulating [^{3}H]InsP formation in the frontal cortex (Fig. 1B), but at least at these doses, DOI failed to induce [^{3}H]InsP formation in the hippocampus (data not shown). All further data are restricted to the frontal cortex. The stimulation of [^{3}H]InsP formation by DOI was abolished by the 5-HT$_{2A}$ receptor antagonist ketanserin (5 mg/kg i.p., 5 min of pretreatment) and was partially reduced by the highest dose of MPEP (3 mg/kg), suggesting that a component of the action of DOI was mediated by the endogenously released glutamate acting at mGlu5 receptors (Fig. 1B). The muscarinic cholinergic receptor agonist pilocarpine (30 mg/kg s.c.) also stimulated [^{3}H]InsP formation in the frontal cortex (Bymaster et al., 2001), and its action was sensitive to the muscarinic receptor antagonist scopolamine (1 mg/kg i.p. injected 15 min before pilocarpine) (Fig. 1C). To

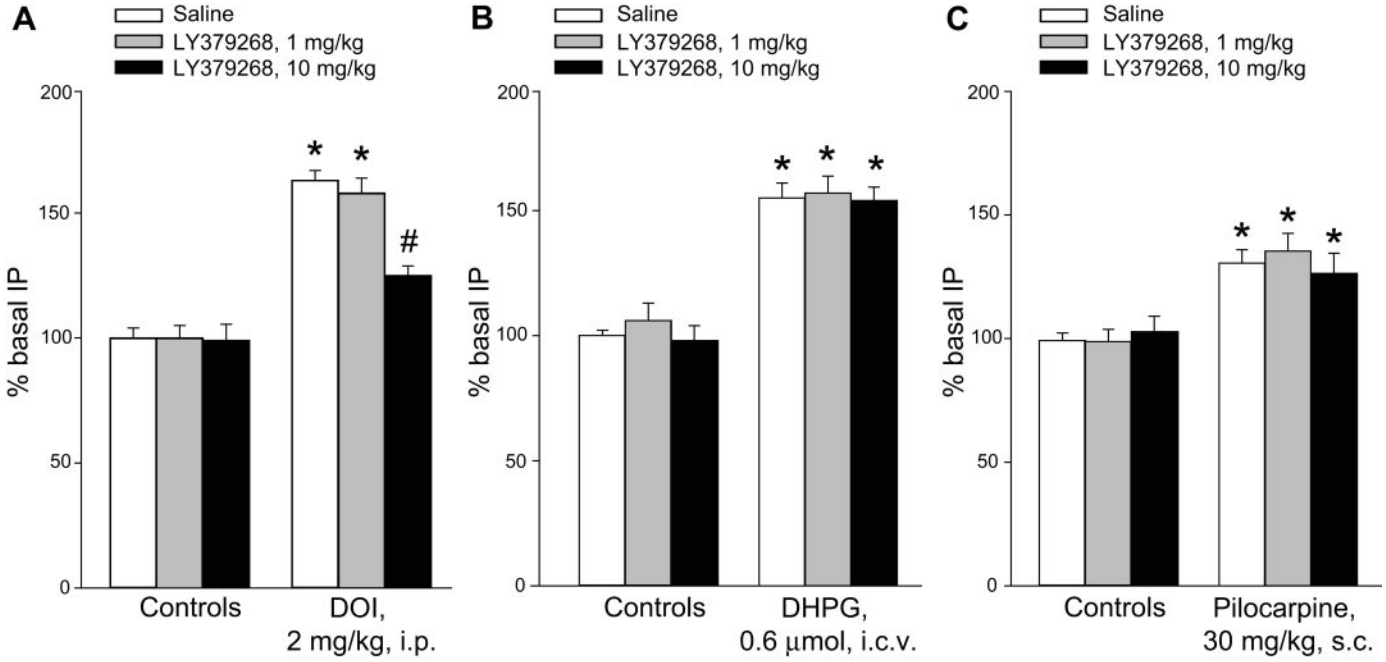

Fig. 2. In vivo stimulation of PI hydrolysis by DOI (A), DHPG (B), or pilocarpine (C) in the frontal cortex of mice pretreated with LY379268. Experiments were carried out as described in Fig. 1. Data (mean ± S.E.M.; n = 6–15) are expressed as the percentage of controls pretreated with saline; $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective control mice (∗) or the respective groups of mice pretreated with saline (#).

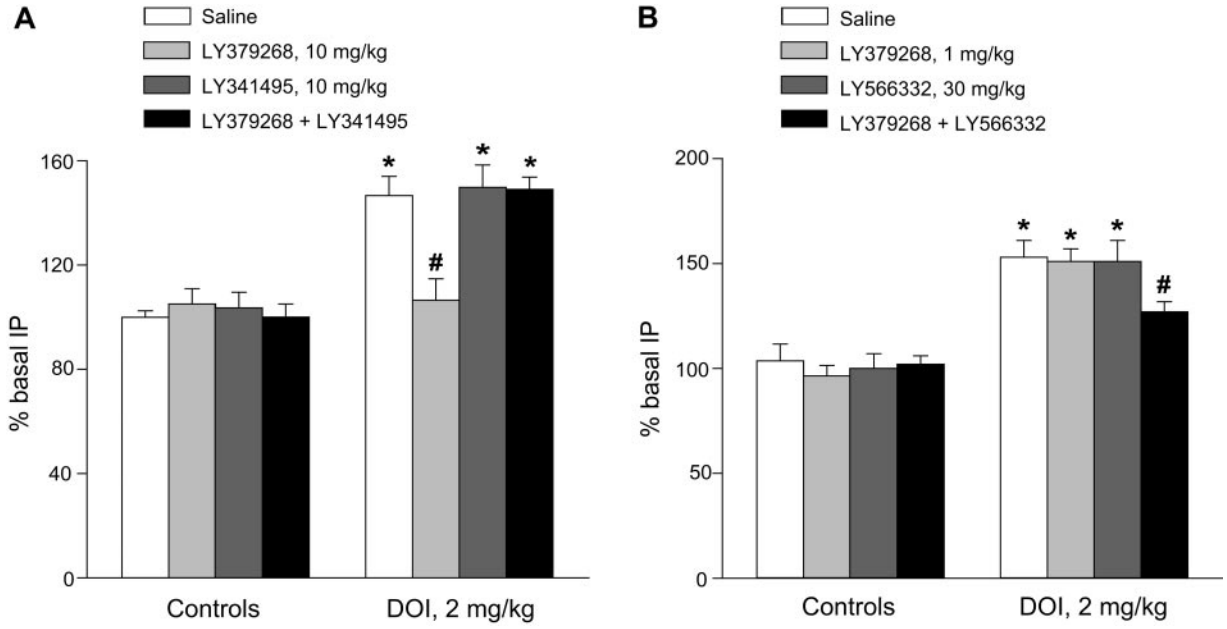

Fig. 3. In vivo stimulation of PI hydrolysis by DOI in the frontal cortex of mice pretreated with LY379268 and LY341495 (A) or LY566332 (B) administered alone or in combination. Experiments were carried out as described in Fig. 1. Data (mean ± S.E.M.; n = 6–9) are expressed as the percentage of controls pretreated with saline. $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective control mice (∗) or the respective groups of mice pretreated with saline (#).

study the interaction between mGlu2/3 and 5-HT$_{2A}$ receptors, we injected mice with two doses of the orthosteric mGlu2/3 receptor agonist LY379268 (1 or 10 mg/kg i.p.) 5 min before the injection of DOI (2 mg/kg i.p.). LY379268 largely reduced the stimulation of PI hydrolysis by DOI at the dose of 10 mg/kg but not at the dose of 1 mg/kg (Fig. 2A). LY379268 was inactive per se and did not affect the stimulation of PI hydrolysis by DHPG or pilocarpine (Fig. 2, B and C). This indicates that 1) activation of mGlu2/3 receptors selectively inhibits the stimulation of PI hydrolysis mediated by the 5-HT$_{2A}$ receptors, and 2) this effect does not involve the mGlu5 receptor component of the overall response to DOI (see above). To examine the pharmacology of the interaction between mGlu2/3 and 5-HT$_{2A}$ receptors in further detail, we combined DOI and LY379268 with the preferential mGlu2/3 receptor antagonist LY341495 (10 mg/kg i.p.) or with the selective mGlu2 receptor enhancer LY566332 (30 mg/kg i.p.). LY341495 and LY566332 were injected at the same time as LY379268 (i.e., 5 min before DOI). LY341495 did not affect the stimulation of PI hydrolysis per se but antagonized the inhibitory action of LY379268 (Fig. 3A). The mGlu2 receptor enhancer LY566332 was also inactive per se but could also reduce DOI-stimulated PI hydrolysis when combined with the subthreshold dose of LY379268 (1 mg/kg) (Fig. 3B). None of the mGlu2/3 receptor ligands had any effect on PI hydrolysis in the absence of DOI (Fig. 3). We then examined the combined action of DOI and LY379268 in the frontal cortex of mGlu2(−/−) mice, mGlu3(−/−) mice, and double mGlu2(−/−)/mGlu3(−/−) mice compared with wild-type mice. Basal PI hydrolysis in the frontal cortex was similar in the four groups of mice (Fig. 4). Stimulation of PI hydrolysis by DOI did not differ among wild-type, mGlu2(−/−), and mGlu3(−/−) mice and was approximately 20% higher in double mGlu2(−/−)/mGlu3(−/−) mice. This difference, however, was not significant (Fig. 4). LY379268 (10 mg/kg i.p.) reduced DOI-stimulated PI hydrolysis to a similar extent in wild-type, mGlu2(−/−), and mGlu3(−/−) mice but failed to reduce PI hydrolysis in the frontal cortex of double mGlu2(−/−)/mGlu3(−/−) mice (Fig. 4).

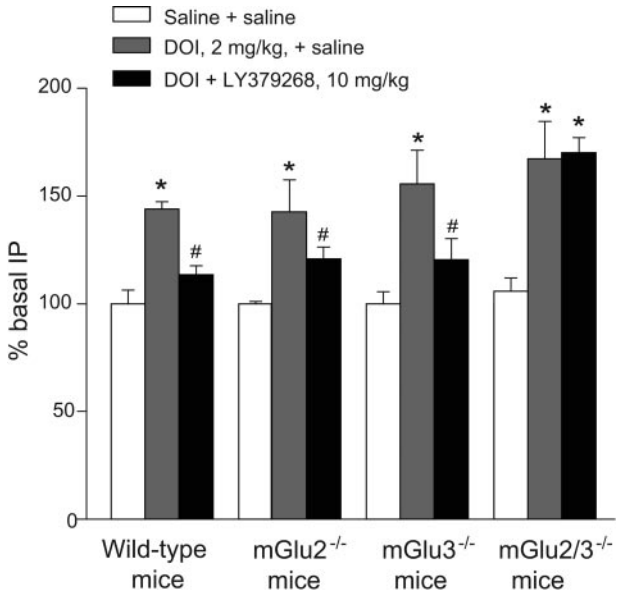

Fig. 4. In vivo stimulation of PI hydrolysis by DOI in the frontal cortex of wild-type, mGlu2(−/−), mGlu3(−/−), and double mGlu2(−/−)/mGlu3(−/−) mice pretreated with saline or LY379268. Experiments were carried out as described in Fig. 1. Data (mean ± S.E.M.; n = 6–9) are expressed as the percentage of the respective groups of mice pretreated with saline. Basal PI hydrolysis (i.e., PI hydrolysis in mice receiving saline + saline) did not differ among the different strains of mice; $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus the respective control mice (saline + saline) (*) or the respective groups of mice treated with DOI + saline (#).

Finally, we examined whether inhibition of DOI-stimulated PI hydrolysis by LY379268 was mediated by a local activation of mGlu2/3 receptors in the frontal cortex. Mice were unilaterally injected with vehicle (PBS) or LY379268 (3 nmol/3 μl of PBS) in the left side of the frontal cortex 5 min before a systemic injection of DOI (2 mg/kg i.p.) or saline. Intracortical infusion of PBS did not change the stimulation of PI hydrolysis by DOI (Fig. 5A). In contrast, local injection of LY379268 attenuated DOI-stimulated PI hydrolysis in the

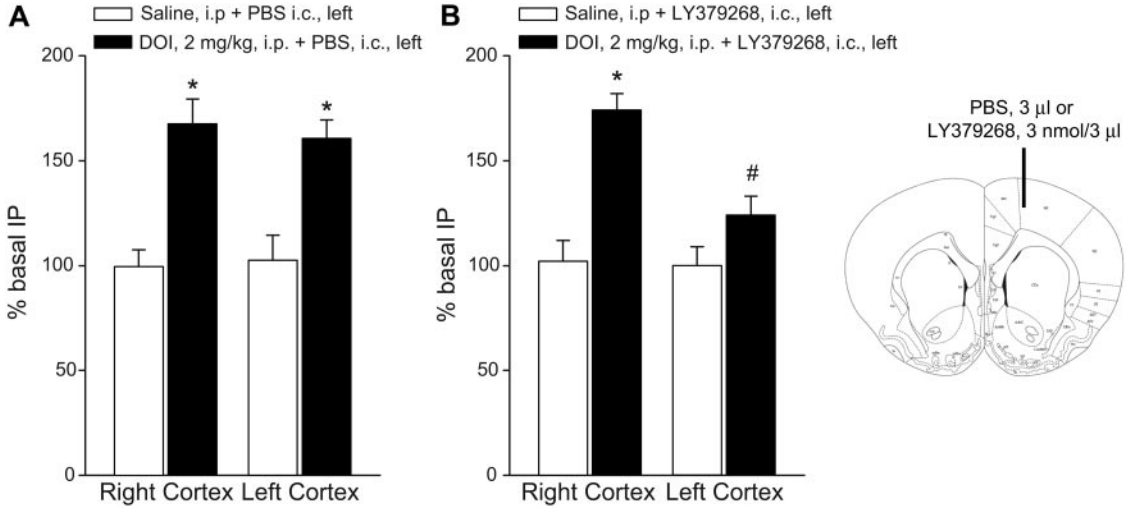

Fig. 5. In vivo stimulation of PI hydrolysis by DOI in mice infused with PBS (A) or LY379268 (B) in the left frontal cortex (see inset). [^{3}H]InsP formation was measured both in the left (injected) and right (noninjected) frontal cortex in each group of mice. Data (mean ± S.E.M.; n = 5–6) are expressed as the percentage of the respective groups of mice receiving saline intraperitoneally; $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective groups of mice treated with saline intraperitoneally (*) or versus the respective values obtained in the right frontal cortex of mice treated with DOI (#).

ipsilateral frontal cortex to an extent similar to that seen in mice systemically injected with LY379268 (Fig. 5B).

Modulation of DOI-Stimulated PI Hydrolysis by mGlu2/3 Receptor Activation in Cortical Slices. We also examined the modulation of PI hydrolysis by LY379268 in slices prepared from the frontal cortex of both neonate and adult mice. In brain slices, at least the PI response to group I mGlu receptor agonists is developmentally regulated and is maximal in the first 10 days of postanatal life (Nicoletti et al., 1986; Casabona et al., 1992). Accordingly, maximally effective concentrations of DHPG (200 μM) stimulated PI hydrolysis by approximately 9-fold in neonate cortical slices but were much less effective (<30% of stimulation) in adult slices (Fig. 6, A and B). We used lower concentrations of DHPG (3 μM) for the analysis of the action of LY379268 in neonate cortical slices. LY379268 (1 μM, applied 10 min before DHPG) markedly amplified the stimulation of PI hydrolysis by DHPG in neonate cortical slices (Fig. 6A). Incubation with the mGlu5 receptor antagonist MPEP completely inhibited the PI response to DHPG alone or combined with LY379268 (Fig. 6A). Similar results were obtained in adult cortical slices challenged with maximally effective concentrations of DHPG (200 μM) applied alone or in combination with LY379268 and/or MPEP (Fig. 6B). This confirms previous findings showing that activation of mGlu2/3 receptors does not stimulate PI hydrolysis per se but amplifies the PI response mediated by mGlu5 receptors (Nicoletti et al., 1993; Genazzani et al., 1994; Schoepp et al., 1996). It is noteworthy that the 5-HT_{2A} receptor agonist DOI (200 μM) also stimulated PI hydrolysis to a much greater extent in neonate (approximately 2-fold) than in adult (approximately 25%) cortical slices (Fig. 6, C and D). The action of DOI was inhibited by ketanserin (1 μM) in both slice preparations (Fig. 6, C and D). In neonate cortical slices, stimulation of PI hydrolysis by DOI was substantially enhanced by LY379268, which was inactive on its own. Coapplication with MPEP did not affect the PI response to DOI but abolished the amplifying effect of LY379268 (Fig. 6C). Similar results were obtained in adult cortical slices, in which the amplification of DOI-stimulated PI hydrolysis by LY379268 (which was smaller than in neonate slices) was also sensitive to MPEP (Fig. 6D). LY379268 never reduced the stimulation of PI hydrolysis by DOI both in the absence and presence of MPEP (Fig. 6).

Reciprocal Functional Antagonism between 5-HT_{2A} and mGlu2/3 Receptors on MAPK Activation. We extended the analysis to the MAPK pathway, which lies along signal propagation in both the G_q-dependent and the G_i/G_o-dependent pathways activated by hallucinogens. Here, we found no difference between in vivo data and data obtained in slice preparations. Treatment of mice with either DOI (2 mg/kg) or LY379268 (10 mg/kg) increased levels of phosphorylated ERK1/2 in the frontal cortex by approximately 50%. It is noteworthy, however, that we found no increase in ERK1/2 phosphorylation in the frontal cortex of mice treated with DOI combined with LY379268 (Fig. 7A). Likewise, both DOI

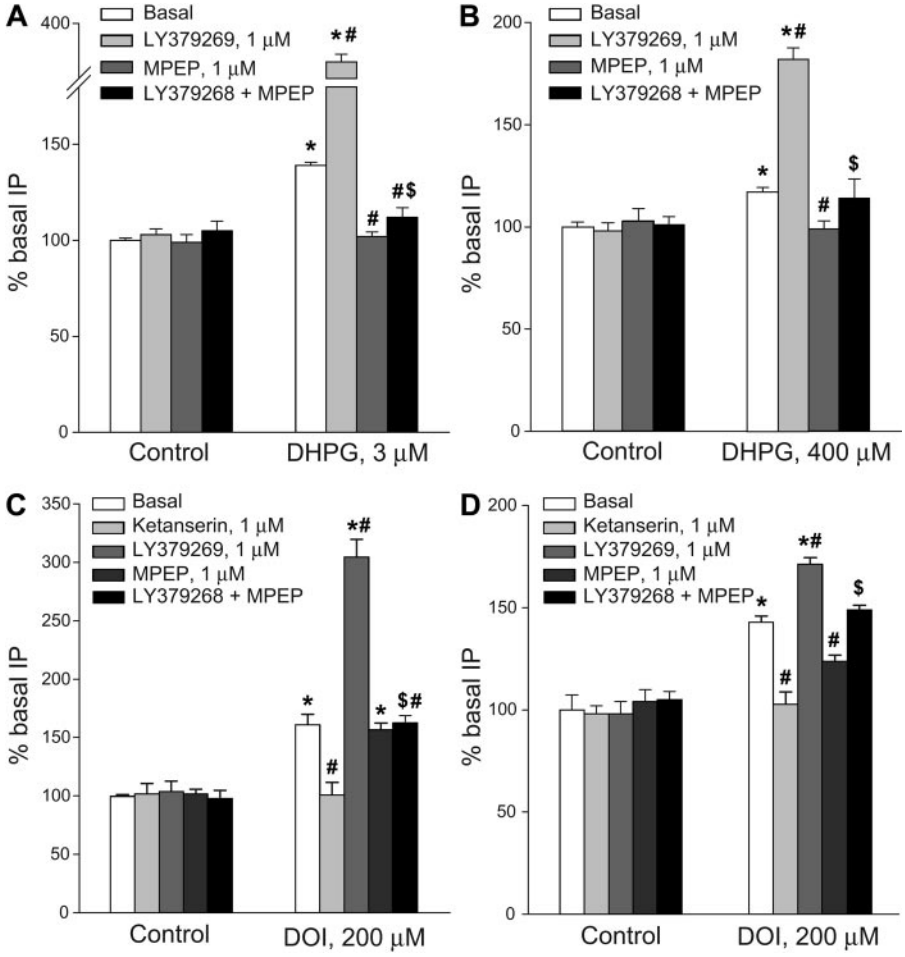

Fig. 6. Stimulation of PI hydrolysis by DHPG or DOI in slices prepared from the frontal cortex of neonate (A and C) or adult mice (B and D). Slices were preincubated with LY379268 (1 μM), MPEP (1 μM), or ketanserin (1 μM), 5 min before DHPG or DOI. Data (means ± S.E.M.; n = 9–15) are expressed as the percentage of the respective control values; $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective controls (*), the respective values obtained with DHPG or DOI alone (#), or the respective values obtained with DHPG or DOI combined with LY379268 ($).

(200 μM) and LY379268 (1 μM) increased ERK1/2 phosphorylation when applied alone to adult cortical slices but failed to activate the MAPK pathway when applied in combination (Fig. 7B).

Discussion

We have studied the modulation of 5-HT$_{2A}$ receptor-mediated PI hydrolysis by mGlu2/3 receptors using two models: 1) cortical slice preparations, which are commonly used for the assessment of agonist-stimulated PI hydrolysis; and 2) an in vivo model in which mice were intracerebroventricularly infused with radioactive inositol and then challenged with receptor ligands in the presence of lithium ions. In vitro and in vivo data were not uniform. In cortical slices, the mGlu2/3 receptor agonist LY379268 amplified DOI-stimulated PI hydrolysis (i.e., the stimulation of PI hydrolysis mediated by 5-HT$_{2A}$ receptors in response to a hallucinogenic drug). This was entirely unexpected because mGlu2/3 receptor agonists inhibit electrophysiological, neurochemical, and behavioral responses to hallucinogens (see Introduction and references therein). Amplification of DOI-stimulated PI hydrolysis in cortical slices was abrogated by the mGlu5 receptor antagonist MPEP. In addition, LY379268 amplified DHPG-stimulated PI hydrolysis in cortical slices (Nicoletti et al., 1993; Genazzani et al., 1994; and Schoepp et al., 1996) but not in the frontal cortex of living mice. This suggests that the slice preparation unmasks an interaction between mGlu2/3 and mGlu5 receptors that may confound data obtained with LY379268 and DOI. For example, DOI-stimulated glutamate release may lead to a secondary activation of mGlu5 receptors, which may be amplified by LY379268. We therefore conclude that cortical slice preparations are not appropriate for the study of the interaction between 5-HT$_{2A}$ and mGlu2/3 receptors.

Using our in vivo model, we demonstrated for the first time that pharmacological activation of mGlu2/3 receptors inhibits the stimulation of PI hydrolysis mediated by 5-HT$_{2A}$ receptors, an intracellular signaling pathway characteristically involved in the behavioral response to hallucinogenic drugs (Garcia et al., 2007). Inhibition of the PI response by LY379268 in the frontal cortex of living mice was specific for DOI (versus DHPG or pilocarpine) and could still be observed when the drug was locally injected into the frontal cortex. This suggests (but not proves) that systemically injected LY379268 inhibits 5-HT$_{2A}$-mediated PI hydrolysis by acting primarily in the frontal cortex. However, it should be highlighted that the in vivo assessment of PI hydrolysis required a short pretreatment with lithium ions, which might have induced changes in serotonergic transmission, including receptor-effector interactions, although these changes usually require days of exposure to lithium ions (Blier and De Montigny, 1985; Price et al., 1990; Blier and de Montigny, 1999; Bauer et al., 2003; Bschor et al., 2003). The use of a lithium-independent method for the in vivo assessment of PI hydrolysis, for example the autoradiographic detection of [^{3}H]-cytidindisphosphate-diacylglycerol after prelabeling with [^{3}H]cytidine (Hwang et al., 1990), might further strengthen our data.

González-Maeso et al. (2008) found that LY379268 inhibits

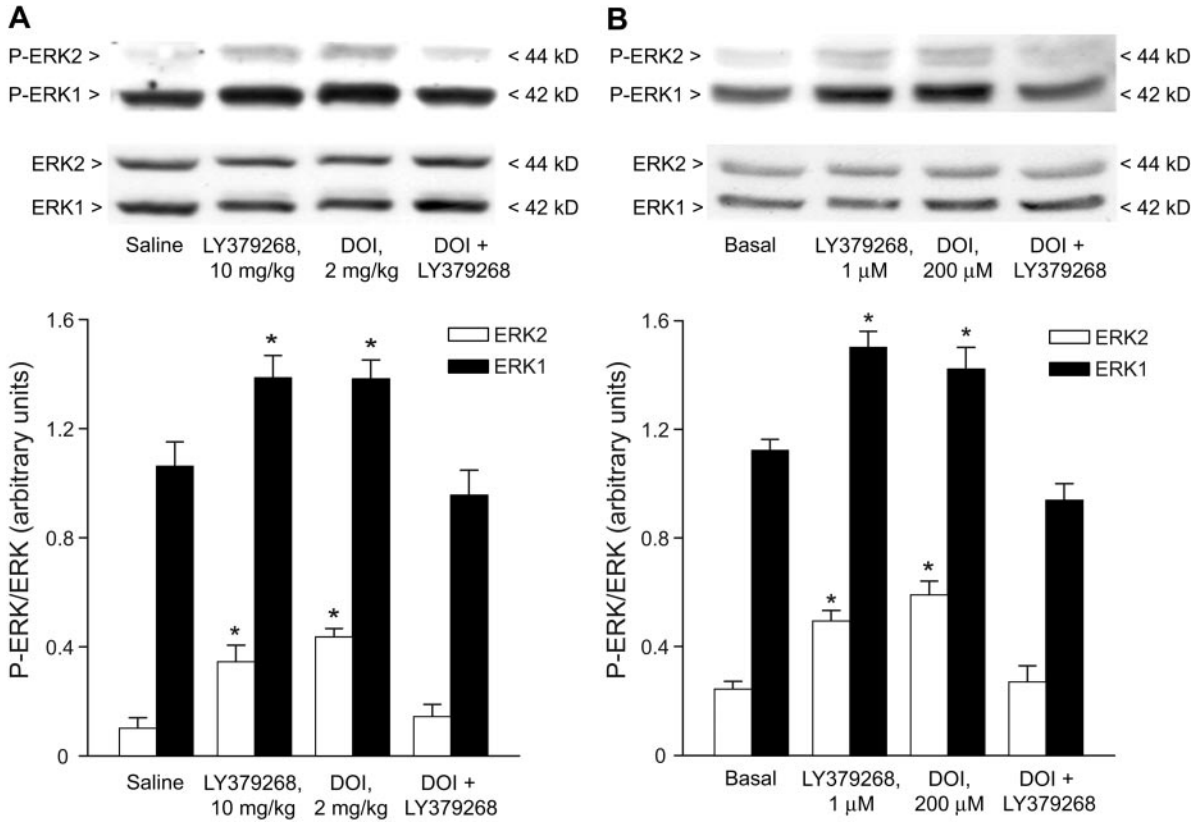

Fig. 7. Levels of phosphorylated ERK1/2 (P-ERK1/2) in the frontal cortex of living mice (A) or in cortical slices (B) after treatment with DOI and/or LY379268. Densitometric values are means ± S.E.M. of six to eight determinations. *, $p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus the respective control values (saline or basal values in A and B, respectively).

the induction of egr-2 but not the induction of c-fos mediated by the activation of 5-HT_{2A} receptors both in neurons of the mouse somatosensory cortex in vivo and in cultured cortical neurons. Egr-2 induction is a downstream event of a 5-HT_{2A}-receptor mediated G_i/G_o-dependent pathway, which is selectively activated by hallucinogens. In contrast, c-fos induction follows the activation of a G_q-dependent pathway, which is activated by both hallucinogenic and nonhallucinogenic 5-HT_{2A} receptor agonists (González-Maeso et al., 2003, 2007, 2008). Our findings suggest that, at least in the frontal cortex, mGlu2/3 receptors negatively regulate the canonical G_q/phospholipase Cβ (PLCβ) pathway activated by 5-HT_{2A} receptors, unless PLCβ is activated by G_i/G_o proteins in response to DOI. The evidence that activation of mGlu2 receptors prevents the induction of c-fos by hallucinogens in the medial prefrontal cortex is in nice agreement with our data (Zhai et al., 2003; Benneyworth et al., 2007).

Several lines of evidence suggest that it is the mGlu2 receptor that specifically interacts with 5-HT_{2A} receptors. For example, 1) selective mGlu2 receptor enhancers mimic the action of mGlu2/3 receptor agonists in inhibiting 5-HT_{2A} receptors (Zhai et al., 2003; Benneyworth et al., 2007); 2) epitope-tagged mGlu2 and 5-HT_{2A} receptors physically interact in recombinant cells (González-Maeso et al., 2008); 3) 5-HT_{2A} receptors colocalize with mGlu2 (but not mGlu3) receptor mRNA in mouse cortical neurons; and 4) mGlu2 and 5-HT_{2A} receptors coimmunoprecipitate in the human cerebral cortex (González-Maeso et al., 2008). Accordingly, we have found that the selective mGlu2 receptor enhancer LY566332 inhibited DOI-stimulated PI hydrolysis when combined with subthreshold doses of the orthosteric agonist LY379268. However, the use of knockout mice generated unexpected results. Systemic injection of LY379268 inhibited DOI-stimulated PI hydrolysis in both mGlu2(−/−) and mGlu3(−/−) receptor knockout mice, although it was inactive in double mGlu2(−/−)/mGlu3(−/−) receptor knockout mice. Taken together, these data suggest that 1) both mGlu2 and mGlu3 receptors negatively regulate 5-HT_{2A} receptor-mediated PI hydrolysis, or 2) alternatively, mGlu3 receptors compensate for the lack of mGlu2 receptors in mGlu2(−/−) receptor knockout mice. The study of the interaction between 5-HT_{2A} receptors and mGlu3 receptors (if any) awaits the availability of selective mGlu3 receptor ligands.

Finally, we have shown a negative interaction between 5-HT_{2A} and mGlu2/3 receptors on the MAPK pathway, which is known to be activated by DOI in a Gq/PLCβ-dependent manner (Schmid et al., 2008). Activation of mGlu2/3 receptors can also stimulate the MAPK pathway (Wang et al., 2004). Both DOI and LY379268 enhanced the levels of phosphorylated ERK1/2 in the frontal cortex of living mice and in cortical slices, as expected. However, the MAPK pathway was no longer activated when DOI was combined with LY379268, indicating a reciprocal functional antagonism between 5-HT_{2A} and mGlu2/3 receptors on the MAPK pathway. Perhaps signal transduction does not propagate through MAPK activation when both 5-HT_{2A} and mGlu2 receptors are activated within the heterodimeric complex (González-Maeso et al., 2008). Alternatively, pharmacological activation of separate 5-HT_{2A} and mGlu2 receptors with DOI and LY379268 may induce mechanisms of cross-desensitization that inhibit receptor signaling. It will be interesting to extend the study to the inhibition of cAMP formation, which is considered the canonical transduction pathway activated by mGlu2/3 receptors.

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