GPR37 and GPR37L1 are receptors for the neuroprotective and glioprotective factors prosaptide and prosaposin

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GPR37 (also known as Pael-R) and GPR37L1 are orphan G protein-coupled receptors that are almost exclusively expressed in the nervous system. We screened these receptors for potential activation by various orphan neuropeptides, and these screens yielded a single positive hit: prosaptide, which promoted the endocytosis of GPR37 and GPR37L1, bound to both receptors and activated signaling in a GPR37- and GPR37L1-dependent manner. Prosaptide stimulation of cells transfected with GPR37 or GPR37L1 induced the phosphorylation of ERK in a pertussis toxin-sensitive manner, stimulated ^{35}S-GTPγS binding, and promoted the inhibition of forskolin-stimulated cAMP production. Because prosaptide is the active fragment of the secreted neuroprotective and glioprotective factor prosaposin (also known as sulfated glycoprotein-1), we purified full-length prosaposin and found that it also stimulated GPR37 and GPR37L1 signaling. Moreover, both prosaptide and prosaposin were found to protect primary astrocytes against oxidative stress, with these protective effects being attenuated by siRNA-mediated knockdown of endogenous astrocytic GPR37 or GPR37L1. These data reveal that GPR37 and GPR37L1 are receptors for the neuroprotective and glioprotective factors prosaptide and prosaposin.

deorphanization | GPCR | MAPK | neurodegeneration | peptide

Approximately half of all drugs in current clinical use act on G protein-coupled receptors (GPCRs) (1). GPCRs are outstanding drug targets for a variety of reasons, including their localization at the cell surface, their natural propensity for being activated by small molecules (neurotransmitters, hormones, sensory stimuli, etc.), and in many cases their discrete patterns of distribution in the body, which can allow for tissue-specific targeting of therapeutics. There are still more than 100 GPCRs with unknown ligands, and these so-called orphan receptors represent a large pool of potential targets for novel therapeutics (2). However, drug development efforts aimed at GPCRs are extremely difficult in the absence of a known ligand.

Two orphan GPCRs that have attracted significant interest over the past 15 years are GPR37 and GPR37L1, a pair of closely related receptors that exhibit more distant similarity to endothelin receptors and other peptide-activated GPCRs (3–9). GPR37 and GPR37L1 are found almost exclusively in the nervous system and are known to be expressed in both neurons and glia (3, 4, 6–10). GPR37 has been studied with particular intensity since it was identified as a substrate of the E3 ubiquitin ligase parkin, earning it the alternative name "parkin-associated endothelin-like receptor," or "Pael-R" (9). Although parkin has many substrates (11), GPR37 may be one of the key substrates related to pathology in patients with parkin mutations, which are associated with autosomal recessive juvenile Parkinson disease (9, 12, 13). The connection between GPR37 and parkin has led to a focus on the dopaminergic system in GPR37 knockout mice, which exhibit a reduced dopaminergic tone and various subtle perturbations in dopaminergic signaling in the brain (14–16). However, GPR37 and GPR37L1 are expressed in many different brain regions beyond just dopaminergic areas, so it seems probable that these receptors play a broader role in the regulation of neuronal and glial physiology beyond simply modulating dopaminergic neurotransmission.

Because GPR37 and GPR37L1 exhibit their strongest sequence similarity to the endothelin receptors and other GPCRs activated by peptides, it has been viewed as likely that these receptors must be peptide-activated. Indeed, it has been reported that GPR37 can bind to a neuropeptide known as "head activator" (HA) (17, 18), which is derived from the invertebrate *Hydra*. However, following a handful of reports three decades ago about the potential existence of an HA ortholog in mammalian brains (19, 20), no further evidence for an HA ortholog in vertebrates has come to light. Several groups have explored the possibility that GPR37 and/or GPR37L1 might be activated by endothelins or endothelin-related peptides, but all studies of this type have yielded negative results (4, 7). Thus, GPR37 and GPR37L1 remain orphan receptors. Here we present evidence that these receptors are activated by the neuroprotective and glioprotective factor prosaposin (also known as sulfated glycoprotein-1) as well as by prosaptide, a peptide derived from prosaposin. Both prosaposin and prosaptide have been reported in numerous studies over the past two decades to exert neuroprotective and glioprotective effects (21–42) via the stimulation of G protein-mediated pathways (25, 28, 35, 37, 43), but the receptor(s) mediating these effects have not been identified. Our data reveal that GPR37 and GPR37L1 are sufficient for evoking prosaptide/prosaposin-mediated responses in HEK-293T cells and necessary for mediating endogenous responses to prosaptide/prosaposin in primary cortical astrocytes.

Results

Prosaptide Binds to and Induces Endocytosis of GPR37 and GPR37L1. A major impediment to identifying the natural ligand for GPR37 has been the poor trafficking of the receptor to the plasma membrane upon transfection into most cell types (44). Because we demonstrated that interaction of GPR37 with the PDZ scaffold protein syntenin-1 greatly promotes trafficking of GPR37 to the plasma membrane in heterologous cells (44), we performed screens for potential GPR37 ligands using HEK-293T cells cotransfected with GPR37 and syntenin-1. In parallel studies, we also screened cells transfected with GPR37L1, which is trafficked to the plasma membrane in HEK-293T cells efficiently without syntenin-1 or other coexpressed chaperone proteins (44). The expression plasmids used in these studies confer Flag tags on the extracellular N termini of GPR37 and GPR37L1. As a screening assay, we examined internalization of the Flag tag from the cell surface in response to ligand stimulation using cell-surface luminometry. Because most GPCRs undergo significant endocytosis in response to agonist

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stimulation (45), ligand-induced endocytosis of a receptor is a strong indication that the ligand may be an agonist for the receptor.

In screens for potential ligand-induced endocytosis of GPR37 and GPR37L1, we examined five neuropeptides— secretoneurin (46), substance P 1–7 (47), cocaine- and amphetamine-regulated transcript (CART) (48, 49), prosaptide (25, 28, 29, 35, 37, 43), and L-prolyl-L-leucyl-glycinamide (PLG) (50)—which are all known to exert physiological effects via G protein-dependent pathways in cell types and/or areas of the brain that express GPR37 and GPR37L1. For four of these five peptides, we observed no effect on GPR37 or GPR37L1 surface expression following a 30-min peptide treatment (1 μM). However, treatment with one of the peptides, prosaptide, induced robust endocytosis of both GPR37 and GPR37L1 (Fig. 1 *A* and *B*). Prosaptide-induced internalization of GPR37L1 was further demonstrated in antibody feeding experiments in which surface-expressed receptors on live cells were labeled with an antibody against the N-terminal Flag tag before stimulation with prosaptide. Treatment with prosaptide (1 μM, 30 min) induced robust endocytosis of the antibody-labeled receptors (Fig. 1 *C* and *D*; internalized receptors are indicated by the green labeling). This prosaptide-induced internalization of GPR37L1 in the confocal microscopy experiments was quantified with regard to the ratio of internalized to cell surface receptor (Fig. 1*E*), thereby confirming the results of the cell surface luminometry studies using a second independent technique.

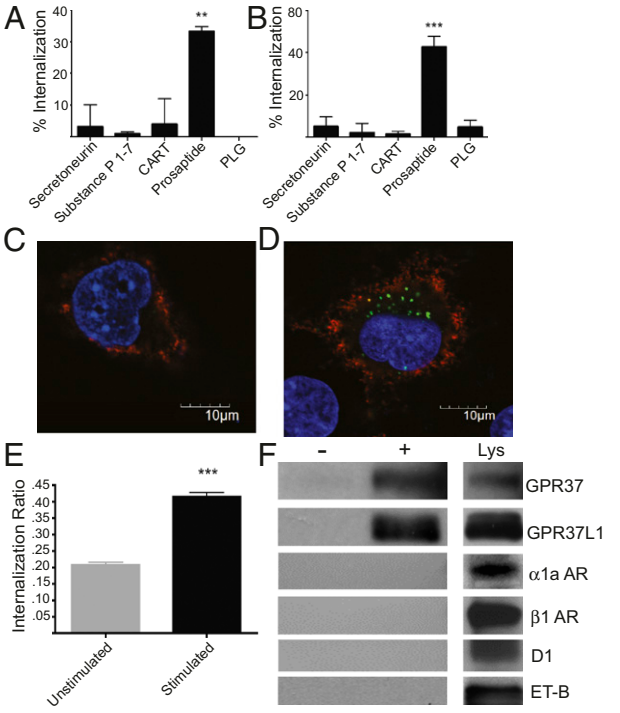

Fig. 1. Prosaptide binds to and induces endocytosis of GPR37 and GPR37L1. (*A* and *B*) Five different peptides were examined for their abilities to induce internalization of Flag-tagged GPR37 (*A*) and GPR37L1 (*B*) in HEK-293T cells. Each peptide was added to the cells at 1 μM for 30 min. Only treatment with prosaptide induced significant receptor internalization (n = 3–5 for each peptide; all points done in triplicate, $^{**}P < 0.01$, $^{***}P < 0.001$). (*C* and *D*) The ability of prosaptide to induce internalization of GPR37L1 in transfected COS-7 cells was visualized using confocal microscopy. Cell surface GPR37L1 is labeled in red, with green labeling indicating internalized GPR37L1. Representative images of GPR37L1 localization in vehicle-treated cells (*C*) and cells treated with 1 μM prosaptide (*D*) are shown. (*E*) Quantification of surface vs. internalized GPR37L1. COS-7 cells were analyzed for ratio of internalization by comparing the quantity of internalized receptor (green) to the total receptor found on the cell surface (red). In this analysis, 20 cells each from vehicle- vs. prosaptide-treated GPR37L1-transfected COS-7 cells were randomly selected in a blinded manner and analyzed to assess the extent of GPR37L1 internalization ($^{***}P < 0.001$). (*F*) The ability of prosaptide to bind to different GPCRs was assessed using biotinylated prosaptide attached to streptavidin beads (+) or streptavidin beads alone (–). Flag-tagged GPR37 and GPR37L1, but not other Flag-tagged (α1A- and β1-adrenergic) or HA-tagged receptors (D1 dopamine and endothelin ET_B), were pulled down with the biotinylated prosaptide adsorbed to the beads, indicating specific binding of the peptide to GPR37 and GPR37L1.

Following our observations of prosaptide-induced internalization of GPR37 and GPR37L1, we sought to determine whether prosaptide could bind to these receptors. Studies with the endothelin receptors, the most closely related receptors to GPR37/GPR37L1, have demonstrated that solubilized endothelin receptors can be efficiently pulled down by biotinylated endothelins coupled to streptavidin (51, 52). Thus, we obtained a biotinylated version of prosaptide and pursued a similar approach for studying prosaptide binding to GPR37 and GPR37L1. As shown in Fig. 1*F*, solubilized GPR37 and GPR37L1 were robustly pulled down by biotinylated prosaptide but not by control streptavidin beads. In contrast, a number of other Flag- or HA-tagged transfected GPCRs (α_{1A}-adrenergic, β_1-adrenergic, D1 dopamine, and endothelin ET_B receptors) were not detectably pulled down by biotinylated prosaptide.

Prosaptide Stimulates G Protein-Mediated Signaling via GPR37 and GPR37L1. Given that the above-described experiments revealed prosaptide could bind to GPR37 and GPR37L1 and induce the internalization of these receptors, we next turned our attention to examining whether prosaptide could activate GPR37 and GPR37L1 to induce intracellular signaling. The most frequently reported signaling pathway activated by prosaptide is the stimulation of ERK phosphorylation (26, 28, 53–55). Thus, we examined the ability of prosaptide to induce ERK phosphorylation in HEK-293T cells transfected with either GPR37/syntenin-1 or GPR37L1. Transfection of the receptors into HEK-293T cells had no significant effect on basal levels of phospho-ERK or total ERK (Fig. S1), but treatment with 100 nM prosaptide induced a significant increase in pERK for both GPR37- and GPR37L1-transfected cells, with no effect observed on mock-transfected cells (Fig. 2 *A* and *B*). Dose–response studies revealed that prosaptide activates GPR37 with an apparent EC_{50} of 7 nM (Fig. 2*C*) and GPR37L1 with an EC_{50} of 5 nM (Fig. 2*D*). Because prosaptide has been reported to activate a pertussis toxin (PTX)-sensitive $G\alpha_{i/o}$-coupled receptor (25, 28, 29, 35, 37, 43), we examined the effects of PTX pretreatment and found that it completely inhibited the ability of prosaptide to stimulate ERK phosphorylation in GPR37- or GPR37L1-transfected cells: pERK induced by prosaptide in the presence of PTX was significantly lower than prosaptide stimulated pERK without PTX (Figs. S2 and S3). These data indicate a $G\alpha_{i/o}$-dependent mechanism of ERK activation for these receptors. We also found that prosaptide significantly inhibited forskolin-stimulated cAMP production in cells transfected with GPR37/syntenin-1 or GPR37L1 (Fig. 2*E*) and enhanced ^{35}S-GTPγS binding to membranes derived from cells transfected with GPR37/syntenin-1/$G\alpha_{i1}$ or GPR37L1/$G\alpha_{i1}$ (Fig. 2*F*), consistent with the idea that prosaptide stimulation promotes coupling of $G\alpha_{i/o}$ to GPR37 and GPR37L1.

Prosaptide Actions on Primary Astrocytes Are Mediated by GPR37 and GPR37L1. All of the experiments described above were performed using cells transfected with GPR37 and GPR37L1 to provide evidence that transfection with these receptors is sufficient to confer physiological responses to prosaptide. To address the issue of whether GPR37 and/or GPR37L1 is necessary for endogenous prosaptide-induced signaling, we turned to primary cultures of cortical astrocytes, a cell type known to express mRNA for both GPR37 and GPR37L1 (10, 56). We confirmed that primary cortical astrocytes express GPR37 and GPR37L1 at the protein level via Western blotting using antibodies specific to each receptor (Fig. 3*A*) using antibodies that were determined in studies on transfected cells to be specific for each receptor (Figs. S4 and S5).

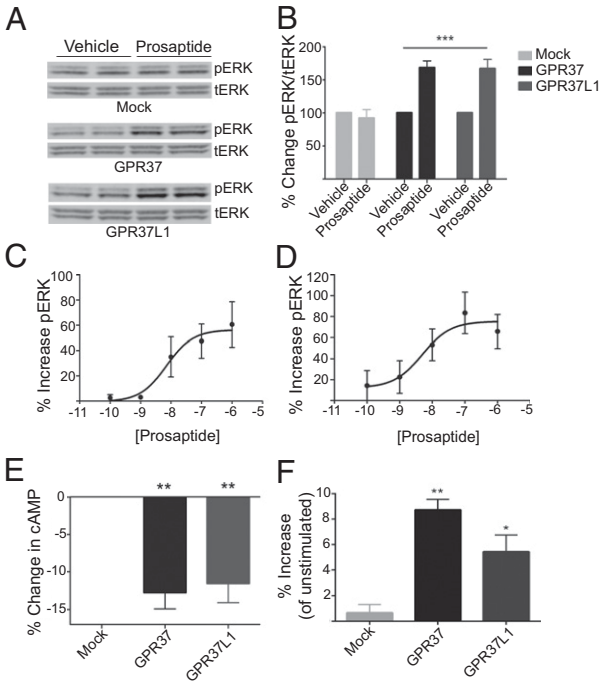

Fig. 2. Prosaptide stimulates G protein-mediated signaling via GPR37 and GPR37L1. (*A*) HEK-293T cells transiently transfected with either empty vector (Mock), GPR37/syntenin-1 or GPR37L1 were treated for 10 min with 100 nM prosaptide and ERK phosphorylation was assessed. pERK, phosphorylated ERK; tERK, total ERK. (*B*) Quantification of changes in pERK levels in response to prosaptide treatment. All experiments determined prosaptide-induced ERK phosphorylation over vehicle treatment ($n = 3$; all points done in duplicate, $^{***}P < 0.001$). (*C* and *D*) Dose–response curves for prosaptide stimulation of HEK-293T cells transfected with either GPR37/syntenin-1 (*C*) or GPR37L1 (*D*) ($n = 6$). (*E*) Effects of prosaptide treatment (30 min, 100 nM) on cAMP levels in HEK-293T cells transfected with empty vector (Mock), GPR37/syntenin-1, or GPR37L1. All experiments determined prosaptide-induced cAMP inhibition compared with vehicle treatment ($n = 5$; all points done in triplicate, $^{**}P < 0.01$). (*F*) Effects of prosaptide treatment on ^{35}S-GTPγS binding to membranes derived from HEK-293T cells transfected with $G\alpha_{i1}$ alone (Mock), GPR37/syntenin-1/$G\alpha_{i1}$, or GPR37L1/$G\alpha_{i1}$. All experiments determined prosaptide-induced ^{35}S-GTPγS binding over vehicle treatment ($n = 3$; all points done in duplicate, $^{**}P < 0.01$, $^{*}P < 0.05$). Total counts for vehicle-treated samples averaged ~6,000 cpm and did not vary significantly between transfection conditions.

These studies revealed that GPR37 is more abundantly expressed in cortical astrocytes relative to whole brain (per unit protein), whereas GPR37L1 is more enriched in whole brain relative to primary cortical astrocytes.

Treatment with prosaptide (100 nM) induced robust ERK phosphorylation in the primary cortical astrocyte cultures (Fig. 3 *B* and *C*). To determine whether this effect was mediated by GPR37 and/or GPR37L1, we knocked down expression of each receptor using a siRNA approach (Fig. S6). Treatment of the cells with either scrambled siRNA or siRNA knocking down GPR37L1 had no significant effect on prosaptide-mediated pERK stimulation. Conversely, knockdown of GPR37 or the joint knockdown of GPR37 and GPR37L1 together completely abolished the ability of prosaptide to induce ERK phosphorylation (Fig. 3 *B* and *C*). It is important to point out that G protein-mediated regulation of the ERK pathway was still intact following siRNA-mediated knockdown of GPR37 and GPR37L1, as assessed by the ability of endogenous lysophosphatidic acid receptors in the astrocytes to stimulate increases in pERK (Fig. S7), thereby suggesting that the siRNA treatments did not induce any nonspecific impairments in the ERK pathway due to off-target effects.

Because prosaptide has been reported to act as a neuroprotective and glioprotective factor (21, 23–40, 42, 57), we also studied the ability of prosaptide to protect primary astrocytes against oxidative stress induced by hydrogen peroxide (H_2O_2). Cortical astrocytes were pretreated with either vehicle or 100 nM prosaptide for 10 min then treated with 500 μM H_2O_2 for 24 h and assessed for the release of lactate dehydrogenase into the media as an indicator of cell death. Prosaptide treatment significantly protected the cortical astrocytes from H_2O_2-induced death in cultures treated with either no siRNA or scrambled siRNA. However, in astrocytes in which either GPR37, GPR37L1, or both receptors had been knocked down, the ability of prosaptide to promote cell survival was significantly attenuated (Fig. 3*D*).

Full-Length Prosaposin Protects Primary Astrocytes via Activation of GPR37 and GPR37L1. Prosaptide is a peptide fragment that mimics the trophic and protective actions of full-length prosaposin (23, 24, 26, 28, 32, 35, 36, 38, 53, 54), but it is widely presumed that full-length prosaposin is the endogenous ligand for any receptor that can be activated by prosaptide. Thus, we purified full-length prosaposin using a slightly modified version of a previously described protocol (58) and assessed whether full-length prosaposin could activate GPR37 and GPR37L1. Cells transfected with empty vector, GPR37/syntenin-1, or GPR37L1 were incubated with purified prosaposin (100 nM). Treatment with prosaposin induced ERK phosphorylation in the cells expressing GPR37/syntenin-1 or GPR37L1, but not in mock transfected cells (Fig. 4*A*), similar to the results obtained earlier in the prosaptide stimulation experiments. Full-length prosaposin also induced internalization of GPR37 and GPR37L1 (Fig. 4*B*), again mirroring our earlier findings with prosaptide.

We next explored the effects of prosaposin on ERK phosphorylation and cell survival in cortical astrocytes. Prosaposin treatment (100 nM) robustly stimulated ERK phosphorylation in primary astrocyte cultures, and this response was not affected by pretreatment of the astrocytes with either scrambled siRNA or siRNA directed against GPR37L1 (Fig. 4 *C* and *D*). However, siRNA knockdown of GPR37, as well as joint knockdown of both GPR37

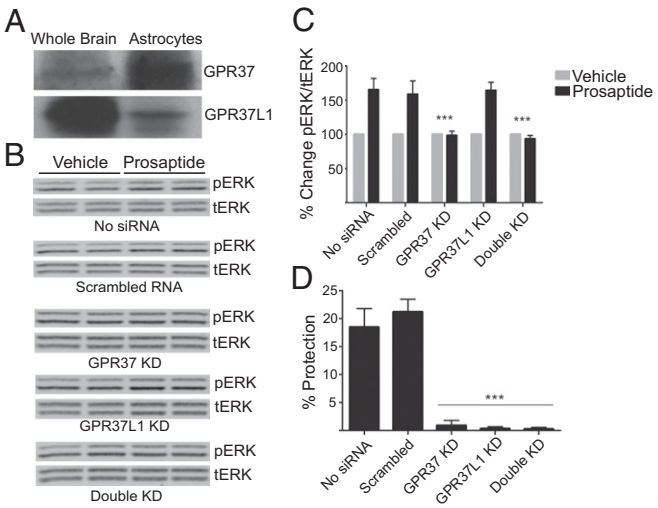

Fig. 3. Prosaptide actions on primary astrocytes are mediated by GPR37 and GPR37L1. (*A*) The relative expression levels of GPR37 and GPR37L1 in primary cortical astrocyte lysates vs. whole-brain lysates were assessed via Western blot. (*B*) Prosaptide-induced ERK phosphorylation in primary cortical astrocytes was assessed under several conditions: no siRNA treatment, treatment with a scrambled siRNA, or treatment with siRNAs directed against GPR37 and/or GPR37L1. KD, knockdown. (*C*) Quantification of the studies illustrated in *B* ($n = 3$; all points done in duplicate, $^{***}P < 0.001$). (*D*) Prosaptide-mediated protection of cortical astrocytes from a hydrogen peroxide insult was assessed by measuring lactate dehydrogenase release. Labels are the same as in *C* ($n = 3$; all points done in triplicate, $^{***}P < 0.001$).

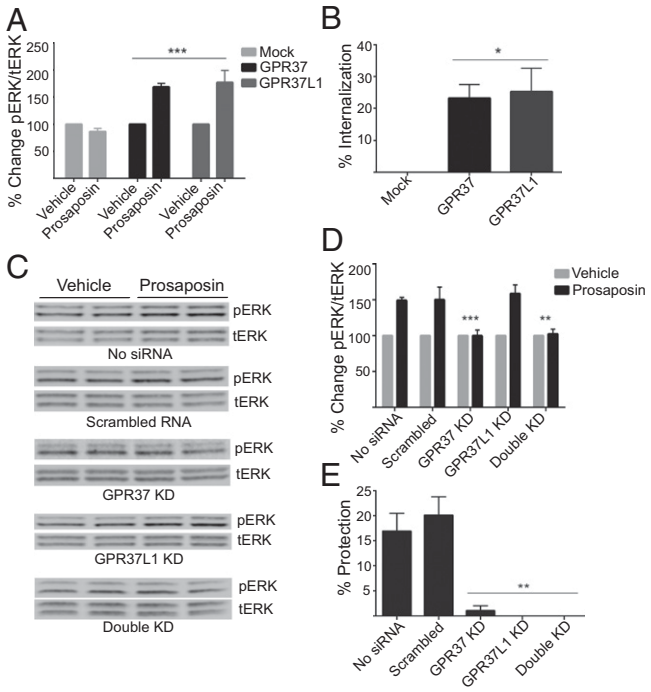

Fig. 4. Full-length prosaposin protects primary astrocytes via activation of GPR37 and GPR37L1. (*A*) Treatment of HEK-293T cells transiently transfected with empty vector (Mock), GPR37/syntenin-1, or GPR37L1 with prosaposin (100 nM, 10 min) resulted in enhanced ERK phosphorylation in only GPR37- or GPR37L1-transfected cells ($n = 3$; all points done in duplicate, $^{***}P < 0.001$). (*B*) Endocytosis of Flag-GPR37 and Flag-GPR37L1 was assessed following treatment with 100 nM prosaposin for 30 min ($n = 3$; all points done in triplicate, $^{*}P < 0.05$). (*C*) Primary cortical astrocytes were treated with either no siRNA, a scrambled siRNA, or siRNAs directed against GPR37 and/or GPR37L1. These astrocytes were then stimulated with prosaposin (100 nM, 10 min) and activation of ERK was assessed ($n = 3$; all points done in duplicate, $^{***}P < 0.001$). (*D*) Quantification of the experiments illustrated in *C*. (*E*) Primary cortical astrocytes were exposed to H_2O_2 and cell death was assessed via measuring the release of lactate dehydrogenase into the media. Prosaposin pretreatment (100 nM) protected the astrocytes from the oxidative stress, but the effects of prosaposin were attenuated when GPR37 and/or GPR37L1 was knocked down via siRNA ($n = 3$; all points done in triplicate, $^{**}P < 0.01$).

and GPR37L1, completely abolished prosaposin-induced ERK phosphorylation. Finally, pretreatment with prosaposin protected cortical astrocytes from H_2O_2-induced cell death, with knockdown of GPR37, GPR37L1, or GPR37 and GPR37L1 together substantially attenuating the protective effects of the full-length prosaposin (Fig. 4*E*), similar to findings made earlier regarding the effects of prosaptide on astrocyte survival.

Discussion

The studies presented here have identified prosaposin and prosaptide, a peptide derived from prosaposin, as ligands for the orphan receptors GPR37 and GPR37L1. These ligands bind to GPR37 and GPR37L1, induce receptor internalization, and stimulate GPR37- and GPR37L1-mediated signaling through PTX-sensitive G proteins. For nearly two decades, it has been known that prosaposin and prosaptide can activate G protein pathways, but the putative receptor(s) mediating these effects have eluded identification. We propose that GPR37 and GPR37L1 mediate at least some and potentially all of these G protein-dependent actions of prosaposin and prosaptide, given the striking similarities between the previously defined properties of the putative prosaposin receptors and the findings reported here for GPR37 and GPR37L1. These properties include binding to prosaposin and prosaptide with nanomolar affinity (21, 25, 26, 53), coupling to PTX-sensitive G proteins (25, 28, 29, 35, 37, 43, 55), stimulation of ERK phosphorylation as a downstream readout (26, 28, 53–55), and protection against cellular stress (27, 30, 31, 38, 41, 42, 55). As demonstrated by our data, all of these criteria are met by GPR37 and GPR37L1. Moreover, the distribution patterns of GPR37 and GPR37L1 correspond exceptionally well to the expected distribution patterns of the putative prosaposin receptors. In addition to the effects of prosaposin and prosaptide on astrocytes demonstrated in this paper, prosaptide and prosaposin have been shown to exert effects on Schwann cells (26, 28, 59), oligodendrocytes (26), and certain populations of neurons (30, 38, 41, 60), cell types that are known to express significant levels of GPR37 and GPR37L1 (10, 56). Furthermore, it is interesting to note that prosaptide and the invertebrate peptide HA, which has previously been reported to activate GPR37 (17, 18), are similar in length and exhibit significant sequence similarity in their C-terminal regions (K-V-I-L for HA vs. K-E-I-L for prosaptide). HA does not seem to be a true ortholog of prosaptide, but nonetheless we propose that this invertebrate peptide may possess the ability to act as a GPR37 agonist owing to its similarity to prosaptide and prosaposin.

GPR37 and GPR37L1 were found in our studies to be both necessary and sufficient for mediating G protein-dependent signaling by prosaptide and prosaposin in the cell types that were examined. HEK-293T cells, for example, do not show any endogenous responses to prosaptide or prosaposin, and these ligands were found to only be capable of stimulating G protein-mediated signaling in HEK-293T cells when the cells were transfected with either GPR37 or GPR37L1. These experiments reveal that either GPR37 or GPR37L1 is sufficient to confer G protein-mediated signaling by prosaptide and prosaposin to HEK-293T cells. Furthermore, primary astrocytes exhibit robust endogenous increases in phospho-ERK when treated with prosaptide or prosaposin, and these effects are dramatically attenuated by siRNA-mediated knockdown of GPR37. These experiments reveal that, at least in primary astrocytes, GPR37 is necessary for stimulation of ERK by prosaptide and prosaposin. With regard to the abilities of prosaptide and prosaposin to protect primary astrocytes from H_2O_2-induced cell death, it is interesting to note that knockdown of either GPR37 or GPR37L1 attenuated the protective actions of prosaptide and prosaposin. This difference in findings between the ERK phosphorylation experiments (where only GPR37 was necessary) and the oxidative stress protection studies (where both GPR37 and GPR37L1 were necessary) suggests that GPR37 and GPR37L1 do not couple to exactly the same set of intracellular signaling pathways and that the protective actions of prosaptide and prosaposin via GPR37/GPR37L1 are dependent on more than just downstream activation of ERK. Perhaps future studies will illuminate the receptor-specific differences between GPR37 and GPR37L1, providing insights into why there seem to be at least two distinct receptors for prosaposin in the CNS.

Prosaposin is an interesting multifunctional protein (61). It can be targeted to lysosomes and cleaved into saposins A–D, which play specific roles in the degradation of sphingolipids (61, 62). Alternatively, prosaposin can be released from cells as an intact protein, which acts as a neuroprotective and glioprotective factor (21, 41, 42, 63). The region of prosaposin required for activating G protein-mediated signaling has been narrowed down to a 14-amino acid sequence in the saposin C region of prosaposin, and this peptide fragment has come to be known as prosaptide (23). Interestingly, knockout mice lacking either full-length prosaposin or the saposin C region exhibit striking patterns of neurodegeneration and loss of motor function, although it is not yet clear which aspects of these phenotypes are due to loss of the lysosomal function of the cleaved saposins and which are due to loss of the neuroprotective and glioprotective actions of secreted full-length prosaposin (64–68). Loss of prosaposin in humans also results in a dramatic phenotype, including profound neurodegeneration in the cerebral cortex and other brain regions (69, 70), indicating a prominent role for prosaposin in promoting neuronal survival in humans.

Our proposal that GPR37 and GPR37L1 represent receptors for the neuroprotective and glioprotective actions of prosaposin might seem upon first consideration to be at odds with much of the literature on GPR37, which has focused on the propensity of overexpressed GPR37 to induce neurodegeneration (9, 12, 71–74). However, it is important to appreciate that published studies linking GPR37 to neurodegeneration have been performed in cells where parkin was knocked out (71, 72) or where GPR37 was highly overexpressed to artificially overwhelm cellular trafficking machinery (9, 12, 73, 74). Parkin is an E3 ubiquitin ligase necessary for targeting misfolded substrates for degradation (11). If parkin is inactivated and its misfolded substrates are allowed to build up and aggregate, this can induce endoplasmic reticulum stress and ultimately cell death (11). Thus, although overexpression of GPR37 can be deleterious in certain cell types if parkin function is lost owing to mutation or knockdown, we propose that GPR37 and GPR37L1 play predominantly protective roles in neurons and glia under normal conditions where parkin is expressed at physiological levels, because our findings provide evidence that GPR37 and GPR37L1 mediate protective actions of secreted prosaposin. Consistent with this idea, GPR37 was recently shown to exert striking protective actions in neuron-like N2a cells, an observation that fits well with our proposal that GPR37 exerts mainly protective effects in cells where the receptor is properly folded and trafficked (75).

Future work may determine the precise roles that GPR37 and/or GPR37L1 play in mediating the various in vivo physiological effects that have been reported for prosaposin and prosaptide. In pursuing such in vivo studies, it is useful to consider a model of the potential physiological significance of this signaling system. It has been demonstrated that the expression of prosaposin is strikingly up-regulated following nerve injury (76). Conceivably, activation of GPR37 and/or GPR37L1 by up-regulated prosaposin following injury could prompt neurons and glia to initiate intracellular signaling pathways that promote survival in the face of the cellular stress associated with injury and inflammation. If future studies support this model, then GPR37 and GPR37L1 may eventually serve as outstanding targets for novel therapeutics aimed at enhancing neuronal and glial survival in response to injury. Prosaposin and prosaptide have been shown to protect dopaminergic neurons in animal models of Parkinson disease (38, 41), facilitate neuronal survival following focal cerebral ischemia (22, 32), reduce neuropathic pain (37, 40, 57), and promote remyelination of nerves following injury (26, 28, 59). Thus, if future work in vivo upholds the idea that the neuroprotective and glioprotective actions of prosaposin and prosaptide are mediated by GPR37 and GPR37L1, the development of small molecule ligands for these receptors may provide new therapeutic possibilities for the treatment of Parkinson disease, stroke, neuropathic pain, and myelination disorders.

Materials and Methods

Detailed materials and methods are given in *SI Materials and Methods*.

Cell Culture. Cells were cultured as described previously (44). Cortical astrocytes were derived from mice 1–2 d old and grown for 7–10 d in culture.

Plasmids, Antibodies, and Peptides. *SI Materials and Methods* gives the origins of antibodies, constructs, and peptides.

siRNA Knockdown. The siRNAs directed against GPR37 and GPR37L1 were incorporated into primary cortical astrocytes. Cells were harvested 72 h following nucleofection.

Prosaposin Production. Prosaposin was expressed and purified using a slightly modified version of a previously published protocol (58).

Western Blotting. Samples were processed and run on gels as previously described (44).

ERK Phosphorylation Assay. Cells were serum-starved for 2–3 h before assay stimulation. Cells were incubated with ligand for 10 min in DMEM at 37 °C.

Cell Surface Luminometry. Assays of receptor surface expression were performed as previously described (44).

cAMP Assay. Transfected HEK-293T cells treated with forskolin and/or prosaptide. Samples were analyzed using a colorimetric cAMP ELISA kit (Cell Biolabs).

Biotin Binding and Pulldown. Biotinylated prosaptide TX14(A) was attached to Neutravadin beads. Soluble lysates were incubated with beads and the resulting samples were examined via Western blot for pulldown of solubilized receptors.

^{35}S-GTPγS Binding. Membranes derived from transfected HEK-293T cells were incubated with ^{35}S-GTPγS and binding was assessed using a scintillation counter.

Microscopy. Transfected COS-7 cells were incubated with rabbit anti-FLAG antibodies, then treated and fixed. Surface-expressed receptors were labeled red and internalized receptors were labeled green.

Cytotoxicity. Forty-eight hours following nucleofection, cells were treated with 500 μM H_2O_2 in DMEM with or without drug for 24 h. Media was harvested and analyzed via a cytotoxicity kit (Promega).

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