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Title Page

REGULATION OF GROUP II METABOTROPIC GLUTAMATE RECEPTORS BY G PROTEIN-COUPLED RECEPTOR KINASES: mGlu2 RECEPTORS ARE RESISTANT TO HOMOLOGOUS DESENSITIZATION

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Running Title Page

Running Title : Desensitization of mGlu2 and mGlu3 receptors

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Abbreviations: GPCR: G protein-coupled receptor; mGlu: metabotropic glutamate receptor; GRK: G protein-coupled receptor kinase; FSK: forskolin; MAPK: mitogen activated protein kinases; 2R,4R-APDC: (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate; LY379268: (–)-2-oxa-4-aminobicyclo[3.1.0]exhane-4,6-dicarboxylic acid; GRK2-K220R: kinase-dead GRK2 mutant; GRK2-Cter: C-terminal domain of GRK2

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Abstract

We examined the regulation of mGlu2 and mGlu3 metabotropic glutamate receptor signalling prompted by the emerging role of these receptor subtypes as therapeutic targets for psychiatric disorders, such as anxiety and schizophrenia. In transfected HEK293 cells, G-protein coupled receptor kinase (GRK)2 and GRK3 fully desensitized the agonist-dependent inhibition of cAMP formation mediated by mGlu3 receptors. In contrast, GRK2 or other GRKs did not desensitize the cAMP response to mGlu2 receptor activation. Desensitization of mGlu3 receptors by GRK2 required an intact kinase activity, as shown by the use of the kinase-dead mutant GRK2-K220R or the recombinant GRK2 C-terminal domain. Overexpression of β-arrestin1 also desensitized mGlu3 receptors and did not affect the cAMP signalling mediated by mGlu2 receptors. The difference in the regulation of mGlu2 and mGlu3 receptors was signal-dependent because GRK2 desensitized the activation of the MAP kinase pathway mediated by both mGlu2 and mGlu3 receptors. *In vivo* studies confirmed the resistance of mGlu2 receptor-mediated cAMP signalling to homologous desensitization. Wild-type, $mGlu2^{-/-}$ or $mGlu3^{-/-}$ mice were treated i.p. with saline or the mixed mGlu2/3 receptor agonist, LY379268 (1 mg/kg) once daily for 7 days. Inhibition of forskolin (FSK)-stimulated cAMP formation by LY379268 was measured in cortical slices prepared 24 hours after the last injection. Agonist pre-treatment fully desensitized the cAMP response in wild-type and $mGlu2^{-/-}$ mice, but had not effect in $mGlu3^{-/-}$ mice in which LY379268 could only activate the mGlu2 receptor. We predict the lack of tolerance when mixed mGlu2/3 receptor agonists or selective mGlu2 enhancers are used chronically in patients.

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Introduction

Agonists of group-II metabotropic glutamate (mGlu) receptors (mGlu2 and mGlu3 receptors) are under clinical development for the treatment of psychiatric disorders, including generalysed anxiety disorder and schizophrenia (Corti et al, 2007b; for review see Swanson et al., 2005). In a recent phase IIa clinical trial, the mixed mGlu2/3 receptor agonist, LY404039 (administered *per os* in form a pro-drug for 28 days) was as effective as the conventional antipsychotic, olanzapine, in relieving both positive and negative symptoms of schizophrenia (Patil et al., 2007). This evidence, which has been highlighted in the US media, gave new impetus to the study of mGlu2 and mGlu3 receptors. We decided to examine the mechanisms that regulate desensitization of mGlu2 and mGlu3 receptors, a process that critically affects the outcome of a long-term treatment with receptor agonists, and is therefore of great clinical relevance. mGlu2 and mGlu3 receptors share >70% of their aminoacid sequence, and are both coupled to Gi proteins in heterologous expression systems (Pin and Duvoisin, 1995). Studies on brain tissue and cultured neurons are usually carried out with orthosteric agonists, such as compounds 2R,4R-APDC, LY354740, and LY379268, which activate both subtypes with equal affinity (reviewed by Schoepp et al., 1999). Activation of native mGlu2 *and* mGlu3 receptors inhibits cAMP formation and stimulates the mitogen-activated protein kinase (MAPK) pathway (De Blasi et al., 2001; Dhami and Ferguson, 2006).

Homologous desensitization of G-protein coupled receptors (GPCRs) is mediated by a family of enzymes called G-protein coupled receptor kinases (GRKs). This family includes three subfamilies: GRK1, which corresponds to rhodopsin kinase (RK) and GRK7 form the RK subfamily; GRK2, and -3, which are ubiquitous and are activated by G-protein $\beta\gamma$ subunits ($G\beta\gamma$), form the βARK subfamily; GRK4, -5, and –6 form the GRK4 subfamily. Phosphorylation of GPCRs by GRKs causes receptor desensitization and internalization through mechanisms that involve additional proteins, such as β-arrestins (Kohout and Lefkowitz , 2003). Arrestins also behave as scaffolding proteins, linking receptors to downstream signalling pathways, including

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MAPK activation (Luttrell and Lefkowitz, 2002). The role of different GRKs in the homologous desensitization of mGlu1 receptors has been elucidated (Dhami and Ferguson, 2006). Recombinant mGlu1 receptors expressed in HEK293 cells are desensitized by GRK4 in an agonist-dependent manner and GRK4 knock-down in cultured cerebellar Purkinje cells (which natively express mGlu1 receptors) impairs receptor desensitization. GRK2 is also involved in desensitization and internalization of mGlu1 receptors, although its action does not require an intact kinase activity (Sallese et al., 2000; Iacovelli et al., 2003; Dhami et al., 2004; Mundell et al., 2003). GRK2 also regulates the expression and function of mGlu5 receptors in heterologous expression systems (Sorensen and Conn, 2003). mGlu4 receptors undergo a complex regulation by GRKs. These receptors are coupled to Gi and their activation inhibits adenylyl cyclase activity and stimulates both the MAPK and the phosphatidyl-inositol-3-kinase (PI-3-K) pathways in cultured cerebellar granule cells (Iacovelli et al., 2002). In heterologous expression systems, mGlu4 receptor signaling is regulated by GRK2, but not GRK4: GRK2 desensitizes the MAPK pathway without affecting the inhibition of cAMP formation (Iacovelli et al., 2004). No data are yet available on the mechanisms mediating the homologous desensitization of mGlu2 and mGlu3 receptors, and whether or not the two receptors desensitize in response to prolonged agonist stimulation. We have addressed this issues both in recombinant cells and in brain slices prepared from knockout mice lacking either mGlu2 or mGlu3 receptors.

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Materials and Methods

Materials. Polyclonal anti-mGlu2/3 receptor was purchased from Chemicon (Temecula, CA); monoclonal anti-GRK2/3 (clone C5/1) and monoclonal anti GRK4-6 (used to probe GRK5) were purchased from Upstate Biotechnology (Lake Placid, NY); polyclonal anti-ERK1/2 and polyclonal anti-GRK6 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-phospho-ERK1/2 was from Cell Signaling Technology (Beverly, MA); monoclonal anti-β-arrestin antibody was from Transduction Laboratories (Lexington, KY)**;** pertussis toxin (PTX) was purchased from Calbiochem (San Diego, CA). (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC) was purchased from Tocris Cookson (Bristol, UK); (–)-2-oxa-4-aminobicyclo[3.1.0]exhane-4,6-dicarboxylic acid (LY379268) was a generous gift from Eli Lilly & Company, (Indianapolis, IN). All other drugs were purchased from Sigma-Aldrich (Milan, Italy). The full-length GRK4 cDNA were prepared as described previously (Sallese et al., 2000). The plasmids encoding for the kinase-dead mutant of GRK2 (GRK2-K220R) and the C-terminal domain of GRK2 (Gly^{495}-Leu^{689}) (GRK2-Cter) were kindly provided by C. Scorer (GlaxoSmithKline, Uxbridge, Middlesex, UK); DynK44A cDNA was kindly provided by J. Benovic; mGlu2 receptor cDNA was kindly provided by J. Blahos (Academy of Science, Prague, Czech Republic); mGlu3 receptor cDNA was kindly provided by F. Ferraguti (Innsbruck Medical University, Innsbruck, Austria).

Cell Culture and Transfection. Human embryonic kidney (HEK)293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). Cells were transfected in 10-mm Falcon dishes using 8 µl of LipofectAMINE2000 in OptiMEM medium (Invitrogen, Carlsbad, CA), and 10 µg of cDNA for 4 h. The cells used for determination of cAMP were co-transfected with 2.5 µg/dish of adenylyl cyclase type V cDNA (Aramori et al., 1997). One day later, cells were seeded into 6-well plates (for

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MAPK assay) or in 48-well plates (for cAMP assay) previously coated with poly(L-lysine) (0.01%), and the experiments were performed 48 h after transfection.

Animals. mGlu2 receptor knockout mice have been provided by S. Nakanishi (University of Kyoto, Japan). mGlu3 receptor knockout mice have been provided by C. Corti (GSK Glaxo, Verona, Italy). All mice were kept under environmentally controlled conditions (ambient temperature, 22°C; humidity, 40%) on a 12 h light/dark cycle with food and water ad libitum. Experiments were performed following the guidelines for animal care and use of the National Institutes of Health. Wild-type, mGlu2$^{-/-}$, and mGlu3$^{-/-}$ mice were treated i.p., once daily with LY379268 (1 mg/kg) for 7 days. Control animals were treated with saline. Animals were killed 24 h after the last LY379268 injection.

Immunoblotting. At the end of the final incubation, cells were rapidly rinsed in ice-cold PBS and solubilized in Triton X-lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β-glycerophosphate) as described previously (Iacovelli et al., 2002). Protein cell lysates (80 µg) were separated by SDS-PAGE electrophoresis, blotted onto nitrocellulose, and probed using specific antibodies. The antibodies were used at the following dilution: anti-phospho-ERK1/2, 1:500; anti-ERK1/2, 1:2000; anti-mGlu2/3 receptor, 1:1000; anti-GRK2/3 (clone C5/1), 1:7000; anti-GRK4, 1:2000; anti-GRK5 and anti-GRK6 were used 1:1000; anti-β-arrestin antibody was diluted 1:2500. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) using horseradish peroxidase-conjugated secondary antibodies.

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cAMP Assay. Cell cultures were incubated in Hanks' balanced salt solution buffer, pH 7.4, containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and bovine serum albumin (0.3%). 2R,4R-APDC (or vehicle) was added 10 min before FSK stimulation (1 μM). After 20 min, the reaction was stopped by substituting the buffer with ice-cold ethanol. Extraction and measurement of cAMP was carried out as described previously (Iacovelli et al., 1996) by RIA (Perkin Elmer). Cortical slices were prepared as described by Nicoletti et al. (1986). In brief, animals were killed by decapitation, cortex were dissected out and cut into 350×350 μm slices with a Mc Ilwain tissue chopper. Slices were incubated in Krebs-Hensleit buffer at 37°C under constant oxygenation to allow metabolic recovery. Forty microliters of gravity packed slices were then incubated in 500 μl of buffer containing 0.5 mM IBMX for 15 min. After addition of FSK and/or mGlu receptor ligands, the incubation was continued for further 20 min. The reaction was stopped by addition of an equal volume of ice-cold 0.8 N $HClO_4$. Samples were then frozen or immediately used. Samples were sonicated and centrifuged at low speed (1500 g for 10 min). One hundred and eighty microliters of the supernatant were added to 20 μl of K_2CO_3 (2 M) and, after further centrifuged in a microfuge (2 min at maximal speed), 20 μl of the supernatant were used for the cAMP assay. Intracellular cAMP levels were assayed by RIA.

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Results

GRKs differentially regulate mGlu2 and mGlu3 receptor signaling in recombinant cells

HEK293 cells were transfected with mGlu2 or mGlu3 receptor cDNA. Immunoblot analysis showed maximal receptor expression 48 hours after transfection, when levels of mGlu2 and mGlu3 receptors were similar (see Fig. 1). Cells were stimulated with compound 2R,4R-APDC (100 μM), which behaves as a selective orthosteric agonist of both receptor subtypes (reviewed by Schoepp et al., 1999). Addition of 2R,4R-APDC inhibited FSK-stimulated cAMP formation in both mGlu2- and mGlu3-expressing cells. Inhibition was prevented by an overnight pretreatment with pertussis toxin (PTX, 1 μg/ml), confirming that both receptor subtypes are coupled to Gi/Go proteins in recombinant cells (Fig. 2A). To examine the regulation of mGlu2 or mGlu3 receptor signaling, cells were co-transfected with individual GRK isotypes, starting with GRK2. Fig. 1 shows that the levels of expression of GRK2 were similar in mGlu2- and mGlu3-expressing cells. The expression of mGlu2 and mGlu3 receptors was not affected by co-transfection of GRK2 or the kinase-dead mutant GRK2-K220R (Fig.1). Overexpression of GRK2 produced markedly different effects on mGlu2 and mGlu3 receptor signalling. In cells expressing mGlu2 receptors, the ability of 2R,4R-APDC to inhibit FSK-stimulated cAMP formation was only slightly desensitised by GRK2. In contrast, overexpression of GRK2 completely blunted the inhibition of cAMP formation mediated by mGlu3 receptors (Fig. 2B).

GRK4, GRK5 and GRK6 mimicked the action of GRK2 in mGlu2 receptor-expressing cells, producing only a slight reduction in the ability of 2R,4R-APDC to inhibit cAMP formation (Fig. 3A). In contrast, only GRK3 shared with GRK2 the ability to fully desensitize the agonist-dependent inhibition of adenylyl cyclase activity in mGlu3 expressing cells, whereas at least GRK5 and GRK6 were inactive (Fig. 3A). Thus, desensitization of mGlu3 receptors in recombinant cells was specifically mediated by GRK2 and GRK3, which are functionally similar, belong to the same GRK subfamily and share >85% similarity in the amino acid sequence (Benovic et al., 1991).

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Desensitization of mGlu2 and mGlu3 receptors required an intact kinase activity of GRK2

GRK2 is a multidomain protein that regulates G-protein-mediated signaling by different mechanisms, as shown for many GPCRs including different mGlu receptor subtypes. GRK2 phosphorylates the agonist-bound GPCR thus leading to homologous desensitization. In addition, GRK2 can inhibit signal propagation through the Gβγ subunits by means of the PH domain located at its C-terminus domain, and can also reduce Gαq signaling by means of the RGS-homology domain located at its N-terminus domain. In order to investigate the mechanisms by which GRK2 desensitises the mGlu3 receptor-mediated signaling, we used the kinase-dead GRK2 mutant GRK2-K220R (in which the kinase activity is disrupted by site-directed mutagenesis) and the recombinant C-terminus domain (GRK2-Cter) which contains the functional PH domain of GRK2 (see Fig. 1 showing the same levels of expression of GRK2-K220R and GRK2-Cter in different experiments). We found that the mGlu3 receptor signalling, which was fully desensitised by wild-type GRK2, was not affected by co-transfection of the GRK2-K220R and GRK2C-ter mutants. The mGlu2 receptor signalling, which was only slightly desensitized by the wild-type GRK2, was not affected at all by both the GRK2-K220R and the GRK2-Cter (Fig. 3B). This indicates that the kinase activity of GRK2 is absolutely required for the desensitization of mGlu3 receptors and also for the slight desensitization of mGlu2 receptors.

GRK2 desensitizes both mGlu2- and mGlu3-receptor mediated MAPK activation in recombinant cells

For the majority of GPCR that are coupled to the inhibitory heterotrimeric Gi protein, agonist stimulation activates multiple signalling pathways, including cAMP inhibition (mediated by Gαi) and ERK1/2 phosphorylation (mediated by Gβγ). Accordingly, addition of 2R,4R-APDC (100 μM) to HEK293 cells expressing mGlu2 or mGlu3 receptors induced ERK1/2 phosphorylation (Fig. 4

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and Table 1). ERK1/2 phosphorylation was rapid, reversible, and prevented by pre-treatment with PTX (Fig. 4 and data not shown), indicating the involvement of Gi/Go proteins. We examined the regulation of mGlu2- and mGlu3-receptor dependent ERK1/2 phosphorylation by GRKs. In both mGlu2- and mGlu3-receptor expressing cells, agonist-stimulated ERK1/2 phosphorylation was fully desensitised by overexpression of GRK2, whereas overexpression of GRK4 and GRK5 was inactive (Fig. 4; Table 1; and data not shown). Co-transfection with GRK2-K220R did not affect agonist-stimulated ERK1/2 phosphorylation mediated by mGlu2 and mGlu3 receptors (Fig. 5; Table 1), indicating that the kinase activity of GRK2 is required for receptor desensitization.

Basal ERK1/2 phosphorylation was not affected when cells were transfected with GRKs alone (Fig. 5B) or co-transfected with the receptors (Figs. 4 and 5). These results show that only agonist-stimulated MAPK activation was desensitised by GRK2 and fulfil the paradigm of receptor homologous desensitisation. Concentration-response curves of agonist-dependent cAMP inhibition and ERK1/2 stimulation in cells expressiong mGlu2 receptors were similar (Fig.6).

β-Arrestin1 differentially affects receptor signaling in cells expressing mGlu2 and mGlu3 receptors

GPCR phosphorylation by GRK allows the binding of β-arrestin to the receptor, thus leading to complete homologous desensitisation. In HEK293 cells expressing mGlu2 receptors, co-transfection of β-arrestin1 slightly reduced the agonist-dependent inhibition of cAMP formation. In contrast, β-arrestin1 abolished this effect in cells expressing the mGlu3 receptor (Fig. 7A). The levels of β-arrestin1 expression were similar in mGlu2- and mGlu3-receptor expressing cells (Fig. 7B). The expression of mGlu2 and mGlu3 receptors was not affected by co-transfection of β-arrestin1 (Fig.1). The effects of GRK2 and β-arrestin1 on mGlu2 receptor desensitisation were additive, although the inhibition of signalling induced by GRK2 *plus* β-arrestin1 was not complete

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(Fig. 7C). This is in contrast to what observed for the mGlu3 receptor, which was fully desensitised by overexpression of either GRK2 or β-arrestin1.

The analysis of mGlu2- and mGlu3-receptor dependent ERK1/2 phosphorylation in the presence of β-arrestin1 revealed an unexpected finding. When β-arrestin1 was co-transfected with either the mGlu2 or the mGlu3 receptor, ERK1/2 phosphorylation was increased by 3-4 fold even in the absence of the agonist. This occluded any possible stimulation of MAPK by 2R,4R-APDC (Fig. 8). As β-arrestin has been reported to act as a signalling protein for GPCR-mediated MAPK activation (Iacovelli et al., 2003; Lefkowitz and Whalen, 2004), we hypothesised that β-arrestin could amplify the stimulation of ERK1/2 phosphorylation by constitutively active mGlu2 or mGlu3 receptors. We examined this possibility by transfecting HEK293 cells with the mGlu2 receptor combined or not with β-arrestin1, and measuring ERK1/2 phosphorylation under basal conditions (i.e. without 2R,4R-APDC). The levels of ERK1/2 phosphorylation in cells transfected with the mGlu2 receptor were similar to those observed in untransfected or mock-transfected cells. In contrast, the levels of ERK1/2 phosphorylation were increased by overexpression of β-arrestin1 (1.4-2.0 fold of mock-transfected cells) and further enhanced by co-transfection of β-arrestin1 and mGlu2 receptors (4-6 fold of mock-transfected cells) (Fig. 9A). In cells co-expressing mGlu2 receptors, β-arrestin and GRK2, basal phosphorylation of ERK1/2 was increased, and the response to 2R,4R-APDC was blunted (Fig. 9B).

To test whether mGlu2 and mGlu3 receptor internalization was required for agonist-dependent MAPK activation, we measured 2R,4R-APDC-stimulated ERK1/2 phosphorylation in HEK293 cells transfected with the dynamin dominant-negative mutant DynK44A. This mutant is able to prevent the internalisation of many GPCRs, by competing with endogenous dynamin that cuts the neck of endocytic vesicles. In the presence of DynK44A, the activation of MAPK by 2R,4R-APDC was almost completely blunted, suggesting that mGlu2 and mGlu3 receptor internalization is involved in this signalling pathway (Fig.10).

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Different pattern of homologous desensitisation in knockout mice lacking mGlu2 or mGlu3 receptor

We have shown that the cAMP signalling activated by mGlu2 and mGlu3 receptors in HEK293 cells is differentially regulated, with the mGlu3 receptor being desensitised by GRK2 and β-arrestin and the mGlu2 receptor being resistant to homologous desensitisation. In order to extend these observations to an *in vivo* experimental model we used knock-out mice lacking either mGlu2 ($mGlu2^{-/-}$ mice) or mGlu3 receptors ($mGlu3^{-/-}$ mice). Mice were pre-treated with saline or with the mixed mGlu2/3 agonist LY379268 for 7 days, and cortical brain slices were prepared 24 hours after the last injection. Slices were challenged with FSK alone or FSK plus LY379268, and used for determinations of cAMP formation. In both wild-type (wt) and $mGlu2^{-/-}$ mice, agonist pre-treatment reduced the ability of LY379268 (1 μM) to inhibit FSK-stimulated cAMP formation. In contrast, $mGlu3^{-/-}$ mice (in which the agonist could only activate mGlu2 receptors) were completely resistant to homologous desensitisation, because the ability of LY379268 to inhibit FSK-stimulated cAMP formation was unaffected by agonist pre-treatment (Fig. 11).

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Discussion

mGlu2 and mGlu3 share >70% of their aminoacid sequence and their differentiation has been problematic because of the lack of selective ligands and antibodies. This contrasts with the extensive development of mixed orthosteric mGlu2/3 receptor agonists, some of which are under clinical development (Swanson et al., 2005; Patil et al., 2007). Established clinical targets for mGlu2/3 receptor agonists are schizophrenia and drug-induced psychosis, anxiety, chronic pain, and drug addiction. The relative contribution of mGlu2 and mGlu3 receptors to the experimental treatment of all these disorders can now be dissected using selective mGlu2 receptor enhancers or mice with genetic deletion of either mGlu2 or mGlu3 receptors. For example, mGlu2 receptor enhancers are effective in models predictive of antipsychotic activity (Johnson et al., 2005; Galici et al., 2005), suggesting that it is the mGlu2 receptor that mediates the therapeutic efficacy of mGlu2/3 receptor agonists in schizophrenia. In addition, mGlu2 receptors form functional complexes with 5-HT_{2A} serotonergic receptors (Gonzàlez-Maeso et al., 2008), and mediate the ability of mGlu2/3 receptor agonists to inhibit cellular and behavioural responses to hallucinogenic drugs (Marek et al., 2000; Gewirtz and Marek, 2000; Marek et al., 2001; Gewirtz et al., 2002; Marek et al., 2006; Gonzàles-Maeso et al., 2008). The individual role of mGlu2 and mGlu3 receptors in the drug treatment of anxiety and panic attacks is more ambiguous because the anxiolytic-like activity of mGlu2/3 receptor agonists is disrupted in both mGlu2- and mGlu3 knockout mice (Linden et al., 2005). Interestingly, the increase in c-Fos expression induced by LY354740 in the central and extended amygdala is mediated by mGlu2 receptors, whereas the suppression of c-Fos expression in the hippocampus is mediated by mGlu3 receptors (Linden et al., 2006). There are only few studies on the individual role of mGlu2 and mGlu3 receptors in drug addiction and chronic pain. mGlu2 receptor knockout mice show an increase in locomotor sensitisation and conditioned place preference in association with repeated cocaine administration, which implicates a critical role for mGlu2 receptors in the reinforcement and addiction of cocaine (Morishima et al., 2005). Acetyl-L-

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carnitine, a drug currently used for the treatment of neuropathic pain, induces analgesia by selectively up-regulating mGlu2 receptors in the dorsal horns of the spinal cord (Chiechio et al., 2002; 2006). Finally, activation of mGlu3 receptors mediates the protective activity of mGlu2/3 receptor agonists against excitotoxic neuronal death (Corti et al., 2007a), although this particular property has not been translated into human studies, as yet.

Our data show for the first time that mGlu2 and mGlu3 receptors are differently regulated in response to agonist stimulation. Similarly to most GPCRs, heterologously expressed mGlu3 receptors were desensitised by GRK2 and GRK3, two protein kinases that have an established role in mechanisms of homologous desensitisation (De Blasi et al, 2001; Kohout and Lefkowitz, 2003, Dhami and Ferguson, 2006). In contrast, mGlu2 receptors showed little or no response to GRK2 or other GRKs when desensitisation was assessed by measuring the inhibition of cAMP formation, i.e. the canonical signalling pathway activated by mGlu2 and mGlu3 receptors. The different behaviour of mGlu2 and mGlu3 receptors was supported by experiments in which HEK293 cells were transfected with β-arrestin, which uncouples GRK-phosphorylated GPCRs from the α subunit of G proteins (Kohout and Lefkowitz, 2003; Lefkowitz and Whalen 2004; Luttrell and Lefkowitz, 2002). Overexpression of ß-arrestin desensitised mGlu3, but not mGlu2 receptors. A partial desensitisation of mGlu2 receptors could only be observed under extreme conditions, i.e. in cells overexpressing both GRK2 and ß-arrestin.

The use of the kinase-dead mutant of GRK2 or the use of the C-terminus domain of GRK2 demonstrated that the kinase activity was necessary for the desensitisation of mGlu3 receptors and that other functions of GRK2 (such as the ability to interact with other proteins through the N-terminal RGS domain or the C-terminal PH domain) have no role in this process. Thus, desensitisation of the cAMP signalling mediated by the mGlu3 receptors proceeds through receptor phosphorylation by GRK2 or GRK3, which facilitates the association of β-arrestin with ensuing uncoupling of the Gi protein. Obviously, this desensitisation process is less efficient with mGlu2 receptors, which are partially refractory to GRK2 or β-arrestin. The evidence that mGlu2 receptor

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cAMP signalling is resistant to homologous desensitization was strengthened by data obtained in knockout mice. Prolonged to the mGlu2/3 agonist, LY379268, efficiently desensitized the cAMP response in mGlu2 receptor knockout mice, while mGlu3 knockout mice, in which the drug could only activate mGlu2 receptors, were fully resistant to homologous desensitization. Thus, the refractoriness to homologous desensitization is a peculiar and intrinsic property of mGlu2 receptors that does not depend on the cellular environment. The complete desensitisation of the cAMP response observed in the hippocampus of wt mice was unexpected because wt mice express both mGlu2 and mGlu3 receptors. One possible explanation is that mGlu2 and mGlu3 receptors may form heterodimers, which rely on the presence of mGlu3 receptors for homologous desensitisation. It should be highlighted, however, no evidence for heterodimerization between mGlu2 and mGlu3 receptors has been provided so far.

Interestingly, studies in recombinant cells showed that, as opposed to cAMP signalling, MAPK activation by mGlu2 receptors was fully desensitised by GRK2. This signal-dependent regulation was unexpected and has no obvious explanation. Control experiments showed that this was not due to changes in the expression of mGlu2 receptors in cells cotransfected with GRK2. In addition, in cells expressing mGlu2 receptors, 2R,4R-APDC inhibited adenylyl cyclase and stimulated ERK1/2 phosphorylation with similar concentration-response curves, and in both cases responses were mediated by Gi/o proteins because they were sensitive to PTX. It is possible that different domains of the mGlu2 receptor trigger the two different signalling pathways, and that GRK2 phosphorylation only affects the domains important for ERK stimulation. Alternatively GRK2 could affect the coupling of the receptor to Gi, but signal propagation through the MAPK pathway is less efficient than the inhibition of adenylyl cyclase, and is therefore more sensitive to homologous desensitization. Although the observed signal-dependent regulation of mGlu2 receptors by GRK2 is unusual, an identical pattern of regulation has been described for native Gi-protein coupled A1 adenosine receptors in FRTL-5 thyroid cell lines (Iacovelli et al., 1999). In these cells,

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the A1 adenosine receptor-mediated ERK1/2 activation is desensitised by GRK2 (but not by GRK2-K220R), whereas inhibition of adenylyl cyclase response is unaffected.

It should be highlighted that in cells expressing mGlu2 or mGlu3 receptors co-transfection of β-arrestin1 enhanced MAPK activation even in the absence of agonist. Previous studies on mGlu1 receptors and other GPCRs have shown that β-arrestin can act as a scaffolding protein mediating receptor-dependent MAPK activation (Iacovelli et al., 2003; Lefkowitz and Whalen, 2004). Thus, it is possible that endogenous activation of mGlu2 or mGlu3 receptors (i.e. receptor activation by the glutamate present in the medium or a not-yet-demonstrated constitutive activity of mGlu2 or mGlu3 receptors) becomes sufficient to activate the MAPK pathway when β-arrestin is overexpressed.

Because inhibition of cAMP formation is the canonical signalling pathway activated by mGlu2 and mGlu3 receptors and contributes to the ability of both receptors to inhibit neurotransmitter release (reviewed by Pin and Duvoisin, 1995), the different sensitivity of the two receptor subtypes to homologous desensitization may have important implications for the therapeutic use of group-II mGlu receptor agonists or enhancers. Given the prominent role of the mGlu2 receptor as a therapeutic target in schizophrenia, we predict the lack of tolerance when mixed mGlu2/3 receptor agonists or selective mGlu2 enhancers are used chronically in patients. In contrast, desensitization of mGlu3 receptors may limit the use of receptor agonists/enhancers as protective agents in neurodegenerative disorders. This hypothesis encourages preclinical and clinical studies on the effects of long-term treatment with mGlu2 or mGlu3 receptor ligands.

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Footnotes

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Legends for Figures

Fig. 1. Immunoblots of mGlu2/3 receptors; GRK2; GRK2 kinase-dead mutant, GRK2-K220R (K220R); GRK5 and GRK6 in HEK293 cells transfected with the respective cDNAs. Mock indicates HEK293 cells transfected with empty vectors. Each lane was loaded with 80 µg of proteins from individual culture dishes. The two bands shown in the immunoblots may correspond to receptor monomers (100 kDa) and dimers (about 200 kDa). Note that expression of the monomeric band is lighter for mGlu3 than mGlu2 receptors. The reason for this difference is unknown.

Fig. 2. A, Inhibition of FSK-stimulated cAMP formation by 2R,4R-APDC is blocked by PTX. HEK293 cells expressing mGlu2 or mGlu3 receptor s were pre-treated with PTX (1 µg/ml for 16-18 h) and then stimulated with 1 µM FSK ± 100 µM 2R,4R-APDC. Values are means ± S.E.M. from 3 determinations each in triplicate. *, $p < 0.05$ versus FSK (one-way analysis of variance + Fisher's least significant difference). **B**, Effects of GRK2 on agonist-dependent adenylyl cyclase inhibition. Inhibition of 1 µM FSK-stimulated cAMP formation by 100 µM 2R,4R-APDC was measured in cells expressing mGlu2 or mGlu3 receptors alone or co-expressed with GRK2. Values are means ± S.E.M. from 3 determinations each in triplicate. * $p < 0.05$, Student's t test.

Fig. 3. Differential regulation of mGlu2- and mGlu3-receptor mediated inhibition of cAMP formation by GRKs. **A**, Inhibition of FSK-stimulated cAMP formation by 2R,4R-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or co-expressed with GRK2, GRK3, GRK4, GRK5 or GRK6 as indicated. **B**, Inhibition of FSK-stimulated cAMP formation by 2R,4R-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or co-expressed with GRK2, GRK2-K220R or GRK2-Cter. Values are means ± S.E.M. from 3 determinations each in triplicate. * $p < 0.05$ vs receptor alone (One-way ANOVA + Fisher's t test).

Fig. 4. Regulation of mGlu2/3-receptor-mediated MAPK activation. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or co-expressed with GRK2 or GRK5 are shown. Membranes were probed with antibodies specific for phosphorylated ERK1/2 (p-ERK1/2) and then washed and re-probed with antibodies recognising total ERK1/2 (ERK). Cells were exposed to 2R,4R-APDC (100 μM) for the indicated times. These experiments were repeated two additional times with similar results. Densitometric analysis are presented in Table 1. Note that a 16-18 hour pre-treatment with pertussis toxin (PTX, 1 μg/ml) blunted the MAPK response to APDC in mGlu2 receptor expressing cells (see first immunoblot on the left). PTX also inhibited MAPK activation by 2R,4R-APDC in mGlu3 expressing cells (not shown).

Fig. 5. Effect of GRK2-K220R on mGlu2/3-receptor-mediated MAPK activation. **A**, Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or co-expressed with GRK2-K220R. Cells were exposed to 2R,4R-APDC (100 μM) for indicated times. Densitometric analysis are presented on Table 1. **B,** Basal ERK1/2 was determined in unstimulated HEK293 cells transfected with the empty vector, GRK2, GRK5 and GRK2-K220R. These experiments were repeated two times with similar results.

Fig. 6. Concentration-response curves of 2R,4R-APDC on cAMP inhibition and MAPK activation. HEK293 cells expressing the mGlu2 receptor were exposed to different concentrations of 2R,4R-APDC, and ERK1/2 phosphorylation and FSK-stimulated cAMP inhibition were measured. Data are means ± S.E.M. from 3 determinations. A representative pERK1/2 immunoblot is also shown.

Fig. 7. Regulation of mGlu2/3 receptor-dependent adenylyl cyclase inhibition by β-arrestin1. **A**, Inhibition of FSK-stimulated cAMP formation by 2R,4R-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or co-expressed with β-arrestin1. Values are means ±

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S.E.M. from 3 determinations each in triplicate. $^{*}p < 0.05$, Student's t test. **B**, The levels of β-arrestin1 co-expressed with the mGlu2 or the mGlu3 receptors are comparable, as assessed by immunoblot. **C**, Inhibition of FSK-stimulated cAMP formation by 2R,4R-APDC was measured in HEK293 cells expressing mGlu2 receptors alone or co-expressed with GRK2, β-arrestin1 or GRK2 plus β-arrestin1. Values are means ± S.E.M. from 3 determinations each in triplicate. $^{*}p < 0.05$ versus receptor alone (One-way ANOVA + Fisher's t test).

Fig. 8. Regulation of mGlu2/3-receptor-mediated MAPK activation by β-arrestin1. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or co-expressed with β-arrestin1 are shown. Cells were exposed to 2R,4R-APDC (100 μM) for the indicated times. Densitometric analysis of 3-5 similar experiments are shown (means ± S.E.M). Data are expressed as ratio of control values obtained in the absence of both 2R,4R-APDC and β-arrestin1 (basal = 1). *p<0.05 vs control values.

Fig. 9. A, β-arrestin1 increases mGlu2/3-receptor-mediated MAPK activation. ERK1/2 was measured in HEK293 cells were untransfected (Un) or transfected with empty vector (Mock), the mGlu2 receptor (mGlu2), β-arrestin1 (βarr1) or mGlu2 plus β-arrestin1 (mGlu2+ βarr1). All the conditions presented were in absence of agonist stimulation. **B**, Effect of β-arrestin1 ± GRK2 transfection on MAPK activation. Cells were exposed to 2R,4R-APDC (100 μM) for the indicated times. Densitometric analysis of 3 similar experiments are shown (means ± S.E.M). Data are expressed as ratio of control values obtained in the absence of both 2R,4R-APDC and β-arrestin1 (basal = 1). *p<0.05 (One-way ANOVA + Fisher's t test) vs control values.

Fig. 10. Effect of dynamin dominant negative mutant (DynK44A) on receptor-mediated MAPK activation. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or

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mGlu3 receptor alone or co-expressed with DynK44A are shown. Cells were exposed to 2R,4R-APDC (100 μM) for the indicated times. Densitometric analysis of 3 similar experiments are shown (means ± S.E.M). control values obtained in the absence of both 2R,4R-APDC and DynK44A (basal = 1).*p<0.05 (One-way ANOVA + Fisher's t test) vs control values.

Fig. 11. Knockout mice lacking mGlu3 receptors are resistant to homologous desensitization induced by repeated administrations of the mGlu2/3 agonist, LY379268.

Wild-type, mGlu2$^{-/-}$ or mGlu3$^{-/-}$ mice were pre-treated with saline or with LY379268 (1 mg/kg, i.p., for 7 days) and cortical slices were prepared 24 hours after the last injection. Slices were challenged with forskolin (FSK) alone or combined with LY379268 (1 μM). Data are expressed as % of FSK-stimulate cAMP formation and were calculated from two independent experiments performed in triplicate. Note that inhibition of FSK-stimulated cAMP formation by LY379268 was no longer visible in cortical slices from wild-type and mGlu2$^{-/-}$ mice pre-treated with LY379268, but persisted in cortical slices from mGlu3$^{-/-}$ mice pre-treated with LY379268. This suggests that native mGlu2 receptors are resistant to homologous desensitization. *p<0.05 (Student's t test) vs. The corresponding values obtained in mice pre-treated with saline.

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Tables

Table 1. Effect of GRKs and GRK mutant on pERK1/2 stimulation: quantification of immunoblots.

Time	**mGlu2R**		**mGlu2R+GRK2**		**mGlu2R+GRK5**		**mGlu2R+K220R**	
	pERK1/ERK1	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***
0’	1	1	1	1	1	1	1	1
5’	2.5±0.3	4.1±0.5	1.4±0.2*	1.6±0.1*	2.4±0.0	4.1±0.3	1.6±0.4	4.2±0.0
15’	1.5±0.4	1.6±0.3	1.0±0.0	0.8±0.1	1.2±0.2	1.1±0.1	1.2±0.1	1.2±0.1
30’	0.9±0.1	1.3±0.2	0.1±0.1	0.8±0.1	1.1±0.1	1.0±0.0	1.0±0.0	1.1±0.1
	mGlu3R		**mGlu3R+GRK2**		**mGlu3R+GRK5**		**mGlu3R+K220R**	
	pERK1/ERK1	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***	***pERK1/ERK1***	***pERK2/ERK2***
0’	1	1	1	1	1	1	1	1
5’	2.7±0.1	3.1±0.4	1.5±0.1*	1.6±0.1*	2.5±0.1	2.8±0.1	2.5±0.1	3.1±0.2
15’	1.9±0.3	2.1±0.5	1.1±0.1	0.9±0.1	1.5±0.3	1.6±0.4	1.5±0.4	1.7±0.3
30’	1.4±0.2	1.5±0.1	1.0±0.0	0.8±0.1	1.0±0.1	1.0±0.1	0.9±0.2	0.9±0.1

Single experiments are shown in Fig. 4 and 5. Data are expressed as ratio of the respective control values (controls = 1). Values are means ± S.E.M. from 3-5 determinations. *p<0.05 (One-way ANOVA + Fisher’s t test) vs control values.

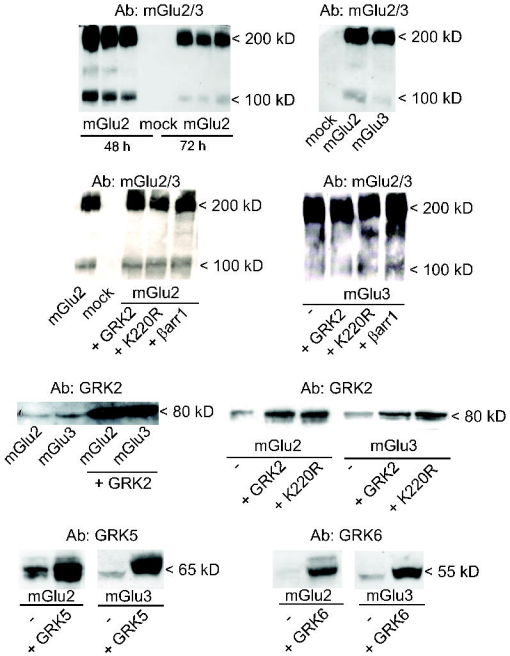
Ab: mGlu2/3
< 200 kD
< 100 kD
mGlu2
mock
mGlu2
48 h
72 h
Ab: mGlu2/3
< 200 kD
< 100 kD
mock
mGlu2
mGlu3
Ab: mGlu2/3
< 200 kD
< 100 kD
mGlu2
mock
mGlu2
+ GRK2
+ K220R
+ βarr1
Ab: mGlu2/3
< 200 kD
< 100 kD
mGlu3
-
+ GRK2
+ K220R
+ βarr1
Ab: GRK2
< 80 kD
mGlu2
mGlu3
mGlu2
mGlu3
+ GRK2
Ab: GRK2
< 80 kD
mGlu2
-
+ GRK2
+ K220R
mGlu3
-
+ GRK2
+ K220R
Ab: GRK5
< 65 kD
mGlu2
-
+ GRK5
mGlu3
-
+ GRK5
Ab: GRK6
< 55 kD
mGlu2
-
+ GRK6
mGlu3
-
+ GRK6

Figure 1

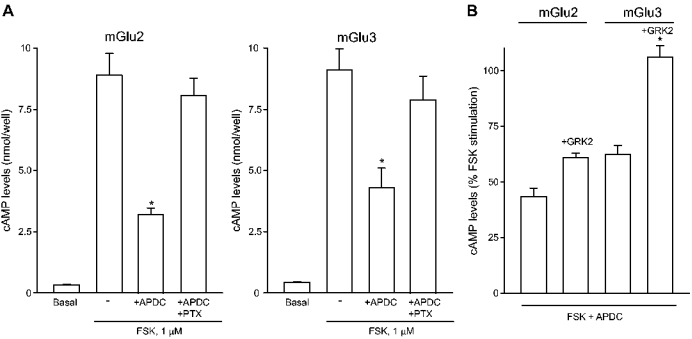
A
mGlu2
cAMP levels (nmol/well)
10
7.5
5.0
2.5
0
Basal
-
+APDC
+APDC
+PTX
FSK, 1 μM
mGlu3
cAMP levels (nmol/well)
10
7.5
5.0
2.5
0
Basal
-
+APDC
+APDC
+PTX
FSK, 1 μM
B
mGlu2
mGlu3
+GRK2
+GRK2
cAMP levels (% FSK stimulation)
100
75
50
25
0
FSK + APDC

Figure 2

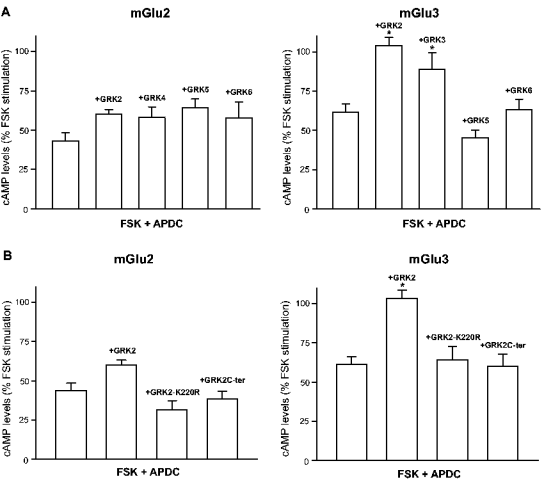
A
mGlu2
cAMP levels (% FSK stimulation)
0
25
50
75
100
+GRK2
+GRK4
+GRK5
+GRK6
FSK + APDC
mGlu3
+GRK2
*
+GRK3
*
+GRK5
+GRK6
FSK + APDC
B
mGlu2
cAMP levels (% FSK stimulation)
+GRK2
+GRK2-K220R
+GRK2C-ter
FSK + APDC
mGlu3
+GRK2
*
+GRK2-K220R
+GRK2C-ter
FSK + APDC

Figure 3

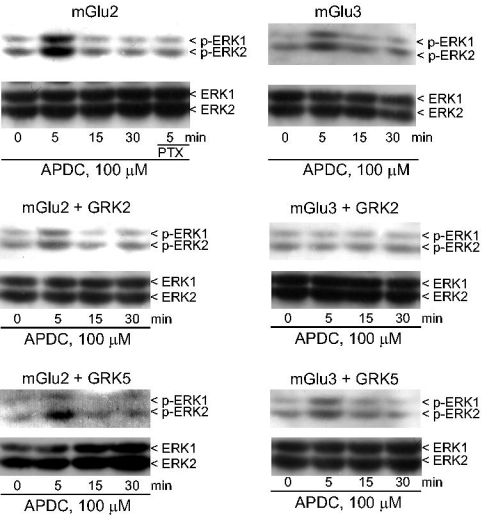
mGlu2
mGlu3
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0 5 15 30 5 min
PTX
APDC, 100 μM
0 5 15 30 min
APDC, 100 μM
mGlu2 + GRK2
mGlu3 + GRK2
mGlu2 + GRK5
mGlu3 + GRK5

Figure 4

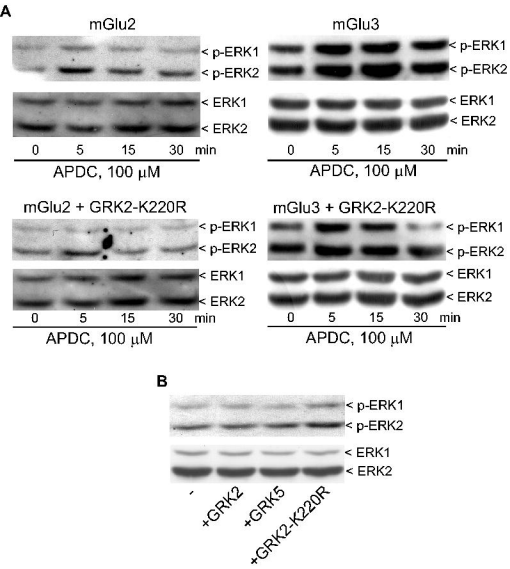
A
mGlu2
mGlu3
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0 5 15 30 min
APDC, 100 μM
mGlu2 + GRK2-K220R
mGlu3 + GRK2-K220R
B
- +GRK2 +GRK5 +GRK2-K220R

Figure 5

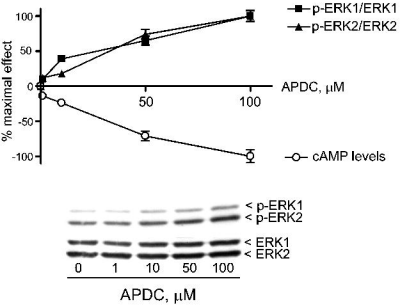
100
50
0
-50
-100
% maximal effect
50
100
APDC, μM
p-ERK1/ERK1
p-ERK2/ERK2
cAMP levels
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0
1
10
50
100
APDC, μM

Figure 6

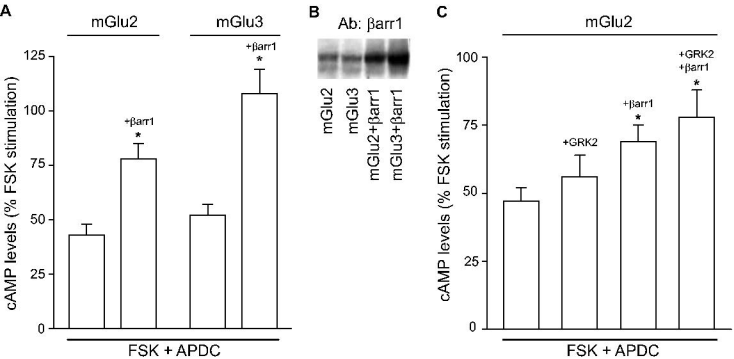
A
mGlu2
mGlu3
+βarr1
*
+βarr1
*
cAMP levels (% FSK stimulation)
125
100
75
50
25
0
FSK + APDC
B
Ab: βarr1
mGlu2
mGlu3
mGlu2+βarr1
mGlu3+βarr1
C
mGlu2
+GRK2
+βarr1
*
+GRK2
+βarr1
*
cAMP levels (% FSK stimulation)
100
75
50
25
0
FSK + APDC

Figure 7

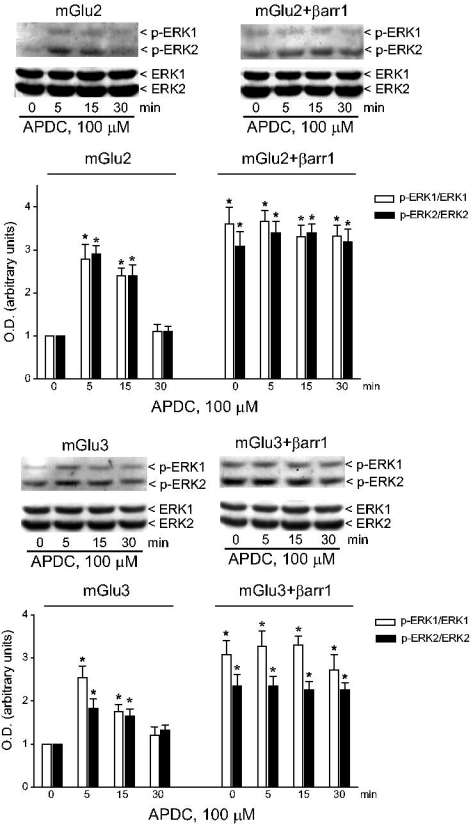
mGlu2
mGlu2+βarr1
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0 5 15 30 min
APDC, 100 μM
mGlu2
mGlu2+βarr1
p-ERK1/ERK1
p-ERK2/ERK2
O.D. (arbitrary units)
0 5 15 30 0 5 15 30 min
APDC, 100 μM
mGlu3
mGlu3+βarr1
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0 5 15 30 min
APDC, 100 μM
mGlu3
mGlu3+βarr1
p-ERK1/ERK1
p-ERK2/ERK2
O.D. (arbitrary units)
0 5 15 30 0 5 15 30 min
APDC, 100 μM

Figure 8

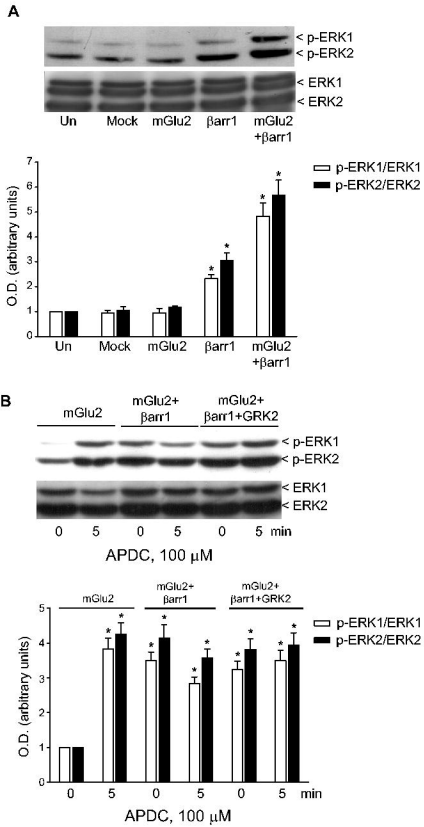
A
< p-ERK1
< p-ERK2
< ERK1
< ERK2
Un
Mock
mGlu2
βarr1
mGlu2 +βarr1
p-ERK1/ERK1
p-ERK2/ERK2
O.D. (arbitrary units)
B
mGlu2
mGlu2+ βarr1
mGlu2+ βarr1+GRK2
0 5 0 5 0 5 min
APDC, 100 μM

Figure 9

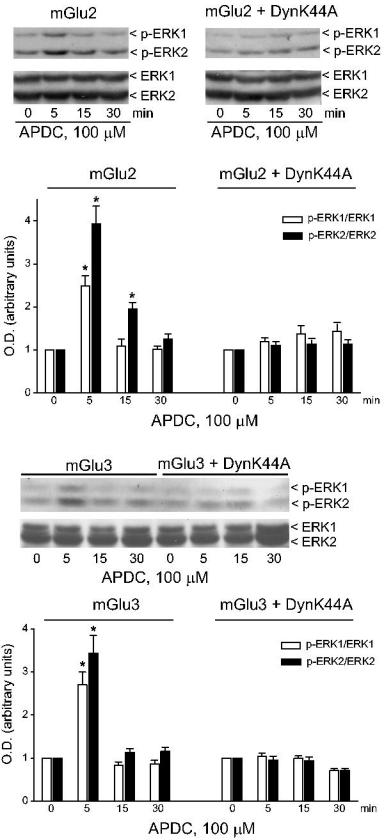
mGlu2
mGlu2 + DynK44A
< p-ERK1
< p-ERK2
< ERK1
< ERK2
0 5 15 30 min
APDC, 100 μM
O.D. (arbitrary units)
p-ERK1/ERK1
p-ERK2/ERK2
mGlu3
mGlu3 + DynK44A

Figure 10

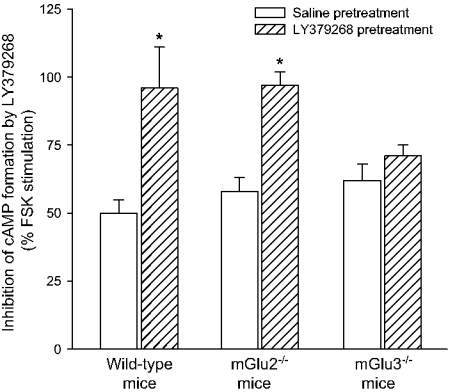
Saline pretreatment
LY379268 pretreatment
Inhibition of cAMP formation by LY379268
(% FSK stimulation)
125
100
75
50
25
0
*
*
Wild-type
mice
mGlu2-/-
mice
mGlu3-/-
mice

Figure 11