Minireview: Recent Developments in the Physiology and Pathology of the Lysophosphatidylinositol-Sensitive Receptor GPR55

Christopher M. Henstridge, Nariman A. B. Balenga, Julia Kargl, Clara Andradas, Andrew J. Brown, Andrew Irving, Cristina Sanchez, and Maria Waldhoer

Institute of Experimental Medicine (C.M.H.), Hungarian Academy of Sciences, Budapest, Hungary HU1083; Institute of Experimental and Clinical Pharmacology (N.A.B.B., J.K., M.W.), Medical University of Graz, Graz A-8036, Austria; Department of Biochemistry and Molecular Biology I (C.A., C.S.), School of Biology, Complutense University, 28040 Madrid, Spain; Division of Screening and Compound Profiling (A.J.B.), Medicines Research Centre, GlaxoSmithKline, Stevenage SG1 2NY, United Kingdom; and Division of Neuroscience (A.I.), Medical Research Institute, University of Dundee, Dundee DD1 9SY, United Kingdom

Emerging data suggest that off-target cannabinoid effects may be mediated via novel seven-transmembrane spanning/G protein-coupled receptors. Due to its cannabinoid sensitivity, the G protein-coupled receptor 55 (GPR55) was recently proposed as a candidate; however, GPR55 is phylogenetically distinct from the traditional cannabinoid receptors, and the conflicting pharmacology, signaling, and functional data have prevented its classification as a novel cannabinoid receptor. Indeed, the most consistent and potent agonist to date is the noncannabinoid lysophospholipid, lysophosphatidylinositol. Here we present new human GPR55 mRNA expression data, providing supportive evidence of GPR55 expression in a vast array of tissues and cell types. Moreover, we summarize major recent developments in GPR55 research and aim to update the reader in the rapidly expanding fields of GPR55 pharmacology, physiology, and pathology. (*Molecular Endocrinology* 25: 1835–1848, 2011)

Classically, cannabinoid ligands interact with two seven-transmembrane spanning (7TM)/G protein-coupled receptors (GPCR); cannabinoid receptor type 1 and type 2 (CB_1 and CB_2); however, pharmacological data and studies exploiting knockout animals suggest that additional cannabinoid-sensitive targets exist (1, 2). Given the prominent role of the endocannabinoid system in normal and pathological conditions, there has been considerable interest in the identification of these novel targets, both within academia and the pharmaceutical industry. Indeed, it was AstraZeneca and GlaxoSmithKline (3) that first thrust G protein-coupled receptor-55 (GPR 55) into the limelight as a novel candidate. However, academic laboratories soon contributed, with the revelation that GPR55 is activated by a novel, endogenous lipid known as lysophosphatidylinositol (LPI) (4). These studies effectively assimilated the fields of cannabinoid, GPR55, and LPI research; however, they quickly conflicted, resulting in a contradictory cannabinoid pharmacology, which has been reviewed extensively elsewhere (5–10).

Since the seminal report by Oka *et al.* (4), LPI has been confirmed with ever-increasing consistency as a potent and direct agonist of GPR55. LPI was first identified in the early 1960s (11, 12), but it was not until 20 yr later that a potential signaling role was suggested, when it was shown to stimulate the release of insulin from pancreatic

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Abbreviations: abn-CBD, Abnormal-cannabidiol; 2-AG, 2-arachidonoyl glycerol; ARA-S, *N*-arachidonoyl serine; CNS, central nervous system; FCA, Freund's complete adjuvant; GASP-1, GPCR-associated sorting protein-1; GPCR, G protein-coupled receptor; GPR55, G protein-coupled receptor-55; HEK, human embryonic kidney; hGPR55, human GPR55; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; MMA, mouse mesenteric artery; PEA, palmitoylethanolamide; PLC, phospholipase C; PTX, pertussis toxin; siRNA, small interfering RNA; S1P, sphingosine-1-phosphate; TM, transmembrane; 7TM, seven-TM spanning.

cells (13). In the 1980s and early 1990s, a number of papers detailing the physiology and production of LPI in various cellular systems, including cancer cell lines, were published (13–19). Interestingly, subsequent studies reinforced the idea that LPI can be released from cells, and one group proposed that blood LPI levels might be a novel biomarker for certain cancers and gynecological diseases, with LPI levels 3-fold higher in patients than controls (20–22). LPI was shown to be neuroprotective in a model of global cerebral ischemia and glutamate excitotoxicity in neuronal cultures (23). When injected 30 min before or after the onset of ischemia, LPI induced a prolonged brain tolerance to ischemic damage and pretreatment with LPI 1–3 d before ischemic challenge resulted in a 98% survival rate of CA1 pyramidal cells (23). The authors suggested the two-pore domain K+ channels TREK and TRAAK (background K^+ channels) may explain the effect because they were previously shown to be sensitive to certain lysophospholipids, including LPI (24), although no direct evidence was presented to confirm this. Finally in 2007, the study by Oka *et al.* (4) revealed the first elusive receptor target for LPI to be GPR55. LPI-dependent GPR55 signaling is now thought to be important in a vast array of physiological and pathological processes including cancer progression, bone regulation, endothelial function, inflammation, and pain (Fig. 1). Therefore, it seems GPR55 should be described as the first recognized LPI receptor, although it undoubtedly retains some cannabinoid ligand sensitivity.

Functional and pharmacological GPR55 data are emerging at an ever-increasing pace, and after some major recent developments, this review aims to summate these findings, bring the reader up to date with the rapidly expanding field of GPR55 physiology, and introduce potential therapeutic strategies exploiting current knowledge of GPR55 pathophysiology.

GPR55 structure

Human GPR55 (hGPR55; NM_005683.3) is a 319-amino acid protein and a member of the rhodopsin-like 7TM/GPCR family. hGPR55 was first isolated and cloned in 1999 and mapped to human chromosome 2q37 (25).

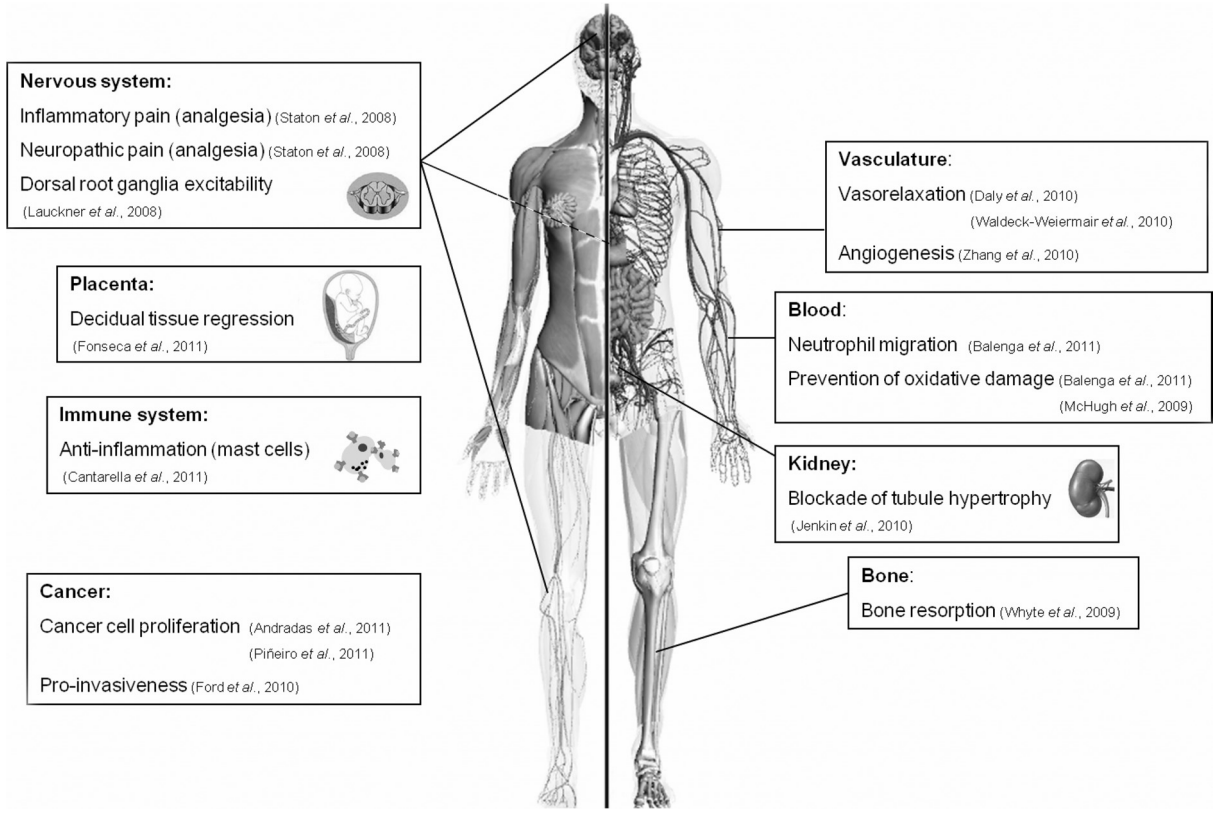

FIG. 1. Pathophysiological relevance of GPR55 expression. Recent evidence suggests that GPR55 is involved in the control of a variety of physiological functions. In the nervous system, GPR55 regulates dorsal root ganglia excitability (49) and controls inflammatory and neuropathic pain (63). In blood, GPR55 regulates neutrophil migration (48) and may prevent oxidative damage (48, 84). GPR55 is also involved in bone metabolism, specifically inducing bone resorption (87). Other studies have suggested additional roles for GPR55 in modulating vascular function [by inducing vasorelaxation (53, 76) and controlling angiogenesis (37), renal tubule hypertrophy (109), decidual tissue regression during pregnancy (105), and mast cell-mediated antiinflammatory actions (104)]. However, additional experimental evidence is required to support these hypotheses. Besides controlling these (and most probably many other) physiological functions, GPR55 seems to play an important role in cancer progression by modulating cancer cell proliferation (52, 102) and migration (43).

Different sequences have been reported for hGPR55, most likely due to polymorphisms or technical errors during cloning or sequencing. So far, orthologs of GPR55 have been identified in the genomes of rat, mouse, dog, cow, chimpanzee, zebrafish, pufferfish, and humans (3, 26, 27). The closest homologs to GPR55, based on its amino acid sequence, are the purinergic receptor P2Y5 (29%, NM_005767.4), the purinoceptor-like orphan receptors GPR23 (30%, NM_005296.2), and GPR35 (27% NM_005301.2) and the chemokine receptor CCR4 (23%, NM_005508.4) (25). Interestingly GPR55 shows low sequence identity with the two traditional cannabinoid receptors CB_1 (13.5%) and CB_2 (14.4%).

Sequence alignment analysis with rhodopsin shows conserved patterns in transmembrane (TM)-1, 2, 4, and 5, but instead of the highly conserved DRY motif (28) in TM3, GPR55 exhibits a DRF motif. GPR55 contains a conserved proline in TM5, which is absent in CB_1 and CB_2. Furthermore, the highly conserved NPXXY motif found in TM7 of rhodopsin, CB_1 and CB_2 sequences, is altered in the GPR55 structure (10, 29). GPR55 comprises several protein kinase A and protein kinase C phosphorylation sites as well as conserved patterns for glycosylation, and conserved cysteines are located in extracellular loops 1 and 2 (25).

Interestingly, although several cannabinoid ligands can activate GPR55, it lacks the classical cannabinoid binding pocket (30). Using sequence to structure alignments with the well-known β_2-adrenergic receptor (PDB: 2RH1) structure (31), a homology model of GPR55 in both its active and inactive state was constructed (29). These models were used to examine the ligand binding pocket of GPR55 and identify important amino acid interactions during ligand binding. The active conformation model of GPR55 was built in a hydrated lipid bilayer, which resulted in a deep, vertical, and highly hydrated binding pocket. This ligand binding region consists of many hydrophilic residues, which is in contrast to the highly hydrophobic CB_1 and CB_2 receptor binding pockets.

Microsecond time-scale molecular dynamic simulations and isothiocyanate covalent labeling studies suggest an entry pathway for lipid ligands between TM6 and TM7 for CB_1 and CB_2 (32–34). The third extracellular loop of GPR55 connects TM6 and TM7 and is significantly longer than the third extracellular loops of the CB_1 and CB_2 receptors. Furthermore, it consists of many charged amino acids, indicating that the loop will be mainly solvated in water thus enabling ligands with large head groups, such as LPI to enter between TM6 and TM7 (35).

Taken together, the data suggest that GPR55 is quite distinct from the traditional cannabinoid receptors and is well primed for receiving the LPI family of lipids as ligands due to its deep elongated binding pocket and three-dimensional protein conformation.

GPR55 pharmacology

The multifarious pharmacology of GPR55 has generated fierce debate over its classification as a novel cannabinoid receptor (36). One argument in support is that GPR55 exhibits sensitivity to distinct cannabinoid ligands in a multitude of cell lines and assays [reviewed extensively elsewhere (5–10)]. However, there is significant inconsistency between studies using cannabinoid ligands, which are variously reported as agonists or antagonists or to have no effect. In contrast, LPI has been confirmed as an agonist in all studies tested. Furthermore, emerging data suggest that other endogenous lipid ligands may possibly interact with GPR55. For example, *N*-arachidonoyl serine (ARA-S) induces a variety of effects in the Human Dermal Microvascular Endothelial Cell line (HMVEC), including modulation of proliferation and migration, which are inhibited upon silencing of GPR55 (37). However, ARA-S exhibited no functional activity in another GPR55 assay (121); thus, its status as a true endogenous GPR55 ligand is debatable.

GPR55 pharmacological studies have proven problematic to date due to lack of ligand specificity, with effects on other targets such as cannabinoid receptors, transient receptor potential vanilloid channels, and peroxisome proliferator-activated receptors. However, recent studies have provided new exciting tools for future GPR55 research. A study by Brown *et al.* (38) reported a number of synthetic compounds that appear highly selective for GPR55. These structurally related benzoylpiperazine compounds are inactive at CB_1 and CB_2 and were initially produced as inhibitors of the glycine transporter subtype 1; however, the most GPR55-specific ligand (GSK57-5594A) is greater than 60-fold selective for GPR55 than glycine transporter subtype 1. Intriguingly, the authors report that LPI is equipotent at human and rodent GPR55, although the new benzoylpiperazine compounds specifically activated human receptors, suggesting that these ligands may have distinct binding domains, which are divergent between species.

Kotsikorou *et al.* (29) have also recently described novel GPR55-specific compounds and have used these to map the putative binding site within GPR55. Importantly, some compounds in this study share a similar structure to those reported by Brown *et al.* (38), providing welcome consistency. LPI adopts an inverted L shape and interacts with the receptor by inserting its fatty acid tail deep within TM helices 2, 3, 5, 6, and 7 [in line with previous reports, suggesting LPI exhibits an extended acyl

chain conformation (35)] and crucially binds K2.60 at the extracellular side of TM2 via its electronegative head group (29). This residue on TM2 is critical for GPR55 activation as all ligands modeled in the study appear to interact here, and furthermore, unpublished mutagenesis data by the group reinforce this significance (29). Although the compounds described in both studies are not identical, Kotsikorou *et al.* (29) have shown that they can adopt similar three-dimensional conformations and interact with the residues critical for ligand-induced activation. Furthermore, their data provide strong evidence that the cannabinoid antagonist AM251, is a *bone fide* GPR55 agonist as previously reported (26, 39–42), which can adopt a similar conformation and interact with the critical residues required for activation.

These new tools and modeling data will ultimately aid future targeted drug design and produce more specific compounds, allowing greater exploration of the pharmacology and physiology of GPR55.

Trafficking and intracellular sorting of GPR55

To date, most GPR55 trafficking studies have been performed in recombinant cell systems, such as human embryonic kidney (HEK)-293, U2OS, and MCF-7 cells, transiently or stably expressing hGPR55. Before agonist treatment the receptor is predominantly found at the cell surface but markedly internalizes following agonist challenge (39–41, 43). The intracellular sorting of internalized 7TM/GPCR is a highly regulated phenomena, typically resulting in recycling of the receptor back to the cell surface or enzymatic degradation and removal. Kargl *et al.* (44) have recently shown that GPR55 is significantly down-regulated via targeted degradation after prolonged agonist stimulation in a process involving the GPCR-associated sorting protein-1 (GASP-1). Furthermore, they demonstrated that in the absence of GASP-1, GPR55 recycles back to the cell surface, largely avoiding intracellular degradation (44). These data suggest GASP-1 dynamically regulates cell surface GPR55, thus playing an important role in GPR55 availability and subsequent cellular physiology (44).

GPR55 signaling

In accordance with the diverse and complex pharmacology of GPR55, the current literature regarding the downstream signaling of the receptor is equally disparate. A vast array of cell lines and assays has been used, potentially explaining some of the controversies. The complex nature of current signaling data may be due to several factors, including endogenous, transient, or stable receptor expression; variation in cellular background; ligand biased signaling or the interaction of endogenous proteins modulating GPR55 function.

In the early study by Ryberg *et al.* (26), GTPγS binding and FLIPR Ca^{2+} assays were exploited to unravel the downstream signaling of GPR55. Preincubation of membranes with blocking peptide (the last 12 amino acids) or antibody against the C terminus of $G\alpha_{13}$ inhibited GTPγS binding in a dose-dependent manner, but those against $G\alpha_{i1/2}$, $G\alpha_{i3}$, and $G\alpha_s$ had no effect. The involvement of $G\alpha_q$ was ruled out because none of the ligands induced Ca^{2+} release in the FLIPR assay. Henstridge *et al.* (39) completed a comprehensive study in HEK-hGPR55 cells, which reinforced the role of $G\alpha_{13}$ in GPR55-mediated Ca^{2+} release from endoplasmic reticulum stores. The single-cell Ca^{2+} imaging approach revealed an oscillatory Ca^{2+} response, a novel finding for 7TM/GPCR, which has previously only been shown for the tyrosine kinase insulin receptor (45, 46). The upstream signaling cascade involved $G\alpha_{13}$-RhoA-ROCK, which in turn induced phospholipase C (PLC)-mediated inositol 1,4,5-triphosphate formation and subsequent release of Ca^{2+} from internal stores. Furthermore, a dynamic mass redistribution assay was used to confirm the pharmacology and G proteins implicated in GPR55 signaling pathways (41, 47). A distinct signature of mass redistribution was shown for GPR55 in comparison with $G\alpha_i$-, $G\alpha_s$-, or $G\alpha_q$-coupled 7TM/GPCR. The GPR55-mediated redistribution was blocked in the presence of dominant negative $G\alpha_{13}$, whereas the $G\alpha_i$ inhibitor pertussis toxin (PTX), $G\alpha_s$ inhibitor cholera toxin, and the $G\alpha_q$ inhibitor YM254890 had no effect. The coupling of GPR55 to $G\alpha_{13}$-RhoA-ROCK and the consequent remodeling of the cytoskeleton was recently described in HEK293 cells and human neutrophils (48). Activation of GPR55 led to the formation of filamentous actin in HEK293 cells, which was dependent on the presence of functional $G\alpha_{13}$, RhoA, and ROCK. Yet further reinforcement of $G\alpha_{13}$ coupling was recently published by Brown *et al.* (38). Using a yeast cell line coexpressing GPR55 and various chimeric G proteins, they show specific coupling of GPR55 to $G\alpha_{13}$ and not to $G\alpha_i$, $G\alpha_s$, or $G\alpha_q$ (38).

However, not all studies have reported specific coupling to $G\alpha_{13}$. Lauckner *et al.* (49) also used single-cell Ca^{2+} imaging to assess GPR55 signaling cascades in HEK293 cells and mouse dorsal root ganglia. Ca^{2+} responses in these cells were mediated by both $G\alpha_q$ and $G\alpha_{12}$ and a phosphatidylinositol-specific PLC. The role of $G\alpha_i$ and its $G\beta/\gamma$ subunits was excluded due to the PTX-insensitive nature of the Ca^{2+} rise. Furthermore, the presence of an intact cytoskeleton and functional RhoA GTPase were suggested as critical elements in the response.

The endogenous GPR55 ligand LPI was discovered by screening a panel of lysolipids and cannabinoids and measuring GPR55-induced ERK1/2 activation (4). ERK phosphorylation has since been confirmed in a number of studies (41, 50–52). Furthermore, Oka *et al.* (50) have also shown that p38 MAPK are activated downstream of GPR55. Henstridge *et al.* (41) analyzed a variety of signaling readouts for GPR55 and in addition to single-cell Ca^{2+} imaging and ERK phosphorylation, nuclear factor of activated T cells, and nuclear factor κB reporter gene assays appear to be robust readouts of GPR55 activation. The GPR55-induced activation of nuclear factor of activated T cells has also been reported in an endothelial cell line (53).

In summary, GPR55 appears primarily a $G\alpha_{13}$-coupled 7TM/GPCR that activates RhoA and ROCK and induces prolonged and oscillatory Ca^{2+} release from intracellular stores, culminating in the induction of a variety of transcription factors with the potential to significantly alter cellular physiology.

(Patho)physiology of GPR55

GPR55 in the nervous system

Given that CB_1 is the most abundant GPCR in the brain and plays a fundamental role in regulating neurotransmission, it is of no great surprise that alterations in the endocannabinoid system have been implicated in a number of severe neurological diseases (54). However, since the discovery of the endocannabinoid system, pharmacological and functional data suggest that additional non-CB_1/non-CB_2 cannabinoid targets exist in the central nervous system (CNS) (1–3). Furthermore, an increasing number of studies describe the importance of glial cannabinoid receptors in regulating neuroimmunological processes and influencing neuronal communication (55, 56).

Anandamide and the synthetic cannabinoid agonist R-(+)-WIN55212 stimulate GTPγS binding in brain homogenates from both wild-type and CB_1 knockout mice (57) and endocannabinoid-induced-long term depression in the CA1 region of the hippocampus is present in both CB_1 knockout mice and wild-type littermates (58). Furthermore, other groups have reported that R-(+)-WIN55212 could equally affect neurotransmission in brain slices from wild-type and CB_1 knockout rodents, and effects were blocked by Rimonabant but not by AM251 (59–61). Also, further studies have shown that primary microglia and astrocytes express unidentified cannabinoid targets that regulate cellular responses to excitotoxicity, cell migration, and cytokine release (56). Together these data suggest the presence of non-CB_1/non-CB_2 receptors within the CNS sensitive to cannabinoids.

Despite the extensive mRNA data showing CNS expression of GPR55 (25, 26, 51, 62, 63), protein localization has yet to be confirmed in the brain. Intriguingly, Ryberg *et al.* (26) suggest that in the striatum, hypothalamus and brain stem, the expression of GPR55 mRNA is comparable with that of CB_1, which may indicate an important role for GPR55 in these regions. Furthermore, we observed high hGPR55 mRNA expression in the striatum and nucleus accumbens and detectable levels in the hippocampus (Fig. 2A). Pietr *et al.* (51) used real-time PCR to confirm the expression of GPR55 in primary microglial cells, signifying a potential role for GPR55 in neuroimmunological regulation. Immunohistochemistry has revealed GPR55 protein expression in mouse large diameter dorsal root ganglia neurons (49) and activation of GPR55 induced significant inhibition of the potassium M-current (49), which can potentially enhance the excitability of sensory neurons.

The endocannabinoid system is believed to be critical for successful brain development and shaping neuronal connectivity (64, 65). A recent study assessed the role of cannabinoid receptors in the development of corticothalamic and thalamocortical axonal projections (66). GPR55 knockout mice showed no perturbation in the targeting or fasiculation of these axonal fibres, although the CB1 receptor was shown to be vital for correct axonal path finding.

Despite finding little or no GPR55 mRNA expression in human cerebellum (Fig. 2A), a recent study has reported GPR55 mRNA in cultured rat cerebellar granule cells (67). Lipopolysaccharide treatment induced significant up-regulation of inflammatory mediators such as IL-1b, IL-6, and TNF-α, and this effect was dose dependently blocked by pretreatment with CP55940. The authors suggest that the effect of CP55940 was not via CB_1 or CB_2; however, also reason that based on their pharmacological evidence, GPR55 is also unlikely to be the CP55940-sensitive target in the cerebellum.

Given that LPI is a potent endogenous agonist of GPR55 and both are found in the brain, it is of interest to note that recent studies have shown LPI application to rat neuroendocrine PC12 cells results in increased exocytosis and subsequent catecholamine release (68). Furthermore, LPI application resulted in a significant intracellular Ca^{2+} rise and LPI-induced effects were abolished after thapsigargin treatment. The effects were not observed with closely related lipid molecules (lysophosphatidylcholine and lysophosphatidylserine); however, GPR55 expression has yet to be assessed in this cell line.

Knowledge of GPR55 protein expression and functional significance in the nervous system are currently

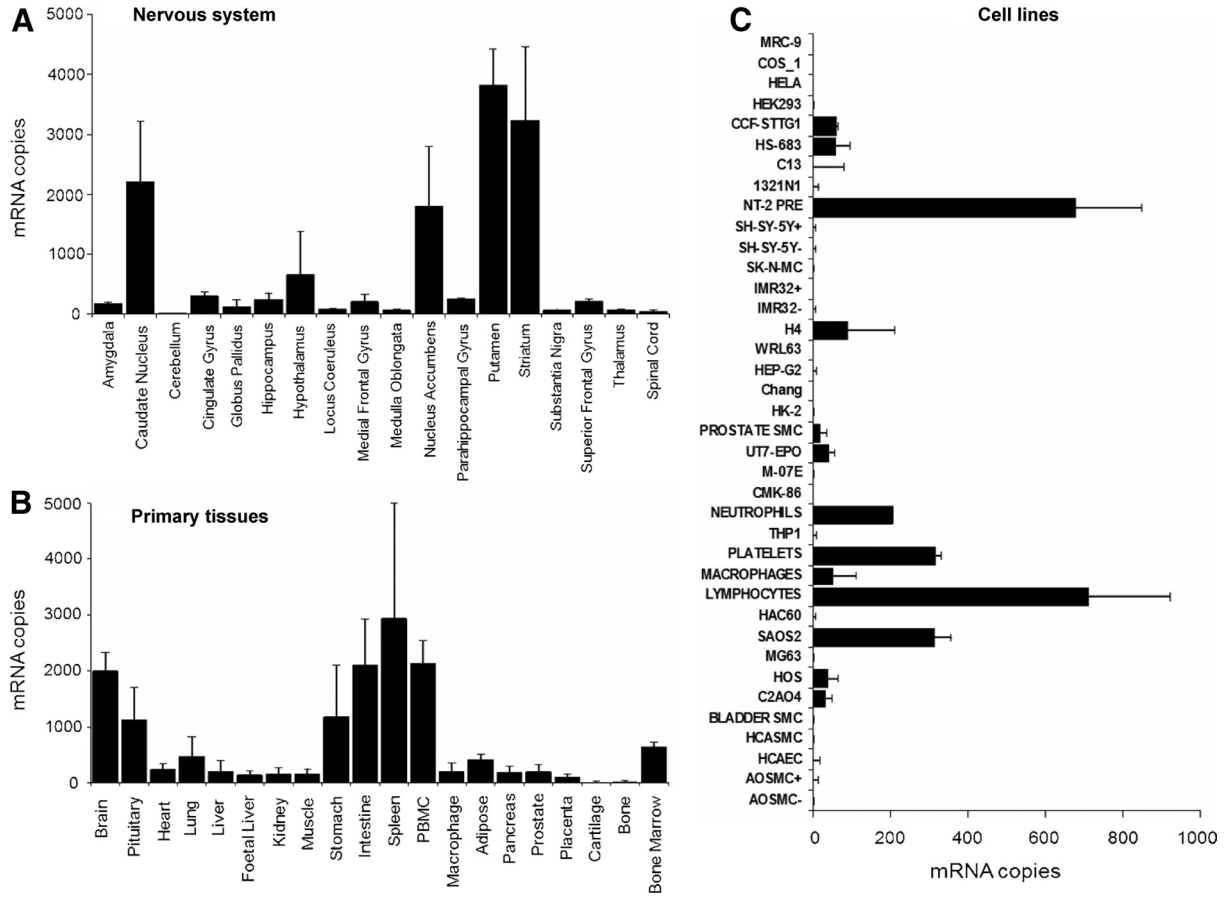

FIG. 2. GPR55 mRNA expression in the CNS, peripheral tissues, and cell lines. TaqMan quantitative RT-PCR analysis of GPR55 in human tissues and cells was conducted. The level of mRNA expression in poly A+ RNA from 18 regions of the brain and nervous system (A) and 20 primary peripheral tissues (B) were determined as described previously (118) using a hGPR55-specific primer set: forward primer, 5′-TCTTCCCGCTGGAGGT GTTT-3′, reverse primer, 5′-CAGGATGTGGATGCTCCTGG-3′, TaqMan probe, 5′-CTTCCTCCTTCCCATGGGCATCATGG-3′. Data show the mean (±SD) copies of mRNA detected in samples from three or four individuals per tissue (two males/two females for all tissues except two males/one female for globus pallidus and four males for prostate). No trends suggestive of sex-specific expression were observed. Levels of β-actin in these samples vary within a normal range and have been described previously (118). The same hGPR55-specific primers were used to measure mRNA levels in 37 distinct human cell lines and primary cells (C). Data show mean (±SD) copies per nanogram mRNA detected in duplicate from a single batch of cells. The mRNA levels of three housekeeping genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin) in these samples vary within a normal range, confirming the integrity of each RNA sample, and has been presented previously (119). +, Differentiated (IMR32 and SH-SY-5Y) or serum-starved (AOSMC) cells; −, undifferentiated or unstimulated cells. The origin of cells has been described elsewhere (120).

limited, however it appears that, GPR55 can regulate dorsal root ganglia excitability and may modulate certain neuroinflammatory conditions. With greater access to GPR55 antibodies and the new selective ligands, plus extensive evaluation of the knockout mice, the true significance of GPR55 in the nervous system will soon be ascertained.

GPR55: a novel target for pain?

Initial GPR55 studies within the pharmaceutical industry provoked great excitement as inflammatory and neuropathic pain were identified as potential therapeutic targets (63). Indeed, GPR55 knockout animals exhibit a striking pain phenotype, being markedly resistant to mechanical hyperalgesia associated with Freund's complete adjuvant (FCA)-induced inflammation or partial nerve ligation, a model of neuropathic hypersensitivity (63). In GPR55 knockout mice, mechanical hyperalgesia was completely absent for up to 2 wk after FCA injection and up to 28 d after ligation. Interestingly, GPR55 does not appear to modulate normal nociceptive pathways because they were largely unaffected in the knockout animals. Although GPR55 is present in dorsal root ganglia, which are an integral component of nociceptive neurocircuitry, it is specifically located in large diameter neurons (49), which typically detect innocuous stimuli. The mechanism of action underlying the effect of GPR55 on inflammatory pain is not clear at present but may reflect an altered immune response. Indeed cytokine profiling re-

vealed increased levels of IL-4, IL-10, interferon-γ, and granulocyte macrophage colony-stimulating factor in paws from FCA-injected GPR55 knockout mice (63). Furthermore, we discovered significant GPR55 mRNA expression in spleen, bone marrow, lymphocytes (peripheral blood mononuclear cells), platelets, and neutrophils consist with a role in inflammatory processes (Fig. 2, B and C). A recent study using noxious rotation of an inflamed rat knee joint, has shown that local administration of O-1602 significantly reduced the mechanosensitivity of the joint afferent fibers (69). More specifically, the effect was observed only in the unmyelinated C fibers was quick in onset and was unaffected by coadministration of CB_1 and CB_2 antagonists (AM281 and AM630, respectively). However, the effect of O-1602 was completely blocked following coadministration of O-1918 (69). Whether these effects involve GPR55 or GPR18 for example, remains to be established, and it would be interesting to repeat these studies with the new GPR55 compounds described by Brown *et al.* (38) and Kotsikorou *et al.* (29).

GPR55 in the vasculature

Studies using rat mesenteric artery preparations suggest that certain cannabinoids target a distinct non-CB_1/non-CB_2 vascular receptor. For example, Δ^9-THC-induced vasorelaxation could not be blocked by the CB_1 antagonist Rimonabant, even at high concentrations (70). Furthermore, abnormal-cannabidiol (abn-CBD) was shown to induce mesenteric vascular relaxation in CB_1 and CB_2 knockout mice (71), and intriguingly, these effects were antagonized by a cannabidiol analog lacking CB_1/CB_2 activity (O-1918) (72). The current data suggest the presence of a non-CB_1/non-CB_2 $G\alpha_i$-coupled receptor that is sensitive to Δ^9-THC, anandamide, and abn-CBD (71, 73, 74). However, Johns *et al.* (75) have shown that abn-CBD induces a similar decrease in mean arterial pressure in both wild-type and GPR55 knockout mice, which could be antagonized by O-1918, suggesting that abn-CBD affects a distinct target from GPR55 in the endothelium. In support of this theory is the well-described data describing GPR55 coupling to $G\alpha_{13}$, which contradicts the PTX-sensitive effects of abn-CBD and anandamide in endothelium (71).

Daly *et al.* (76) have recently shown using a fluorescent ligand binding approach that GPR55 may be expressed in mouse mesenteric arteries (MMA). T1117 is a derivative of AM251 with a fluorescent tetramethylrhodamine group added. The addition of this fluorescent group renders the compound unable to bind CB_1 yet retain its agonist properties at GPR55 (76). T1117 bound to all three vascular layers of the MMA, and preincubation of excess unlabeled AM251 inhibited T1117 binding. Furthermore, prior treatment of the MMA preparation with O-1602 resulted in a significant loss of T1117 labeling, suggesting that O-1602 internalizes GPR55, thus removing the T1117 binding target.

Interestingly, endothelial cells may regulate GPR55-mediated physiology via an autocrine release of LPI. Early studies show that endothelial cells can release LPI into the extracellular milieu after intracellular PLA_2-mediated lipid breakdown (77). Anandamide is also produced by vascular endothelial cells (78) and activates GPR55 in some recombinant systems, suggesting that in principle, anandamide can bind both CB_1 and GPR55 in endothelial cells. Waldeck-Weiermair *et al.* (53) have shown in human umbilical vein endothelial cells (HUVEC), that anandamide induces an increase in intracellular Ca^{2+}, which can be blocked by the CB_1 antagonist Rimonabant and CB_1-insensitive O-1918. Furthermore, O-1602 evoked GPR55-mediated Ca^{2+} elevation, thus suggesting that both CB_1 and GPR55 may contribute to anandamide-induced Ca^{2+} release in endothelium.

Integrins play a major role in the anandamide response, as revealed with the use of Mn^{2+}, fibronectin, and the ROCK inhibitor Y27632, which all modulate integrin clustering (53). If CB_1 and GPR55 are both found within the same cell type and can be activated by the same ligands, this suggests there may be interactions between their downstream signaling cascades, as observed in neutrophils (48), and this appears to be the case in the endothelium. When integrins are unclustered, anandamide activates the CB_1-$G\alpha_i$-Syk pathway, which inhibits the GPR55-phosphatidylinositol 3-kinase -Bmx-PLC-Ca^{2+} cascade at the level of Syk (53). However, when integrins cluster, this uncouples CB_1 from β1 integrin and unleashes the uninhibited GPR55-phosphatidylinositol 3-kinase-Bmx-PLC-Ca^{2+} cascade after anandamide stimulation.

Intriguingly, a recent study has shown that LPI-induced activation of nonselective cation channels and endothelial cell depolarization are independent of GPR55 (79). The authors followed up this observation with new reports describing further direct effects of LPI on endothelial cation channels (80, 81); however, these non-GPR55-mediated effects currently appear to be endothelium specific.

GPR55 in blood

Cannabinoids induce diverse responses in blood cells, such as migration, proliferation, cytokine production, apoptosis, reactive oxygen species production, and chemotaxis. Indeed, certain phytocannabinoids and synthetic CB_2 ligands exert potentially therapeutic immu-

nosuppressant actions (82, 83). The role of cannabinoids in regulating neutrophil chemotaxis and migration is contentious, and although Balenga *et al.* (48) recently demonstrated 2-arachidonoyl glycerol (2-AG)-induced chemotaxis via CB_2, other studies have not reported this effect (84). Furthermore, increasing evidence in the immune system suggests the presence of non-CB_1/non-CB_2 cannabinoid and associated lipid targets (82, 84, 85). Two lipid-sensitive orphan 7TM/ GPCR have been implicated as major candidates, GPR18 and GPR55.

Our mRNA expression data confirm GPR55 in a number of diverse human immune cells, including neutrophils (Fig. 2C). However, it was recently shown in a model of experimental colitis that O-1602 could inhibit neutrophil recruitment, independently of GPR55, implicating the potential role of the O-1602-sensitive receptor GPR18 in the effects (86). Despite this, Balenga *et al.* (48) have shown for the first time that LPI and AM251 act as GPR55 agonists in neutrophils, promoting RhoA-dependent chemotaxis. Furthermore, Balenga *et al.* identified novel downstream interactions between GPR55 (activated with LPI or AM251) and CB_2 (activated with 2-AG) receptor signaling in neutrophils. When both receptors were stimulated in tandem, pathways involving RhoA and Cdc42 were significantly enhanced with clear synergistic potentiation observed between AM251 and 2-AG induction of RhoA-mediated chemotaxis (48). Thus, the data suggest that CB_2 and GPR55 initially cooperate by mutually enhancing RhoA-induced migration to hunt for inflammatory loci.

Interestingly, a negative interaction between GPR55 and CB_2 was observed during neutrophil respiratory burst at the level of reactive oxygen species generation. Also, LPI significantly reduced the 2-AG-induced activation of the GTPase Rac2. Intriguingly, previous data have described a novel Rimonabant-sensitive non-CB_1/non-CB_2 target in neutrophils, exhibiting negative cooperativity with CB_2 receptors (84). Thus, after initial functional cooperation in inducing chemotaxis, GPR55 and CB_2 disengage, and in a refined process of functional repression, GPR55 restricts excessive CB_2-mediated oxidative damage. Microglia express both GPR55 and CB_2; thus, in future studies it would be interesting to assess GPR55-CB_2 receptor cross talk and any functional relevance of such interaction.

GPR55 in bone

Recently GPR55 has been found in cells that regulate bone metabolism (87) and thus may represent a novel target for osteoporosis and bone loss associated with arthritis. GPR55 is present in cells that both generate (osteoblasts) and resorb (osteoclasts) bone. In osteoclasts, GPR55 activation results in osteoclastogenesis, cell polarization and bone resorption; however, the function of GPR55 in osteoblasts is unclear (87). Male GPR55 knockout mice exhibit a clear osteopetrotic phenotype with high bone mass, although this is not observed in females; the reasons for this striking difference are intriguing but remain to be established (87). In male GPR55 knockouts, osteoclast numbers are increased significantly; however, osteoclast function is impaired overall, which is consistent with other osteopetrotic phenotypes and may be a homeostatic response (88). Furthermore, the authors also show that in GPR55 knockout mice, cartilaginous remnant (chondrocytes) levels are doubled within the trabecular bone. In addition, a recent conference abstract has confirmed the presence of GPR55 in human chondrocytes (89). Using RT-PCR, the authors suggest that GPR55 is present in normal human chondrocytes and those extracted from osteoarthritic patients. However, to date the role of GPR55 in these cells or whether the receptor is important in osteoarthritis development remains to be determined. Despite finding very low levels of GPR55 mRNA in human bone and cartilage samples, cell lines derived from these tissues expressed significant levels of the receptor in our hands (Fig. 2, B and C).

GPR55 signaling in osteoclasts is in line with most other cell types, in that there is prominent activation of RhoA and ERK1/2, together with effects on the actin cytoskeleton (87). Much of the work on bone metabolism was carried out using the ligands O-1602 and LPI, which exhibit bell-shaped concentration-response relationships in this system. However, the effects of O-1602 and LPI on RhoA signaling are absent in GPR55 knockout mice and O-1602 responses are inhibited by the putative GPR55 antagonist cannabidiol (87).

GPR55 in cancer

Several lines of evidence point to a potential role for GPR55 in the control of cancer cell proliferation. First, certain cannabinoids can modulate GPR55 activity (5, 7, 9, 10, 36, 90), and it is well established that cannabinoids can control cancer cell proliferation (91–93). This property has been demonstrated in several models of cancer, ranging from the simplest (cell cultures) to the most complex [genetically engineered mice (94–96)], and in tumors from very different origins [brain, breast, pancreas, hematopoietic system, *etc.* (91–93)]. Second, the levels of the endogenous GPR55 ligand LPI (4) are augmented in plasma and ascites from patients with ovarian cancer compared with healthy women or with women with noncancerous pathologies (22, 97). Interestingly, the highest elevated LPI species found in cancer patients is arachidonoyl-LPI (22), and this particular LPI is the most bio-

logically active at GPR55 (98). Moreover, Falasca and Corda demonstrated some 15 yr ago that epithelial cells (18) and fibroblasts (99) are able to generate LPI when transformed with ras oncogene and that this LPI induces cell proliferation. Finally, GPR55 predominantly couples to $G\alpha_{12/13}$ proteins (26, 38, 39, 48, 49), which are known to signal oncogenesis (100, 101). For example, overexpression of these $G\alpha$ proteins induces fibroblast transformation, and their activation (by binding of ligands to their corresponding GPCRs) enhances invasive potential and angiogenic responses (100, 101).

The expression of GPR55 has been confirmed in a diverse range of human cancer cell lines including breast (43, 102), ovary, prostate (52), brain, skin, cervix, liver, blood, pancreas (102), and bile ducts (103). Interestingly, in human tumors the expression of GPR55 correlates with their aggressiveness. Higher GPR55 mRNA levels were found in high histological grade breast, pancreas, and brain tumors compared with low-grade tumors and healthy tissue (102). Moreover, elevated GPR55 mRNA was associated with decreased overall glioma patient survival (102). These data suggest that GPR55 expression and/or activation confers an oncogenic capability on cancer cells. In fact, this hypothesis has been proven correct and results obtained so far indicate that this capability is invested in an increased proliferative potential. In addition, genetic blockade of GPR55 [using selective small interfering RNA (siRNA)] in ovarian, prostate (52), breast and brain (102) cancer cells decreased their proliferation in culture, whereas GPR55 overexpression in breast and brain cancer cells had the opposite effect (102). Importantly, *in vivo* silencing of GPR55 reduced tumor growth in a xenograft model of glioblastoma, an effect that was accompanied by a decrease in the number of proliferating cells within the tumors (102).

Together these results indicate that GPR55 expression enhances the proliferative capability of cancer cells. However, a recent paper shows that activation of this receptor in cholangiocarcinoma cells exerts an antiproliferative action both *in vitro* and in *vivo* (103). In this case, anandamide and O-1602 activated GPR55, induced coupling to $G\alpha_{12}$ proteins, activation of c-Jun N-terminal kinase, cell death by apoptosis, and the consequent decrease in the number of viable cancer cells (103). This process was accompanied by the recruitment of Fas death receptor into lipid rafts (103). Additional research is required to determine whether this discrepancy with previous data is the reflection of the complex pharmacology of GPR55; an indirect, cell type-, ligand-, or dose-specific effect; or the combination of these and other factors.

It is important to note that experiments describing a proliferation-inducing effect were performed in the absence of exogenously applied GPR55 agonists, which raises the question of whether GPR55 is activated by endogenously produced ligands or is constitutively active. Although the latter possibility cannot be ruled out, experimental evidence supports the existence of an endogenous GPR55 agonist tone. Pharmacological or genetic inhibition of the cytosolic phospholipase A_2, an enzyme that was previously described to generate mitogenic LPI in ras-transformed cells (18, 99), reduced proliferation in prostate cancer cells (52) and GPR55-overexpressing HEK293 cells (102). Both *in vitro* and *in vivo* approaches point to the ERK pathway as a major GPR55 downstream signaling pathway involved in its proliferation-promoting action. For example, Piñeiro *et al.* (52) demonstrated that LPI induces ERK activation in prostate cancer cells in culture and that this effect can be prevented by genetic (siRNA) or pharmacological (cannabidiol) blockade of GPR55. Andradas *et al.* (102) also demonstrated the modulation of this GPR55 signaling cascade by silencing and overexpressing the receptor in breast and brain cancer cells, both in cell cultures and *in vivo*.

Interestingly, GPR55 expression/activation may confer additional capabilities to cancer cells apart from an increased proliferation potential. For instance, it has been reported that MCF-7 breast cancer cells increase their migration toward chemoattractants when GPR55 is ectopically overexpressed and that these cells migrate in response to LPI via a mechanism selectively blocked by GPR55 silencing (43). Furthermore, activation of GPR55 induces endothelial cell proliferation, migration, tube formation, and an increase in vascular endothelial growth factor in culture (37). Although further research is needed, these results suggest that GPR55 may enhance the metastatic potential of cancer cells and induce/sustain angiogenesis in tumors.

Recent GPR55 expression data in other tissues and cell lines

Mast cells

Human mastocytic cells treated with phorbol 12-myristate 13-acetate induce significant release of nerve growth factor, which is thought to further enhance inflammatory responses via angiogenesis (104). Inflammation-induced nerve growth factor release from a human mast cell line (HMC-1) was specifically attenuated by preincubation with the cannabinoid-like molecule palmitoylethanolamide (PEA). PEA has been shown in one study to be a potent GPR55 agonist (26), and the receptor is expressed in HMC-1 cells, thus potentially regulating the antiinflammatory effect of PEA. CB_1 and CB_2 receptors were not found in HMC-1 cells, and targeted GPR55

siRNA could specifically knock down the antiinflammatory effect.

These data further enforce the idea that GPR55 may play a role following an inflammatory insult.

Placenta

Recently GPR55 expression in uterine tissues from d 10 until the end of pregnancy was assessed using real-time quantitative PCR, Western blot, and immunohistochemistry (105). PCR revealed peak GPR55 transcript expression at d 12, although protein levels peaked slightly later, at d 14. Delayed protein expression in uterine tissue is not unprecedented and has been previously described for the important endocannabinoid regulating enzyme fatty acid amide hydrolase (106).

GPR55 was significantly expressed in decidual tissue, which is crucial for pregnancy success and undergoes a regressive process during pregnancy development (105). Intriguingly, the authors suggested that GPR55 may play a role in these critical regression processes by inducing apoptotic death of decidual cells because the GPR55 agonist AM251 promoted cell death.

Kidney

Diabetes is becoming a major health issue in our modern indulgent society, and diabetic patients often exhibit elevated endocannabinoid levels (107). The onset of diabetic nephropathy is often preceded by proximal tubule hypertrophy, and research has revealed a role for CB_1 in inducing hypertrophic events in the renal tubules (108). A recent study has used RT-PCR and Western blotting to reveal GPR55 expression in human proximal tubule cells [HK2 (109)]. Treating HK2 cells with anandamide resulted in significant cell hypertrophy. To assess the role of CB_1, the authors pretreated the cells with AM251. AM251 alone reduced hypertrophy below baseline and blocked anandamide-induced hypertrophy. However, this effect could be attributed to GPR55 interaction because AM251 is a GPR55 agonist at the concentrations applied (0.5–5 μM) (41). To assess the role of GPR55, the authors attempted to block the effects of anandamide with O-1918. This is a cannabidiol analog that may antagonize GPR55 (53) or act via GPR18 (85). Therefore, GPR55 effects in this cell line cannot be ruled out, although further pharmacological evaluation is required.

Summary and concluding remarks

Interaction with GPR55 may explain some of the off-target effects of certain cannabinoid ligands; however, other novel cannabinoid targets in various tissues remain to be characterized. For example, the R-(+)-WIN55212-sensitive brain receptor (57, 59) awaits isolation, and the vascular system retains the so called abn-CBD-sensitive endothelial receptor awaiting precise characterization. Furthermore, intriguing data, especially from endothelial cells, suggest LPI and the putative GPR55 ligand ARA-S have non-GPR55 targets, distinct from CB_1 and CB_2 (37, 79). Moreover, another putative GPR55 ligand O-1602 has non-GPR55 effects in experimental colitis and neutrophil migration, potentially acting via GPR18 (86). Therefore, although our current knowledge of GPR55 pharmacology and physiology has significantly improved our understanding of lipid signaling, a number of questions still remain unanswered.

Lysophospholipid receptors and the traditional cannabinoid receptors are widely expressed in a variety of tissues and cells throughout the body and have considerable influence over vast physiological processes (36, 110). There are currently more than 4000 publications detailing the similar lipid-sensitive lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) receptor families, which are implicated in numerous pathophysiological states ranging from cancer, multiple sclerosis, cardiovascular disease, and asthma (111, 112). Intriguingly, LPA and S1P can be released from the host cell in a similar manner to LPI and can act on specific receptors in an autocrine or paracrine fashion and play a significant role in disease prognosis (113). Given that LPI can be released in a similar manner and exerts similar autocrine actions (52), it raises the exciting yet speculative hypothesis that LPI/GPR55 signaling may play an equally important role in cellular physiology. Furthermore, the role of these lipid-sensitive receptors in human pathology is currently under consideration for novel therapeutic exploitation (111, 114). For example, the S1P ligand fingolimod has recently been approved in a number of countries as a treatment for relapsing forms of multiple sclerosis (115).

Therefore, GPR55 should also be considered as a novel therapeutic target in the numerous pathologies in which it is involved. For example, GPR55 plays an important role in tumor progression and could be exploited as a new biomarker and/or therapeutic target in oncology. GPR55 antagonists may prove beneficial in slowing tumor proliferation, angiogenesis, and cancer pain (116). Furthermore, the ability of GPR55 to regulate bone metabolism could be exploited therapeutically. However, because the osteopetrotic phenotype is absent in female GPR55 knockout mice, this may be of limited utility. Interestingly, in male mice, an 8-wk *in vivo* treatment paradigm with cannabidiol significantly reduced serum type 1 collagen C-terminal telopeptide fragments, a biochemical marker of bone resorption (87). Arthritis may prove to be a more tractable target because GPR55 has the potential to act at multiple levels in this pathological state, regulat-

ing the underlying immune response as well as bone resorption and pain. Indeed, *in vivo* studies suggest that cannabidiol is an effective oral antiarthritic therapeutic in murine collagen-induced arthritis (117). The role of GPR55 antagonism in this effect of cannabidiol remains to be established. Additionally, there is need for more academic research into the role of GPR55 in inflammatory pain, and it will be of interest to discover whether the sites of pain exhibit elevated levels of LPI. Whether GPR55 itself continues to be explored as a therapeutic pain target by the pharmaceutical industry may be limited by the inherent drugability of the receptor rather than the underlying physiology, although phytocannabinoids such as cannabidiol may offer potential here. Other aspects of the LPI-GPR55-pain pathway may be worth exploring, for example, regulating LPI metabolism or reducing the availability of endogenous ligands in plasma using molecular sequestration approaches.

In summary, GPR55 has emerged as an intriguing addition to the lipid-sensitive receptor family and is involved in numerous cellular processes and pathologies, which may represent areas for novel therapeutic intervention in the future.

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Address all correspondence and requests for reprints to: Dr. Chris Henstridge, Institute of Experimental Medicine, Hungarian Academy of Sciences, 43 Szigony Utca, HU1083 Budapest, Hungary. E-mail: henstridge@koki.hu, or Dr. Maria Waldhoer, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz A-8036, Austria. E-mail: maria.waldhoer@medunigraz.at.

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Current address for M.W.: Hagedorn Research Institute, Novo Nordisk A/S, Gentofte, Denmark.

Current address for N.A.B.B.: Molecular and Signal Transduction Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases/National Institutes of Health, 10 Center Drive, Room 11N242, Bethesda, Maryland.

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