The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor

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Nucleotides and cysteinyl-leukotrienes (CysLTs) are unrelated signaling molecules inducing multiple effects through separate G-protein-coupled receptors: the P2Y and the CysLT receptors. Here we show that GPR17, a Gi-coupled orphan receptor at intermediate phylogenetic position between P2Y and CysLT receptors, is specifically activated by both families of endogenous ligands, leading to both adenylyl cyclase inhibition and intracellular calcium increases. Agonist-response profile, as determined by $[^{35}S]GTP\gamma S$ binding, was different from that of already known CysLT and P2Y receptors, with EC_{50} values in the nanomolar and micromolar range, for CysLTs and uracil nucleotides, respectively. Both rat and human receptors are highly expressed in the organs typically undergoing ischemic damage, that is, brain, heart and kidney. *In vivo* inhibition of GPR17 by either CysLT/P2Y receptor antagonists or antisense technology dramatically reduced ischemic damage in a rat focal ischemia model, suggesting GPR17 as the common molecular target mediating brain damage by nucleotides and CysLTs. In conclusion, the deorphanization of GPR17 revealed a dualistic receptor for two endogenous unrelated ligand families. These findings may lead to dualistic drugs of previously unexplored therapeutic potential.

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Introduction

The seven transmembrane domain G-protein-coupled receptors (GPCRs) constitute the largest membrane receptor family, and, owing to their crucial role in cell-to-cell communication and involvement in a variety of physiological phenomena, represent the most common target of pharmaceutical drugs (Howard *et al*, 2001). The progress of human genome sequencing has revealed the existence of several hundred orphan GPCRs (Fredriksson *et al*, 2003), that is, molecularly identified receptors that still lack a defined physiologically relevant ligand. The 'deorphanization' of these receptors and the identification of their roles is expected to clarify novel regulatory mechanisms of physiological phenomena and to unveil novel drug targets (Howard *et al*, 2001; Fredriksson *et al*, 2003; Mori *et al*, 2005).

In addition to established metabolic roles, adenine (ATP, ADP), uracil (UTP, UDP) and sugar nucleotides (e.g., UDP-glucose and UDP-galactose) are universal and phylogenetically ancient signaling molecules involved in a multitude of biological processes, from embryogenesis to adult homeostasis (Burnstock and Knight, 2004). Actions of extracellular nucleotides on target cells are mediated by specific membrane receptors: the seven ligand-gated purinergic P2X channels and the eight G-protein-coupled P2Y receptors (the $P2Y_{1,2,4,6,11,12,13,14}$ receptors) (Abbracchio *et al*, 2003; Burnstock and Knight, 2004). Owing to their involvement in the regulation of many physiological phenomena, dysfunctions of nucleotides and their receptors have been associated to various human diseases, including immune and ischemic/inflammatory conditions (Abbracchio *et al*, 2003; Burnstock and Knight, 2004). Conversely, cysteinyl-leukotrienes (CysLTs, such as LTC_4 and LTD_4) are inflammatory lipid mediators generated by 5-lipoxygenase metabolism of arachidonic acid (Samuelsson, 2000) acting through G-protein-coupled $CysLT_1$ and $CysLT_2$ receptors (Brink *et al*, 2003) and implicated in bronchial asthma (Drazen, 2003), stroke (Ciceri *et al*, 2001) and cardiovascular diseases (Brink *et al*, 2003).

Recent data highlight the existence of a functional crosstalk between the nucleotide and the CysLT systems in orchestrating inflammatory responses (Capra *et al*, 2005). Both types of mediators accumulate at the sites of inflammation, and inflammatory cells often co-express both P2Y and CysLT receptors. In rat microglia, the brain immune cells involved in the response to cerebral hypoxia and trauma, the activation of $P2Y_1$ and CysLT receptors mediate co-release of nucleotides and CysLTs (Ballerini *et al*, 2005), which might, in turn, contribute to neuroinflammation and neurodegeneration. In human monocyte/macrophage-like cells, $CysLT_1$ receptor function is regulated by extracellular nucleotides via heterologous desensitization (Capra *et al*, 2005), and, in the same cells, montelukast and pranlukast, two selective $CysLT_1$ receptor antagonists (Brink *et al*, 2003), functionally interact

with P2Y receptor signaling pathways (Mamedova *et al*, 2005). Moreover, there are close structural and phylogenetic relationships between P2Y and CysLT receptors, which cluster together into the 'purine receptor cluster' of the rhodopsin family of GPCRs, which also includes a large number of orphan GPCRs (Fredriksson *et al*, 2003). Mellor *et al* (2001) proposed that, in human mast cells, both the $CysLT_1$ receptor and a yet unidentified elusive receptor upregulated by treatment with the pro-inflammatory cytokine interleukin-4 (IL-4) were responsive to both CysLTs and UDP. Here, we challenged the hypothesis that GPR17, an orphan receptor at an intermediate phylogenetic position between P2Y and CysLT receptor families, may represent the yet unidentified elusive receptor responding to both nucleotides and CysLTs. We show that the heterologous expression of GPR17 in a variety of different cell lines results in the appearance of highly specific and concentration-dependent responses to both families of signaling molecules. We also show that *in vivo* knockdown of GPR17 by either CysLT/P2Y receptor antagonists or by antisense technology markedly prevents evolution of ischemic brain damage in a rat focal ischemia model, suggesting GPR17 as a common molecular target mediating the inflammatory effects induced *in vivo* by nucleotides and CysLTs. We thus propose that novel pharmacological agents targeting both signaling components of dualistic receptors may prove very effective in halting or preventing human diseases, such as inflammation (Mellor *et al*, 2001) and ischemia (the present study).

Results

GPR17, a close relative of both P2Y and CysLT receptors, is highly expressed in organs undergoing ischemic damage

The relationships among P2Y, CysLT receptors and GPR17 are shown in Figure 1A. Phylogenetically, $CysLT_1$ and $CysLT_2$ receptors cluster together, whereas P2Y receptors cluster in two phylogenetically distinct subgroups, one encompassing $P2Y_{1,2,4,6,11}$ and the other encompassing $P2Y_{12,13,14}$ (Abbracchio *et al*, 2003). GPR17 is equally distant from the $P2Y_{12,13,14}$ subgroup and the $CysLT_1$ and $CysLT_2$ group, and thus, its ligand specificity cannot be predicted simply based on its phylogenetic position. As a first step to the 'deorphanization' of GPR17, we cloned and analyzed the coding sequences from the human and rat receptors. The previously unidentified rat ortholog (GenBank accession no. DQ777767) displayed an 89% amino-acid identity with hGPR17 (Figure 1B). The hydropathic profile of deduced putative proteins was consistent with the typical seven transmembrane (7TM) structure of a GPCR (Abbracchio *et al*, 2003). Alignment of rat, mouse and human proteins showed almost complete overlapping of TM3, TM6 and TM7 and conservation of a typical amino-acid motif in TM6 (**H**-X-X-**R)** that is present in several GPCRs including all known P2Y and CysLT receptors and is believed (at least for nucleotide receptors) to be essential for ligand binding (Erb *et al*, 1995; Jiang *et al*, 1997; Jacobson *et al*, 2002) (Figure 1B).

In line with previous expression data (Blasius *et al*, 1998), both human and rat GPR17 were highly present in the brain (the present study) and in other organs typically undergoing ischemic damage (kidney and heart) with very low expression in the liver and lung (Figure 1C). hGPR17 mRNA was

also found in some of the cell lines tested (see Figure 1 legend).

Functional characterization in heterologous systems unveils the dual pharmacology of GPR17

In search for the natural ligand of GPR17, the cDNAs from human and rat GPR17 were cloned into the expression vector pcDNA3.1 and transfected in 1321N1, COS-7 and HEK-293 cells. As GPCR stimulation results in increased binding of GTP to G-proteins (which can, in turn, be quantified by measuring [^{35}S]GTPγS binding to purified membranes; Marteau *et al*, 2003), GPR17 activation was determined by testing the increase of [^{35}S]GTPγS binding by exogenously added agonists in transfected cells (Kotani *et al*, 2001; Marteau *et al*, 2003; Fumagalli *et al*, 2004). Optimal [^{35}S]GTPγS binding conditions were determined in preliminary experiments (see Supplementary Materials and methods, Supplementary Results and Supplementary Figure 7). In 1321N1 cells, which do not express any P2Y or CysLT receptors (Communi *et al*, 2001; GE Rovati and MP Abbracchio, unpublished data, 2005), hGPR17 expression (Figure 2B) induced the appearance of concentration-dependent responses to LTD_4 and LTC_4 (with $LTC_4 \gg LTD_4$) and to UDP, UDP-glucose and UDP-galactose (with UDP-galactose = UDP > UDP-glucose) (Figure 2A). ATP, ADP, 2-methyl-thio-ADP, UTP, α,β-methylene ATP and guanosine had no effect (data not shown). Thus, the agonist-response profile of GPR17 is different from that of $CysLT_1$ and $CysLT_2$ receptors (Brink *et al*, 2003; Capra, 2004), and, for nucleotides, is intermediate between $P2Y_6$ and $P2Y_{14}$ receptors (Abbracchio *et al*, 2003; Burnstock and Knight, 2004). Interestingly, half-maximal response concentrations (EC_{50}) for agonist stimulation were in agreement with the characteristics of known CysLT and P2Y receptors (Brink *et al*, 2003; Burnstock and Knight, 2004; Capra, 2004), that is, in the nanomolar (nM) and micromolar (μM) range for CysLTs and nucleotides, respectively (Figure 2 and Supplementary Table I). Expression of rGPR17 in 1321N1 cells (Figure 2D) also induced responses to nM LTD_4 and LTC_4 and to μM UDP and UDP-glucose (Figure 2C). However, at the rat receptor, UDP-glucose was more potent than UDP, and UDP-galactose had no effect; moreover, the relative potency of CysLTs was inverted (Figure 2 and Supplementary Table I). The ligand specificity of hGPR17 was also confirmed in additional cell lines (i.e., COS-7 and HEK-293 cells). Transfection in COS-7 cells (which do not constitutively respond to CysLTs; Capra *et al*, 2005) induced responses to LTD_4 and LTC_4 (Figure 2E). COS-7 cells do express some P2Y receptors (Herold *et al*, 1997), so the 'purinergic' component of GPR17 could not be studied in these cells. Transfection of hGPR17 in HEK-293 (which do not express P2Y receptors interfering with our analysis; see Supplementary Discussion) (Figure 2F) or in CHO cells (not shown) also induced responses to UDP, UDP-glucose and UDP-galactose, with EC_{50} values similar to those observed in 1321N1 cells. In all cell systems, no response was ever observed in cells transfected with corresponding empty vectors (Supplementary Figure 1).

The ability of some known purinergic and leukotriene antagonists to counteract agonist-induced [^{35}S]GTPγS binding in 1321N1 cells expressing the human or rat receptor was also assessed. Both cangrelor (formerly AR-C69931MX), a $P2Y_{12}/P2Y_{13}$ antagonist (Ingall *et al*, 1999; Marteau *et al*,
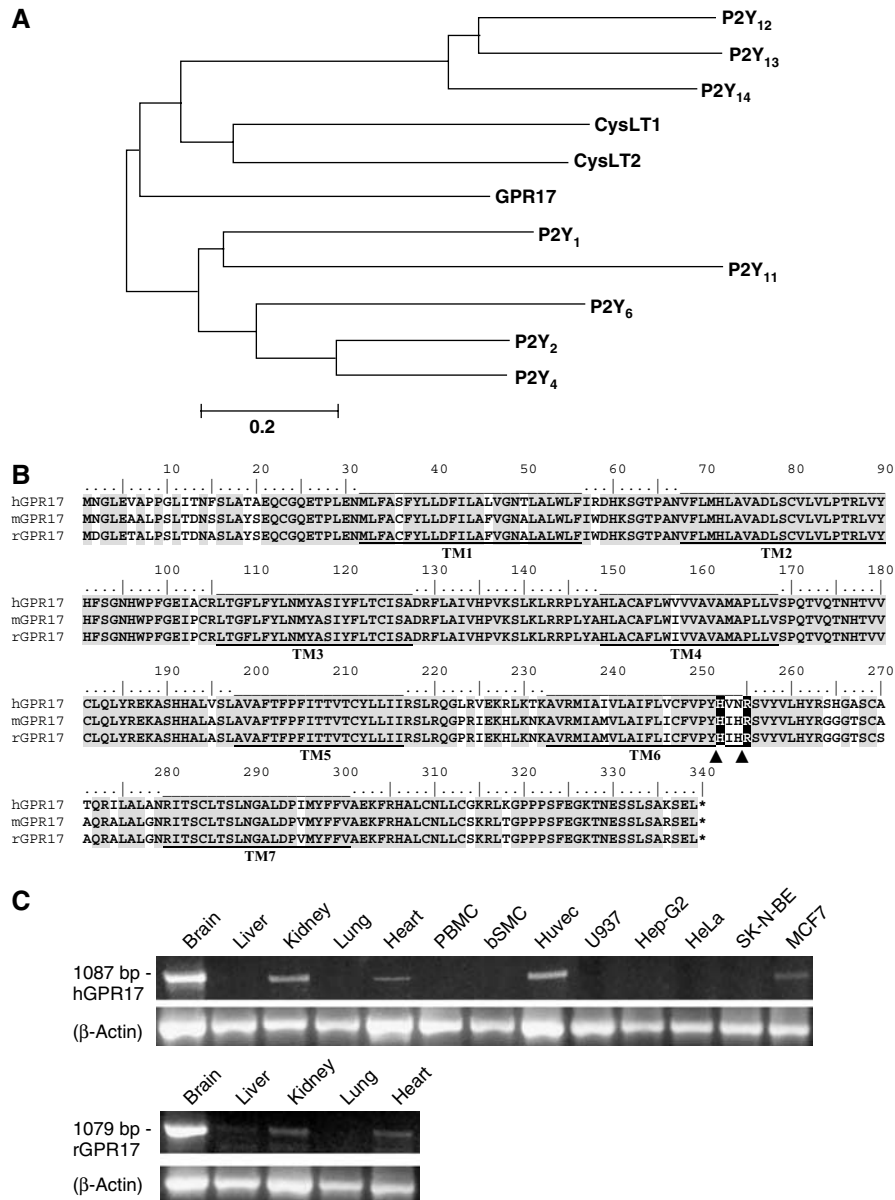
Figure 1 Sequence analysis of GPR17 and expression of the human and rat receptors. (**A**) Phylogenetic tree showing the relationships between GPR17 and P2Y and CysLT receptors. (**B**) Alignment of human, mouse and rat amino-acid GPR17 sequences highlighting the seven TM domains and the conserved H-X-X-R motif in TM6. (**C**) RT–PCR amplification of the human (1087 bp) or rat (1079 bp) cDNA sequences in brain, kidney, heart and in human umbilical vein endothelial cells (HUVEC) and breast adenocarcinoma MCF7 cells. No signal was found in human peripheral blood mononuclear cells (PBMC), bronchial smooth muscle cells (bSMC), myeloid U937, hepatocellular carcinoma Hep-G2, cervix carcinoma (HeLa) and neuroblastoma SK-N-BE cells. Parallel expression of the housekeeping gene beta-actin is shown.

2003; Fumagalli *et al*, 2004), and the P2Y$_1$ receptor antagonist MRS2179 (Jacobson *et al*, 2002) concentration-dependently inhibited [^{35}S]GTPγS binding stimulated by 50 μM UDP-glucose in cells expressing hGPR17, with half-maximal inhibition (IC$_{50}$) in the nM range (Figure 3A and Supplementary Table I). These values are comparable with the IC$_{50}$ of cangrelor (Ingall *et al*, 1999; Marteau *et al*, 2003) and MRS2179 (Nandanan *et al*, 1999) at human P2Y$_{12}$/P2Y$_{13}$ and P2Y$_1$ receptors, respectively. These antagonists were considerably more potent in inhibiting the effects induced by 50 μM UDP-glucose on rGPR17 (Figure 3B and Supplementary Table I), with IC$_{50}$ values in the picomolar (pM) range. Moreover, MRS2179 was more potent than

cangrelor (Figure 3B). For the latter, affinity at rGPR17 is 1000-fold higher than that at rP2Y$_{13}$ (Fumagalli *et al*, 2004). Such species differences in agonist/antagonist-response profiles will have to be taken into account when using rodents to study the pathophysiological roles of GPR17. Conversely, the CysLT$_1$ antagonists montelukast and pranlukast (Brink *et al*, 2003; Capra *et al*, 2006) concentration-dependently inhibited activation of human (Figure 3C) and rat receptors (Figure 3D) by 100 nM LTD$_4$, with nM IC$_{50}$ values and similar potencies (Supplementary Table I). Montelukast and pranlukast have similar affinity at hCysLT$_1$ receptors (Capra *et al*, 2006). Antagonists had no effect in cells transfected with the pcDNA3.1 empty vector (see flat curves in Figure 3A–D).

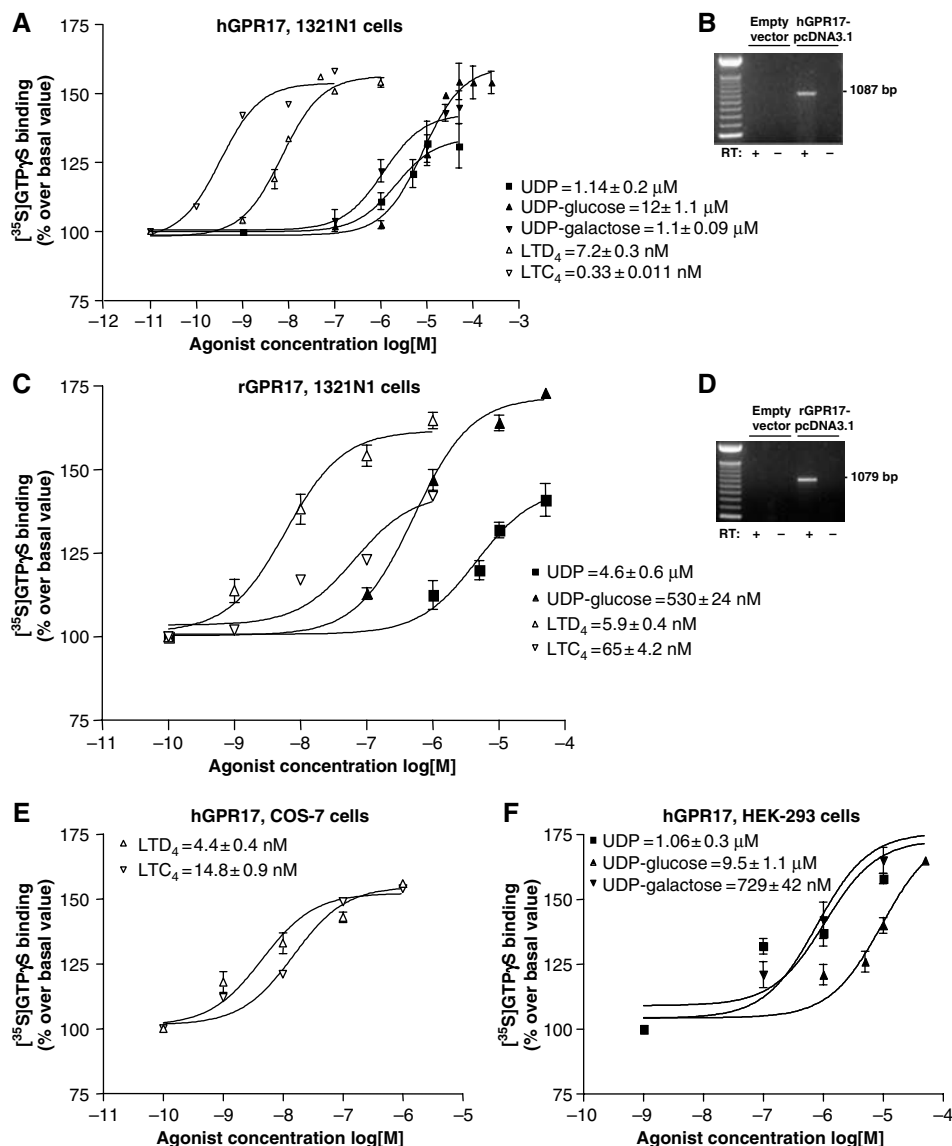**Figure 2** GPR17 agonist specificity in $[^{35}S]GTP\gamma S$ binding. (**A, B**) Agonist-response curves to CysLTs and nucleotides in 1321N1 cells expressing hGPR17 (A), as shown by the presence of a specific 1087 bp amplification product (B). No products were detected in the absence of retrotranscription (RT) (indicated as –RT), nor in cells transfected with the empty vector. (**C, D**) same as (A) and (B) for rGPR17 (RT–PCR product of 1079 bp). (**E**) Responses to CysLTs in COS-7 cells expressing hGPR17. (**F**) Responses to nucleotides in HEK-293 cells expressing hGPR17. Each point is the mean $\pm$ s.d. of 4–9 independent experiments run in triplicate. For each agonist, EC_{50} values are also reported.

Two distinct binding sites (one for nucleotides and the other for CysLTs) seem to be present on GPR17 (Supplementary Figures 2 and 3 and Supplementary Results).

As both P2Y (Communi *et al*, 2001; Abbracchio *et al*, 2003; Fumagalli *et al*, 2003) and CysLT receptors (Brink *et al*, 2003; Capra *et al*, 2006) may couple to G-proteins of the Gi subfamily, 1321N1 cells expressing hGPR17 were incubated with the Gi-protein inhibitor pertussis toxin (PTX) before membrane preparation and $[^{35}S]GTP\gamma S$ binding. PTX strongly inhibited $[^{35}S]GTP\gamma S$ binding stimulated by UDP, UDP-galactose, UDP-glucose and LTD_4, thus establishing an essential role for Gi proteins in GPR17 responses (Figure 4A). As Gi proteins inhibit adenylyl cyclase and cAMP formation (Milligan and Kostenis, 2006), we next evaluated the possible coupling of GPR17 to cAMP inhibition and the effect of PTX on this coupling. In 1321N1 cells expressing hGPR17, UDP, UDP-glucose, UDP-galactose and LTD_4 concentration-depen-

dently inhibited the cAMP formation elicited by $10\,\mu M$ forskolin (FK) (Figure 4B). These effects were detected at μM concentrations of nucleotides and nM concentrations of LTD_4, in line with the $[^{35}S]GTP\gamma S$ binding data. No inhibition of FK-stimulated cAMP levels was obtained in cells transfected in parallel with the empty plasmid (Figure 4C), confirming that agonist responses are specifically due to GPR17. In line with Figure 4A data, pretreatment of GPR17-expressing cells with PTX fully obliterated the agonists effects on FK-induced cAMP formation (Figure 4D). Finally, as $P2Y_{12,13,14}$ and $CysLT_1$ receptors can also couple to phospholipase C and increase intracellular calcium $([Ca^{2+}]_i)$ likely via Gi-protein $\beta\gamma$ subunits (Communi *et al*, 2001; Abbracchio *et al*, 2003; Fumagalli *et al*, 2003; Hardy *et al*, 2004; Capra *et al*, 2005, 2006), responses of GPR17 were also investigated by single-cell calcium imaging. Approximately 30% of 1321N1 cells expressing hGPR17 responded to UDP-glucose,
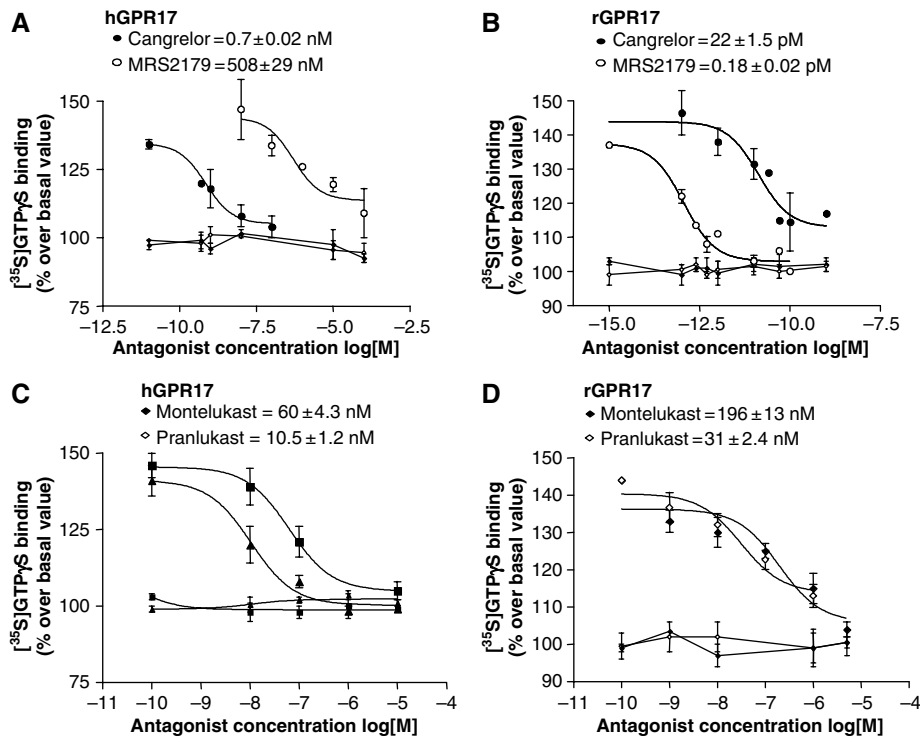
Figure 3 Effect of P2Y and CysLT$_1$ receptor antagonists on the activation of recombinant GPR17 in [^{35}S]GTPγS binding. (**A**) Antagonism of UDP-glucose stimulation of [^{35}S]GTPγS binding by the indicated P2Y antagonists in 1321N1 cells expressing hGPR17. (**B**) Same as in (A), in 1321N1 cells expressing rGPR17. (**C**) Antagonism of LTD$_4$ stimulation of [^{35}S]GTPγS binding by indicated CysLT$_1$ antagonists in 1321N1 cells expressing hGPR17. (**D**) Same as in (C), in 1321N1 cells expressing rGPR17. Flat concentration–response curves in (A–D) refer to cells transfected with the pcDNA3.1 empty vector. Each point is expressed as percentage of basal [^{35}S]GTPγS-specific binding set to 100% and is the mean $\pm$ s.d. of 4–7 independent experiments run in triplicate. For each antagonist, IC$_{50}$ values are reported.

UDP, UDP-galactose or LTD$_4$ with increases of [Ca^{2+}]$_i$ (Figure 5A–D). No responses were detected in cells transfected with the empty vector (Figure 5E–H). In approximately 50% of COS-7 cells expressing hGPR17, LTD$_4$ induced a [Ca^{2+}]$_i$ response (Figure 5J) similar to that observed in cells expressing hCysLT$_1$ receptor, here utilized as a positive control (Figure 5I). These responses are independent of extracellular calcium and due to calcium release from intracellular stores, as expected for a GPCR (Supplementary Figure 4 and Supplementary Results). Taken together, these results demonstrate that GPR17 is a dualistic receptor specifically responding to two unrelated families of inflammatory molecules, nucleotides and CysLTs. This novel dualistic receptor seems different from that studied by Mellor *et al* (2001), as it was not found in a human mast cell line (HMC-1 cells) nor in mast cells isolated from cord blood mononuclear cells (see Supplementary Figure 5 and Supplementary Results). GPR17 is coupled to Gi proteins and, in a similar way to many GPCRs (Milligan and Kostenis, 2006), leading to both adenylyl cyclase inhibition and [Ca^{2+}]$_i$ increases.

Neuronal GPR17 expression in brain is markedly increased after ischemia

To shed light on GPR17 pathophysiological roles, we next developed an anti-GPR17 antibody for immunohistochemical studies. Rabbits were immunized with a peptide derived from the C-terminal region of the human receptor, which is highly similar (94% identical) to the corresponding region of the rat receptor. Sera from immunized rabbits specifically recognized both human and rat GPR17 and did not crossreact

with other receptors of the P2Y family (e.g., P2Y$_1$ and P2Y$_{13}$; data not shown). In line with the pharmacological data (Figure 2), positive immunoreactivity to antisera was observed in 1321N1 (Figure 6B) or COS-7 cells (Figure 6E) expressing rGPR17, but not in corresponding control cultures transfected with the empty vector (Figure 6A and D). In both cell systems, labeling was abolished by preabsorption of antisera with the peptide used for immunization (Figure 6C and F), confirming specificity of immunostaining. Based on expression data (Figure 1), we next utilized this antibody to study the cellular localization of GPR17 in rat cortical slices. Confocal microscopy showed positive immunoreactivity (green fluorescence) in the soma of a consistent number of cortical cells (Figure 6G). No colocalization with glial fibrillary acidic protein (GFAP)-positive cells (red fluorescence in Figure 6G) was found (see also Supplementary Results), suggesting that GPR17 is not expressed by astrocytes. Conversely, several GPR17-expressing cells also positively stained for neuronal markers, such as SMI-311 or β-III tubulin (yellow fluorescence in Figure 6H and I, respectively), which, however, did not systematically colocalize with GPR17 (suggesting that receptor expression is restricted to specific neurons; see also Supplementary Results). As CysLTs and nucleotides accumulate to a greater extent in ischemic brain (Ohtsuki *et al*, 1995; Ciceri *et al*, 2001; Burnstock and Knight, 2004), based on the present data demonstrating the *in vitro* activation of GPR17 by both families of endogenous ligands (Figure 2), we next analyzed the role of GPR17 in an established model of ischemic damage (the permanent mono-lateral middle cerebral artery occlusion in the rat, MCAo).
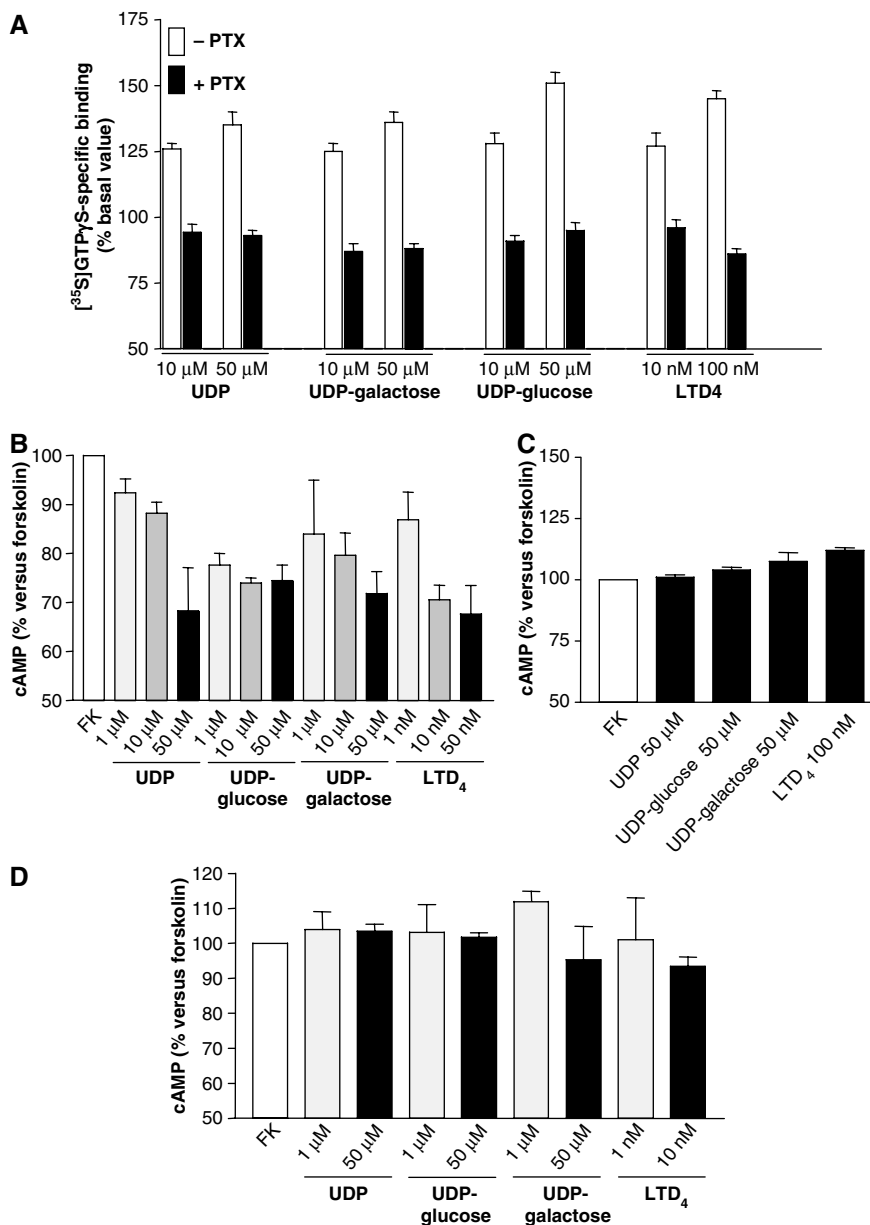
Figure 4 A key role for Gi proteins and adenylyl cyclase in GPR17 function. (**A**) Pre-incubation of 1321N1 cells expressing the human receptor with the Gi-protein inhibitor PTX (black columns) significantly reduced stimulation of [^{35}S]GTPγS binding by the indicated agonists with respect to untreated cells (white columns) ($P < 0.001$). Data are the mean of three experiments run in triplicate. (**B, C**) Effect of uracil nucleotides and LTD$_4$ on FK-stimulated cAMP accumulation. 1321N1 cells transfected with either hGPR17 (B) or with corresponding empty plasmid (C) were treated with the indicated agonists in the presence of 10 μM FK. cAMP levels (means ± s.e.m.; $n = 6$) were quantified and expressed as percentage of cAMP in the presence of FK alone set to 100% (white bar in graphs). A concentration-dependent inhibition of FK-stimulated cAMP production was detected only in hGPR17-expressing cells. (**D**) Preincubation of 1321N1 cells expressing hGPR17 with PTX under the same conditions reported in (A) before agonist treatment fully obliterated the effects of uracil nucleotides and LTD$_4$ on FK-stimulated cAMP formation. Basal and FK-stimulated cAMP levels were 11.5 ± 1.2 and 89.5 ± 6.5 pmol/ml, respectively (values refer to 10 μg of protein/ sample and a 10 min incubation of intact cells with either medium alone or medium + FK).

Forty-eight hours after ischemia, in the cortex of ischemic rats, GPR17-immunoreactivity was markedly increased both within (Figure 6J) and at the borders (Figure 6K) of the ischemic infarct (gray area in the brain section drawing of Figure 6), in comparison with corresponding cortical areas of the unlesioned contralateral hemisphere (Figure 6L and M). Within the ischemic lesion, GPR17 was highly expressed in cells resembling pyramidal neurons (see inset of Figure 6J), as also confirmed by costaining with SMI-311 (Figure 6N) (see also Supplementary Results).

In vivo knockdown of dualistic GPR17 prevents evolution of ischemic brain damage

Based on increased GPR17 expression after MCAo (Figure 6), we next hypothesized that GPR17 activation during ischemia may contribute to injury development. If this were true, agents such as montelukast or cangrelor, which effectively antagonize GPR17 *in vitro* (Figure 3), should contrast the *in vivo* actions of GPR17 and attenuate brain damage evolution. Moreover, we anticipated that agents interfering with either one or both the signaling components of a dualistic receptor
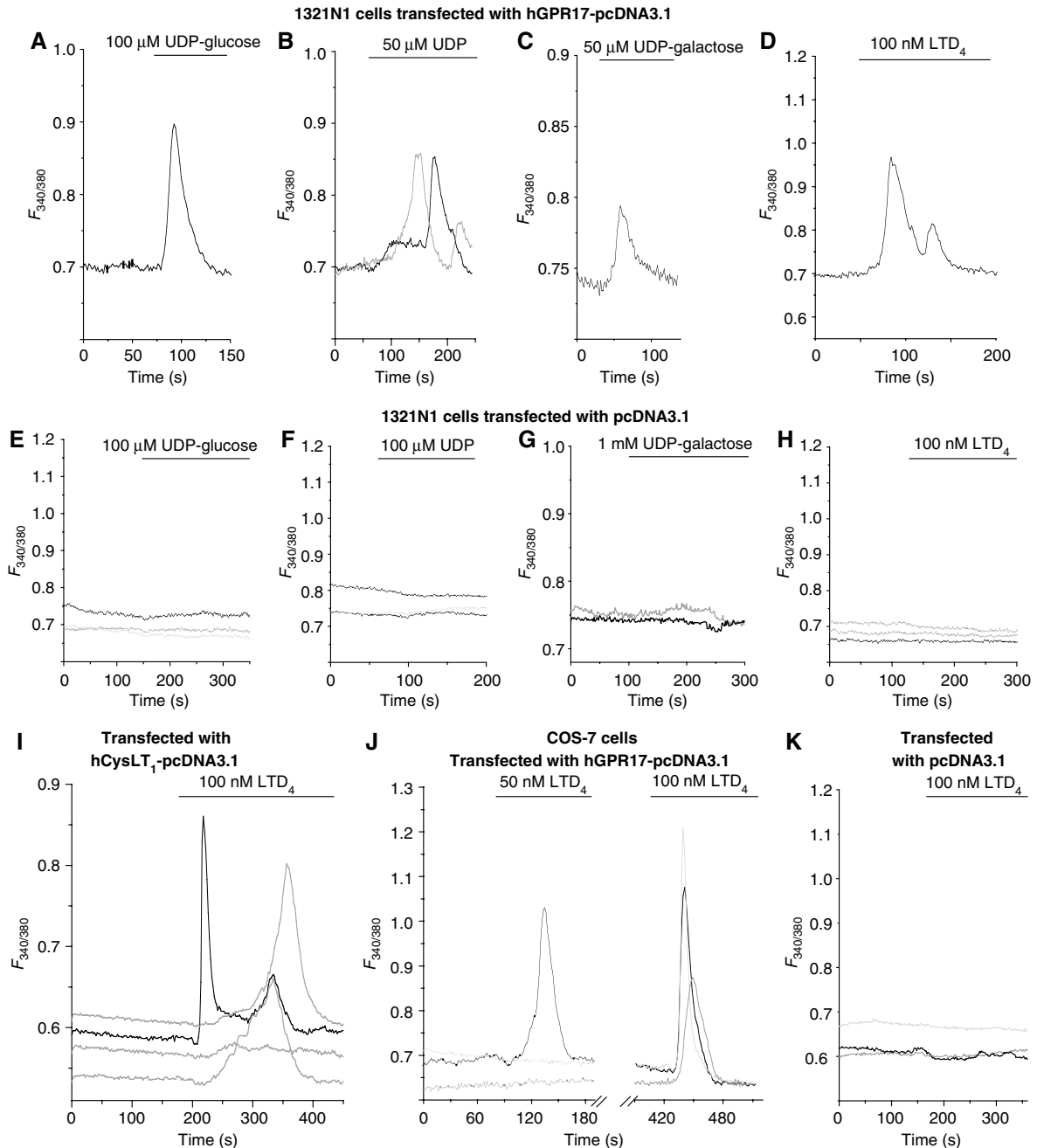
Figure 5 Single-cell calcium imaging in cells expressing hGPR17. Each trace shows response recorded from one single cell. (**A–C**) Approximately 30% of 1321N1 cells expressing hGPR17 showed responses to uracil nucleotides (mean calcium response to UDP-glucose: $\Delta F_{340/380} = 0.23 \pm 0.06$, mean $\pm$ s.e.m., $n = 15$; mean calcium response to UDP: $\Delta F_{340/380} = 0.12 \pm 0.01$, $n = 11$; mean calcium response to UDP-galactose: $\Delta F_{340/380} = 0.05 \pm 0.003$, $n = 3$) or (**D**) to LTD$_4$ ($\Delta F_{340/380} = 0.26 \pm 0.05$, mean $\pm$ s.e.m., $n = 18$). (**E–H**) The same agonists induced no responses in cells transfected with the empty plasmid. (**I**) Approximately 45% of COS-7 cells transfected with hCysLT$_1$ receptor showed calcium transients to LTD$_4$ (mean calcium response: $\Delta F_{340/380} = 0.3 \pm 0.08$, mean $\pm$ s.e.m., $n = 17$). (**J**) Similar responses were recorded from approximately 50% of COS-7 cells expressing hGPR17 (mean calcium response: $\Delta F_{340/380} = 0.18 \pm 0.03$, mean $\pm$ s.e.m., $n = 22$). (**K**) No responses to LTD$_4$ were recorded in cells transfected with the empty plasmid.

should be able to very robustly modulate its *in vivo* activity. Magnetic resonance imaging (MRI) of developing damage showed that, after MCAo, brain infarct volume in lesioned hemispheres increased dramatically between 2 and 48 h with respect to the contralateral unlesioned side (see C1, C2 and C3 in Figure 7). *In vivo* treatment of ischemic animals with either montelukast or cangrelor markedly prevented increase of damage at 24 and 48 h with respect to 2 h (Figure 7A and B), suggesting that activation of GPR17 during ischemia

indeed contributes to injury development. However, as montelukast and cangrelor are also antagonists at CysLT$_1$ and P2Y$_{12,13}$ receptors, respectively, and some of these receptors are expressed in the brain (Ingall *et al*, 1999; Brink *et al*, 2003; Marteau *et al*, 2003; Fumagalli *et al*, 2004), to prove the specific involvement of GPR17 in ischemia and to test the efficacy of selective *in vivo* receptor knockdown, we utilized an antisense oligonucleotide strategy (Stein, 2001), which has been proven to very efficiently downregulate other GPCRs in
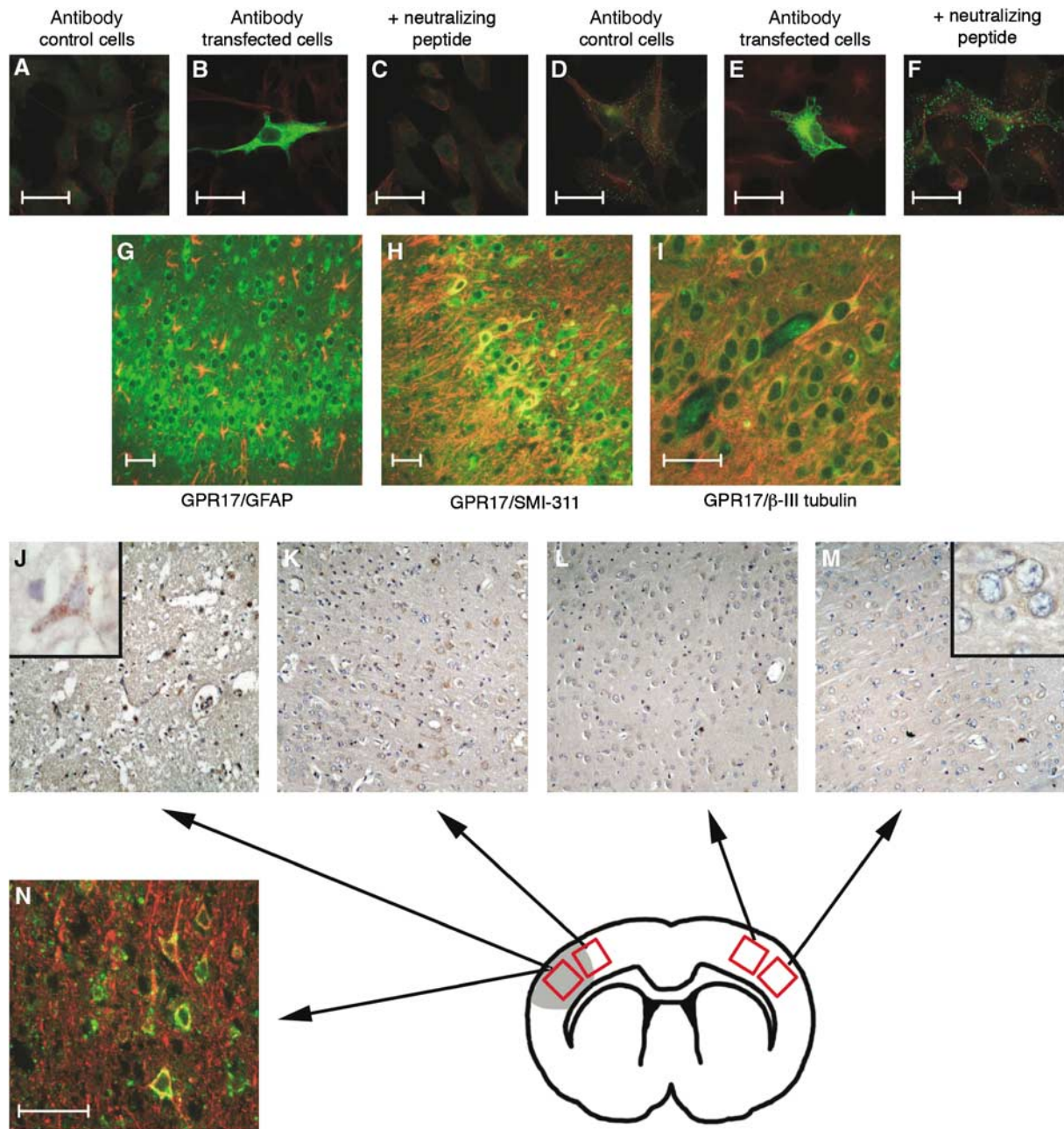
Figure 6 GPR17 immunoreactivity in transfected cells and in rat brain. (**A–C**) 1321N1 cells transfected with empty vector (A, control cells) or rGPR17-pcDNA3.1 (B, transfected cells) were incubated with anti-GPR17 antisera in the absence or presence (C) of the peptide used for rabbit immunization (neutralizing peptide). (**D–F**) Same as in (A–C) on COS-7 cells. (**G–I**) Double-labeling of rat cortical slices with anti-GPR17 antibody (green fluorescence) and astroglial (G, GFAP) or neuronal markers (H, SMI-311 or I, β-III tubulin) (red fluorescence). (**J–N**) At 48 h after MCAo, rat brain slices were incubated with anti-GPR17 antisera followed by either horseradish peroxidase staining (J–M) or double-staining with anti-SMI-311 (N). Micrographs were taken inside the ischemic area (J, gray area in left lesioned hemisphere) or at its borders (K), or in the corresponding healthy brain areas of the contralateral unlesioned right hemisphere (L, M), as indicated by squares in the drawing of coronal brain section. Inside the ischemic infarct, GPR17 immunoreactivity was found on pyramidal-like cells (higher magnification inset in J), which were also positive for SMI-311 (N). Similar data were obtained in five experiments (24 brain sections in total). Scale bars 30 μm.

the brain (Tepper *et al*, 1997; Van Oekelen *et al*, 2003). Of several antisense oligonucleotides designed on rGPR17 sequence, only oligo616 and, to a lesser extent, oligo241 reduced the *in vitro* expression of rGPR17 in HEK-293 cells (Supplementary Figure 6), and were thus selected for the *in vivo* MCAo study. In a similar way to montelukast and cangrelor, intracerebroventricular (i.c.v.) injections of oligo616 to ischemic rats 48 and 24 h before and 10 min after MCAo markedly attenuated infarct size evolution

(Figure 7). A smaller protective effect was observed with oligo241 (data not shown), which targeted a different sequence on rGPR17. Bioinformatic analysis showed that the two antisense oligonucleotides that effectively prevented brain infarct evolution were not homologous to any other GPCR (see Materials and methods). This, together with the absence of activity of a 'scrambled' oligonucleotide both *in vivo* (Figure 7) and *in vitro* (Supplementary Figure 6), strongly indicates that GPR17 is indeed the specific target of
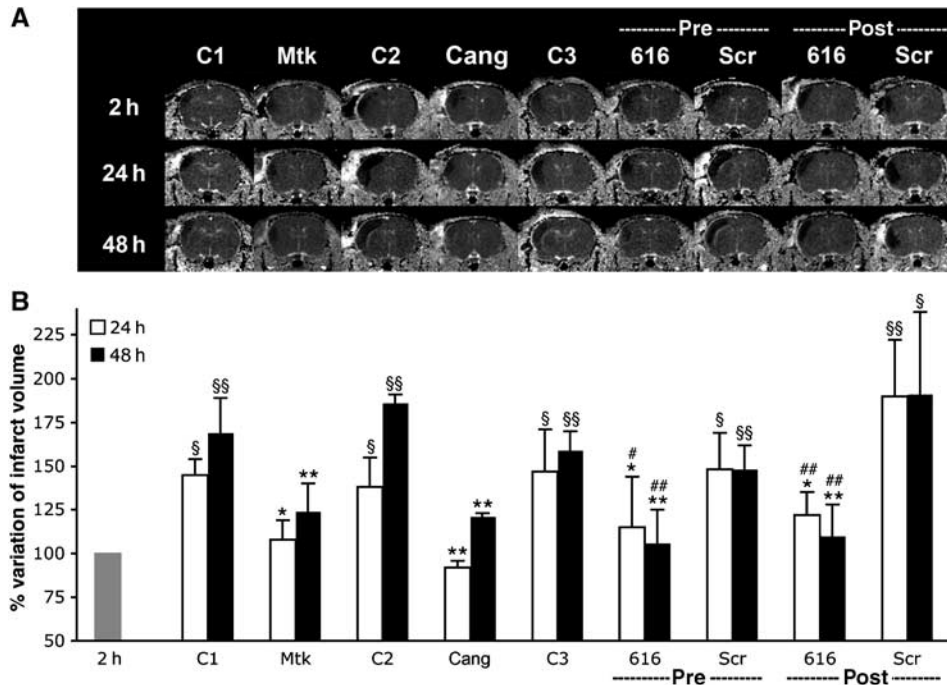
Figure 7 Effect of montelukast (Mtk), cangrelor (Cang) and oligo616 on evolution of brain infarct size as determined by MRI at 2, 24 and 48 h after MCAo. (**A**) Representative Tr(D) images of coronal brain sections from ischemic rats treated with either Mtk, Cang, oligo616 (616) or scrambled oligonucleotide (Scr) in comparison with corresponding control animals treated with vehicle only (C1, C2, C3). Antisense oligonucleotides were administered 48 and 24 h before and 10 min after MCAo (indicated as 'pre'); in some experiments, animals only received a single dose of antisense oligonucleotide (indicated as 'post', also see below). In left lesioned hemispheres, ischemia-associated brain damage is shown by black areas. (**B**) Quantitative analysis of infarct size volume at 24 and 48 h after MCAo from rats receiving vehicle (C1, $n=5$) or Mtk (2 mg/kg intravenously (i.v.), 10 min after MCAo; $n=6$); vehicle (C2, $n=5$) or Cang (4.5 μg/animal, i.c.v., 10 min after MCAo, $n=5$), vehicle (C3, $n=5$) or either oligo616 or scrambled-oligo (400 ng/animal, i.c.v., 48 and 24 h before and 10 min after MCAo, $n=5$, indicated as 'pre'). Some animals received a single i.c.v. dose of 4.5 μg of either oligo616 ($n=6$) or scrambled-oligo ($n=6$) 10 min after ischemia (indicated as 'post'). Data are expressed as percentage variation of infarct volume at 24 and 48 h after MCAo compared to 2 h considered as 100%. $^{\S}P<0.05$, $^{\S\S}P<0.01$ versus 2 h; $^{*}P<0.05$, $^{**}P<0.01$ versus corresponding control animals; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus corresponding Scr animals.

oligo616 and oligo241. Importantly and rather surprisingly, GPR17 knockdown by a single i.c.v. injection of oligo616 10 min after MCAo also resulted in comparable brain protection (Figure 7). This result might have important therapeutic implications for human stroke, also in consideration of the mechanism of action of antisense oligonucleotides, which, at variance from antagonist receptor ligands, need time to exert their effects. Thus, GPR17 may represent a novel target for development of new relevant therapeutic approaches to human stroke. Taken together, these data suggest that GPR17 activation by CysLTs and nucleotides during ischemia contributes to development of brain damage. In line with this hypothesis, GPR17 knockdown by either pharmacological agents or antisense technology results in robust protection against brain damage, even when receptor inhibition is accomplished after ischemia is induced.

Discussion

It is generally believed that each neurotransmitter/hormonal receptor specifically and selectively responds to one single endogenous ligand or a family of structurally related ligands (Kenakin *et al*, 1992; Kenakin, 2004). Recently, two GPCRs (the ALX and ChemR23 receptors) responding to lipid-derived ligands have been reported to also recognize specific peptides (Chiang *et al*, 2000; Arita *et al*, 2005). Mellor *et al* (2001) were the first to postulate the existence of dualistic

receptors, by showing that both CysLT$_1$ and a yet unidentified LTC$_4$-preferring receptor (there referred to as the CysLT$_3$ receptor) mediated dual responses to both CysLTs and UDP. Here, by heterologous expression of the previously orphan receptor GPR17 in several cell lines (see Results and Supplementary Discussion), we identify and fully characterize this receptor as a dualistic GPCR responsive to two unrelated families of non-peptide small ligands (nucleotides and CysLTs). Phylogenetically, GPR17 is located at an intermediate position between ligand-specific receptor families and is the closest receptor to a common ancestor that also gave rise to the P2Y and CysLT families. In a similar way to P2Y$_{12,13,14}$ and CysLT$_1$ receptors, GPR17 is coupled to Gi, and its activation can inhibit cAMP formation and also stimulate intracellular calcium release. The need for more selective, organ- and cellular-specific responses to either extracellular nucleotides or CysLTs may have driven the evolution of this family towards the more specialized P2Y and CysLT receptors, in parallel with a restriction of ligand selectivity. In line with this hypothesis, both montelukast and pranlukast, two 'specific' CysLT$_1$ antagonists, also interfere with P2Y receptor signaling (Mamedova *et al*, 2005). GPR17 does not seem to be the elusive receptor described by Mellor and co-workers, as it was not found in mast cells, suggesting that other dualistic receptors remain to be characterized. GPR17 was instead found to be highly expressed in organs that can typically undergo ischemic injury (brain, heart and kidney) and may

thus represent a 'primitive', evolutionarily conserved, receptor able to respond to gross tissue injuries (e.g., trauma and ischemia) which do not require a very selective response. The present data also add complexity to the already established 'crosstalk' between the purinergic and the leukotriene receptor systems (see Introduction), suggesting GPR17 as an additional means by which these two signaling systems interact with each other.

The existence of dualistic receptors profoundly change the current concept of receptor specificity, suggesting that the same receptor may respond to either one or two families of signaling molecules depending upon specific pathophysiological conditions and/or ligand concentrations. In the case of GPR17, not only are agonist-response profiles different from those of both CysLTs (Brink *et al*, 2003; Capra *et al*, 2006) and nucleotides (Abbracchio *et al*, 2003; Burnstock and Knight, 2004) (which suggests peculiar functions) but, most notably, agonist EC_{50} values are in the nM range for CysLTs and in µM range for uracil nucleotides. Specifically for brain ischemia, this might reflect the different modalities and times of accumulation of these ligands after MCAo. In the same ischemia model, levels of CysLTs in the lesioned cortex were sharply increased 4 h after MCAo and rapidly declined afterwards (Ciceri *et al*, 2001); extracellular concentrations of ATP were instead constantly elevated starting from 20 min after MCAo throughout a 220 min of microdialysis sampling, with a time course similar to that of excitatory amino-acids (Melani *et al*, 2005). During this period, nucleotide release is likely to originate from depolarized neurons or glial membrane channels (Melani *et al*, 2005). However, the local concentrations of both ATP and other nucleotides in ischemic brain are likely to further increase in the subsequent hours and days, as a result of massive degradation of nucleic acids from damaged and dying cells (Neary *et al*, 1996). Thus, at the site of injury, neurons are likely exposed to high concentrations of nucleotides for very prolonged periods of time. These data are also consistent with the present results demonstrating that inhibition of GPR17 protects against brain damage even when receptor knockdown is accomplished after the induction of ischemia. Based on increased GPR17-expressing cells both within and at the borders of the ischemic lesion 48 h after MCAo, we speculate that GPR17 represents the common molecular target of CysLTs and nucleotides that sensitizes neurons to ischemic damage, thereby contributing to injury propagation in the hours and days after ischemia. The possibility of interfering with ischemia progression after the ischemic insult has obvious relevant implications for the development of innovative therapeutic approaches for management of human stroke. Previous enthusiasm on anti-stroke agents has been tempered by demonstration that the efficacy of most neuroprotective agents diminishes quite rapidly if they are given after the onset of ischemia (Huang and McNamara, 2004). The present results might, thus, generate novel neuroprotective strategies to counteract damage evolution even after ischemia. More in general, we anticipate that the existence of receptors responding to more than one family of endogenous ligands will lead to the development of novel dualistic pharmacological agents with previously unexplored therapeutic potential. New chemical entities targeting both components of dualistic receptors may indeed prove extremely more effective in halting or preventing a variety of human diseases.

Materials and methods

Cell culture, transfection and treatments

Human astrocytoma cells (ADF cells), 1321N1, COS-7 and HEK-293 cells were cultured as described (Brambilla *et al*, 2000; Fumagalli *et al*, 2004). For [^{35}S]GTPγS, 10^6 1321N1, COS-7 or HEK-293 cells were seeded on 75 cm^2 flasks and transfected by the calcium phosphate precipitation method (Fumagalli *et al*, 2004). For calcium imaging studies, 1321N1 and COS-7 cells were seeded on 2.4 cm diameter glass coverslips (100×10^3 cells). In selected experiments, cells were exposed to 100 ng/ml PTX (Sigma) for 18 h before membrane preparation. For immunocytochemistry, 1321N1 and COS-7 cells were seeded on 1.3-cm diameter glass coverslips (1.5×10^4 cells) and transfected with FuGENE 6, according to the manufacturer's instructions.

For treatment of cultured cells with antisense oligonucleotides, HEK-293 cells were utilized, based on their high transfection efficiency. Briefly, 13×10^4 HEK-293 cells were seeded on 9 cm^2 dishes. On day 2 after plating, cells were transfected with pcDNA3.1 containing the construct encoding for rGPR17 together with the neomycin resistance gene, here used as a reporter gene. The various oligonucleotides described in Supplementary Figure 3 (all used at 0.3 µM final concentrations) were added to cells twice in a small Fugene volume (125 µl): 16 and 40 h after transfection of rGPR17. Twenty-four hours after the last Fugene addition, RNA was extracted from cells as described below, and the transcripts for both rGPR17 and the neomycin resistance gene determined as specific RT–PCR amplification products of 1079 and 357 bp, respectively.

Reagents

Culture media and sera were from Celbio. Reagents for RT–PCR, cloning and transfection were from Invitrogen, with the exception of FuGENE 6, which was from Roche Diagnostics. LTD$_4$ was purchased from Cayman Chemical Co. (Ann Arbor, MI). Cangrelor was kindly provided by The Medicines Company (Parsippany, NJ, USA). Montelukast and pranlukast were a kind gift from MERCK & Co (USA). The hCysLT$_1$-pcDNA3.1 was a kind gift from Dr J Evans, MERCK & Co (USA). Antisense oligonucleotides were selected according to the general criteria for oligo design and synthesized by MWG-Biotech AG. Thermodynamic criteria were set according to Matveeva *et al* (2003) and care was taken to avoid internal loop, palindrome of 6 or more base pairs, nucleotide repetition (more than 3 base pairs), and where possible, an AGGG consensus sequence shown to target RNase degradation was included (see oligo616 and oligo241 in Supplementary Figure 3) (Smith *et al*, 2000). Oligo antisense sequence was mapped on GPR17 RNA secondary structure predicted using GeneBee service (http://www.genebee.msu.su/services/rna2_reduced.html) to choose oligo mapping in the loop part of the hairpin; the scrambled oligonucleotide was randomly generated on the basis of oligo616. We chose to use unmodified oligonucleotides to avoid possible toxicity, while the stability issue was faced by a multiple delivery experimental design (see Figure 7 legend and text). Each oligo antisense sequence was challenged with rat GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to exclude the presence of multiple target sequences in the rat genome. All other reagents were from Sigma-Aldrich.

Total RNA isolation and PCR analysis

Total RNA was extracted using the TRIZOL® Reagent (Invitrogen) according to the manufacturer's instructions. Retrotranscription to cDNA and PCR reactions were carried out as described previously (Fumagalli *et al*, 2004). The following primers were used to detect the expression of human and rat GPR17, respectively:

Fw 5′-GACTCCAGCCAAAGCATGAA-3′ and
Rw 5′-GGGTCTGCTGAGTCCTAAACA-3′;
Fw 5′-TAGACTTCTGCCTTCAGC-3′ and
Rw 5′-TGACAGGACCTCCCCGAG-3′.

Cloning of human and rat GPR17

By using specific oligonucleotide PCR primers external to the open reading frame (ORF) of the previously reported human receptor sequence (GenBank accession no. U33447), we amplified a 1087 bp product from human astrocytoma cells (ADF cells) and cloned it into a pcDNA3.1 expression vector using the pcDNA3.1/V5-

His©TOPO® TA Expression Kit (Invitrogen, Milan, Italy). With the same strategy, we cloned the putative 1020 bp ORF of the rat sequence (included in clone CH230-239E15, GenBank accession no. AC112062) from rat brain. The sequence of the new cloned rat receptor has been submitted to GenBank (accession no. DQ777767). Constructs were verified by sequencing with the Applied Biosystems Terminator cycle sequencing kit. A partial sequence of the mouse ortholog of GPR17 is reported in GenBank (AY255543), and the complete sequence, 98% identical to the rat receptor, was found in a BAC clone (AC131761). Phylogenetic trees were generated with the program Mega 2.1.

[^{35}S]GTPγS binding assay

Control and transfected cells were homogenized in 5 mM Tris–HCl and 2 mM EDTA (pH 7.4) and centrifuged at 48 000 g for 15 min at 4°C. The resulting pellets (plasma membranes) were washed in 50 mM Tris–HCl and 10 mM MgCl$_2$ (pH 7.4) and stored at −80°C until used. Nucleotide-stimulated [^{35}S]GTPγS binding in membranes of cells expressing the human or rat receptor was performed as described previously (Kotani *et al*, 2001; Marteau *et al*, 2003; Fumagalli *et al*, 2004). For more information, see Supplementary Materials and methods.

cAMP assay

Control and hGPR17-transfected 1321N1 cells were treated for 10 min with purinergic or leukotriene agonists in serum-free medium containing the phosphodiesterase inhibitor Ro201724 (20 μM) in the absence or presence of 10 μM FK. Aliquots of cells were pretreated with PTX before agonist treatments. Cells were then harvested, lysed and assayed for cAMP accumulation using the cAMP enzyme immunoassay system kit (Sigma-Aldrich), following the manufacturer's instructions.

Functional calcium imaging assay

Intracellular calcium concentrations ([Ca^{2+}]$_i$) were measured as described previously (Fumagalli *et al*, 2003). At 48 h after transfection, 1321N1, COS-7 and HEK-293 cells were loaded with 2 μM Fura-2 pentacetoxy methylester in Krebs–Ringer solution, washed and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, NY) equipped with a calcium imaging unit. In some experiments, agonists were applied in calcium-free medium. Polychrome IV (TILL Photonics, Germany) was used as the light source. Fura-2 and EGFP fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, Forest City, CA) and analyzed with the Axon Imaging Workbench 2.2 software (Axon Instruments). Images were acquired at 1–4 340/380 ratios/s.

Generation of an antibody against GPR17

Polyclonal antibodies recognizing human and rat GPR17 receptor were raised in rabbits using a synthetic peptide corresponding to the C-terminal region of the human receptor (amino acids (cg)SFEGKT-NESSLSAKSEL) and 94.12% identical to the rat sequence (Figure 1). The peptide was coupled to keyhole-limpet hemocyanin and injected intradermally in rabbits using 200 μg of peptide/treatment.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, detergent permeabilized and incubated with anti-GPR17 polyclonal antibody (1:200) and anti-α-tubulin monoclonal antibody (1:5000; Sigma-Aldrich) followed by anti-rabbit-FITC-and anti-mouse-TRITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) (Verderio *et al*, 1999). For negative controls, anti-GPR17 antibody and neutralizing peptide were preincubated overnight at 4°C before addition to cells.

Histology and horseradish peroxidase immunohistochemistry

After dewaxing and rehydration, paraffin-embedded sections were permeabilized in 10 mM sodium citrate and 0.05% Tween 20 (pH 6) for 20 min at 90°C and incubated in 0.01 M phosphate-buffered saline pH 7.4 (10% normal goat serum, 0.1% Triton X-100). Sections were then incubated with primary anti-GPR17 antibody (1:200; see above) and stained for horseradish peroxidase immunohistochemistry as described previously (Sironi *et al*, 2003).

Fluorescence immunohistochemistry

Sections treated and permeabilized as described above (Sironi *et al*, 2003) were double stained with anti-GPR17 polyclonal antibody and anti-GFAP (1:1000; Sigma-Aldrich) or anti-SMI-311 (1:300; Sternberger Monoclonal Inc.) or anti-β-III tubulin (1:1000; Promega) monoclonal antibodies at 4°C overnight and then incubated with anti-rabbit-FITC- and anti-mouse-TRITC-conjugated secondary antibodies (Verderio *et al*, 1999).

Confocal microscopy

Labeled cultures or brain sections were examined with a Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hemel Hempstead, UK) mounted on a light microscope (Eclipse TE2000-S; Nikon, Tokyo, Japan). Images were acquired using LaserSharp 5 software. For quantification of GPR17-expressing cells, see Supplementary Materials and methods.

Induction of focal brain ischemia in the rat

Male Sprague–Dawley rats (Charles River) underwent permanent MCAo as described previously (Tamura *et al*, 1981; Sironi *et al*, 2003). Procedures involving animals and their care at the Department of Pharmacological Sciences of the University of Milan respected the Institution's guidelines, which comply with the national and international rules and policies. Drug treatments were as follows: montelukast (2 mg/kg, i.v., single bolus of 200 μl in physiological solution) and cangrelor (4.5 μg/animal, i.c.v., 5 μl in physiological solution) were administered 10 min after MCAo. Cangrelor was used here to simply test the involvement of GPR17 in brain ischemia, and, being a very polar molecule likely to very poorly permeate the blood–brain barrier, it was administered i.c.v. Oligo616 and scrambled-oligo (400 ng in 5 μl of physiological solution) were administered i.c.v. three times to each rat 48 and 24 h before and 10 min after MCAo. Some animals received a single i.c.v. (4.5 μg of either oligo616 or scrambled-oligo) after ischemia induction. Control groups received corresponding vehicle.

MRI analysis

MRI measurements were taken 2, 24 and 48 h after MCAo using a 4.7 T, vertical superwidebore magnet of a Bruker AMX3 spectrometer with microimaging accessory. Animal preparation, image acquisition, trace of the diffusion tensor map computation, ischemic volume determination and progression of the ischemic damage over time were as described (Guerrini *et al*, 2002).

Statistical analysis

For [^{35}S]GTPγS binding data, analysis and graphic presentation was performed by the nonlinear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad). For cAMP experiments, data are mean±s.e.m. of six replicated from three independent experiments and statistical analysis was performed by either Student's *t*-test or one-way analysis of variance (ANOVA) (multiple comparison test).

For calcium imaging, data were normalized to the mean $F_{340/380}$ increase recorded in control cells. Data are presented as mean± s.e.m. of 4–18 experiments run in triplicate. Statistical analysis was performed by either Student's *t*-test or one-way ANOVA (multiple comparison test). Significance refers to results where $P < 0.05$ was obtained.

For NMR studies, data on progression of ischemic damage over time were evaluated by ANOVA for repeated measures. For each animal, the ischemic area at 2 h after ischemia induction was set to 100%, and the extension of the ischemic area at all other time points was proportionally calculated, thus providing an internal control of ischemia development. Then, variations of ischemic volumes between animals and groups were compared. Data are expressed as mean values±s.em. *P*-values <0.05 were considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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