The emerging pharmacology and function of GPR35 in the nervous system

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Abbreviations: AHO, Albright's hereditary osteodystrophy; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CNS, central nervous system; cGMP, cyclic guanosine 3'5' monophosphate; DRG, dorsal root ganglion; EC_{50}, half-maximal effective concentration; GPR35, G protein-coupled receptor 35; GPCR, G protein-coupled receptor; NMDA, N-methyl-D-aspartate; PDE, phosphodiesterase; PKG, protein kinase G; RET, rearranged during transfection; SNP, single nucleotide polymorphism; TRPV1, transient receptor potential cation channel subfamily V member 1

Abstract

G protein-coupled receptor 35 (GPR35) is an orphan G protein-coupled receptor (GPCR) that can be activated by kynurenic acid at high micromolar concentrations. A previously unappreciated mechanism of action of GPR35 has emerged as a $G\alpha_{i/o}$-coupled inhibitor of synaptic transmission, a finding that has significant implications for the accepted role of kynurenic acid as a broad-spectrum antagonist of the NMDA, AMPA/kainite and α7 nicotinic receptors. In conjunction with previous findings that link agonism of GPR35 with significant reduction in nociceptive pain, GPR35 has emerged as a potential effector of regulation of mechanical sensitivity and analgesia of the Ret tyrosine kinase, and as a receptor involved in the transmission of anti-inflammatory effects of aspirin– potentially through affecting leukocyte rolling, adhesion and extravasation. Single nucleotide polymorphisms of GPR35 have linked this receptor to coronary artery calcification, inflammatory bowel disease and primary sclerosing cholangitis, while chromosomal aberrations of the 2q37.3 locus and altered copy number of GPR35 have been linked with autism, Albight's hereditary osteodystrophy-like syndrome, and congenital malformations, respectively. Herein, we present an update on both the pharmacology and potential function of GPR35, particularly pertaining to the nervous system. This review forms part of a special edition focussing on the role of lipid-sensing GPCRs in the nervous system.

1. Introduction

GPR35 is a poorly characterised, 7-transmembrane domain, GPCR that transmits function via interaction with $G\alpha_{i/o}$, $G\alpha_{13}$, and ² -arrestin (Figure 1) (Milligan, 2011; Mackenzie et al., 2011; Divorty et al, 2015; Shore and Reggio, 2015). In terms of sequence similarity, GPR35 is related to the purinergic receptor LPA_4 (32 %), the hydroxycarboxylic acid binding receptors HCA_2 and HCA_3 (30 %), and the cannabinoid and lysophosphatidylinositol-binding GPR55 receptor (30 %) (O'Dowd et al., 1998). As a consequence of the ligand-binding properties and shared sequence identity with GPR55, various groups have focussed on GPR35 as a putative lysophosphatidic acid-sensing GPCR (Oka et al., 2010; Zhao and Abood, 2013). This is of interest to further investigate experimentally,

although at present it is difficult to draw any conclusions based on the original findings (Oka et al., 2010). Although certainly able to be activated by high concentrations of kynurenic acid, questions of which effects of this ligand, a well-studied metabolite of tryptophan, can be attributed to activation of GPR35 remain some of the major undefined issues in understanding the function of this receptor. This is vital to examine closely because kynurenic acid is clearly neuroactive and produces a broad range of effects in the central nervous system (CNS). However, many of these effects can be attributed to blockade of ionotropic receptors for the excitatory amino acid glutamate. Specific challenges in exploring the roles of GPR35 in the CNS relate to (a) the low potency of kynurenic acid at both rodent, and particularly the human, orthologues of the receptor, (b) that although many ligands with activity at GPR35 have been reported, the vast majority of these display modest potency and are known to also have a range of non-GPR35 mediated effects and, (c) although antagonists from two distinct chemical classes have been identified, at least in transfected cell systems these appear to display exquisite selectivity for human GPR35 and lack significant affinity at either mouse or rat GPR35 (Jenkins et al., 2012). Moreover, although a line of GPR35 knock-out mice has been generated and reported on (Min et al., 2010), these have not been employed widely and, currently, no information on the elimination of expression of GPR35 on effects of kynurenic acid in cells or tissue from the CNS has been released into the public domain. Each of these issues will be considered within the current review.

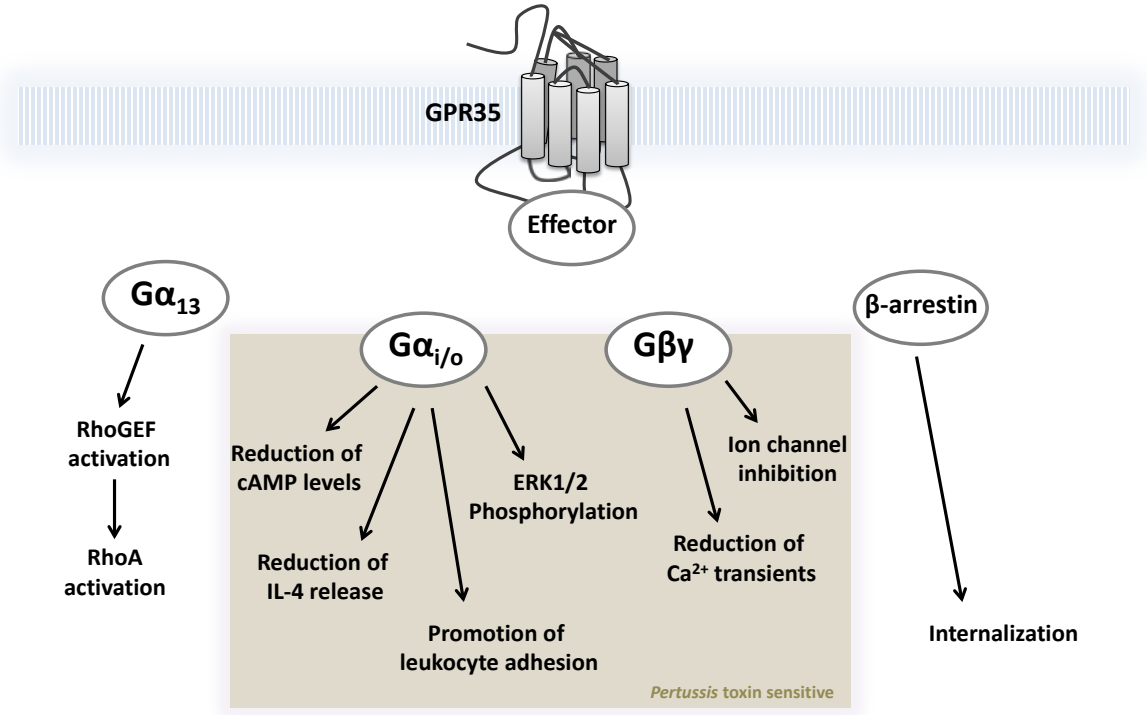

Figure 1. GPR35 couples to various effectors following agonist stimulation. RhoGEF, Ras homologue guanine nucleotide exchange factor; RhoA, Ras homologue gene family member A; cAMP, 3'-5'-cyclic adenosine monophosphate; IL-4, interleukin-4; ERK1/2, extracellular-signal regulated kinase 1/2; Ca^{2+}, calcium.

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from the CNS has been released into the public domain. Each of these issues will be considered within the current review.

2. Pharmacology

2.1. Putative endogenous agonists of GPR35

GPR35 retains "orphan" GPCR status despite being able to be stimulated by high concentrations of a number of endogenously produced small molecules, including kynurenic acid, 2-oleoyl lysophosphatidic acid, DHICA (5,6-dihydroxyindole-2-carboxylic acid), reverse T3 (3,3 ,5-triiodothyronine), cGMP (cyclic guanosine 3' 5' monophosphate), (Wang et al., 2006; Oka et al., 2010; Deng et al., 2012b; Southern et al., 2013), and, most recently, more modest levels of the chemokine CXCL17 (Maravillas-Montero et al., 2014). This reflects that reported estimates of ligand concentration in man, under normal physiological conditions at least, are less than those required to modulate the activity of the receptor substantially (e.g. kynurenic acid, DHICA, reverse T3 and cGMP (Divorty et al., 2015)), or have been described in single publications that have not yet been verified by independent sources (e.g. CXCL17 and derivatives of lysophosphatidic acid). The linkage of endogenously produced molecules with GPR35 activation is further complicated by marked differences in concentrations required to activate species homologues of this receptor (Milligan, 2011). This has led to the suggestion that kynurenic acid could feasibly be an/the endogenous ligand of rat but not human GPR35 (Mackenzie et al., 2011). A further point to note is that additional studies are required to verify the finding that CXCL17 is an/the endogenous ligand of GPR35 before the suggested systematic nomenclature of "CXCR8" (Maravillas-Montero et al., 2014) is agreed upon. Although this terminology has already appeared in subsequent literature (Shore and Reggio, 2015), definition of receptor de-orphanisation and adoption of a new nomenclature requires acceptance by the relevant subcommittee of the International Union of Basic and Clinical Pharmacology (IUPHAR). This has not yet occurred.

2.2. Synthetic agonists of GPR35

Since there is no consensus on the endogenous ligand(s) of this receptor, a large and concerted effort in both academic (Jenkins et al., 2010; Zhao et al., 2010; Funke et al., 2013; Thimm et al., 2013) and industrial (Taniguchi et al., 2006; 2008; Yang et al., 2010; 2012; Deng et al., 2011a; 2011b; 2012b) sectors; in addition to working collaborations between the two (Neetoo-Isseljee et al., 2013; Mackenzie et al., 2014), has resulted in reports of a wide range of novel and previously reported small molecule agonists from both distinct, and overlapping, chemical series that are able to activate GPR35. Such ligands include zaprinast, pamoic acid, YE-120, YE-210, tyrphostin-51, compound 1/TC-G 1001, PSB-13253, lodoxamide, bufrolin, amlexanox, furosemide and cromolyn (Taniguchi et al., 2006; Zhao et al., 2010; Deng et al., 2011a, 2011b, 2012c; Neetoo-Isseljee et al., 2013; Funke et al., 2013; Mackenzie et al., 2014; Jenkins et al., 2010; Yang et al., 2010, 2012). Because screening for GPR35 active ligands has predominantly used the human orthologue, all of these compounds have some level of potency at human GPR35. However, despite a number of ligands displaying little potency at rodent orthologues of GPR35, recently a number of rodent-selective and high and species equipotent ligands, e.g. lodoxamide and bufrolin, have been reported (Mackenzie et al., 2014). Thus, there is now a wide selection of ligands available that have agonist activity at GPR35. A challenge for those who have not followed developments in this field closely is to select ligands with the appropriate pharmacological activity for the species of cell or tissue being studied. Furthermore, various agonist ligands display marked differences in efficacy in distinct screening assays, and the implications of this for biological activity in cells and tissues that express GPR35 endogenously remain unclear. Moreover, as the vast majority of ligands with GPR35 agonist activity have derived from screening of commercially available compound libraries, many of the ligands identified from these screens are known to have significant and prominent effects at biological targets other than GPR35. It is vital, therefore, that potential non-GPR35-mediated effects of such ligands are considered.

2.3. Synthetic antagonists of GPR35

Although substantial progress has been made in identification of agonists of GPR35, the identification and/or reporting of GPR35 antagonists has lagged behind. Indeed, representatives from

only two chemical series are widely available. Key exemplars of these series are CID-2745687 (1-(2,4-difluorophenyl)-5-[[2-[[(1,1-dimethylehyl)amino]thioxomethyl]hydrazinylidene]methyl]-1*H*-pyrazole-4-carboxylic acid methyl ester) and ML-145 (2-hydroxy-4-[4-(5*Z*)-5-[(*E*)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]butanoylamino]benzoic acid) (Heynen-Genel et al., 2010; 2011; Zhao et al., 2010). Although far from fully characterised, there are no reports of substantial off-target effects of these compounds when used at modest concentrations. Indeed, in both these examples there is marked selectivity between GPR35 and GPR55, the most closely related member of the GPCR family. These ligands should, therefore, be expected to offer an avenue to define contributions of GPR35. However, the use of these compounds is complicated substantially by their marked species selective behaviour. Although not evident initially (Zhao et al., 2010), in transfected cell expression systems both CID-2745687 and ML-145, although derived from entirely distinct chemical series, were both found to display high affinity at human GPR35 but to display no significant affinity for either the rat or mouse orthologues. This was the case whether monitoring their capacity to block agonist function at GPR35 in transfected cell systems using either ^{2}-arrestin-2 recruitment, receptor internalisation, or G protein-mediated signalling pathways (Jenkins et al., 2012). Thus, as all reported studies on potential CNS function of GPR35 have used cell and tissues from rodents, the use of these antagonists would appear to be inappropriate. However, as discussed later, this has not precluded their use in certain studies. Despite the apparent clarity of the *in vitro* pharmacological studies (Jenkins et al., 2012), initial experiments did report GPR35 antagonism with CID-2745687 at the mouse orthologue in a transfected cell system (Zhao et al., 2010) and, subsequently, a number of functional studies have employed these antagonists in rodent models to prevent apparent GPR35 agonist responses (Berlinguer-Palmini et al., 2013; Alkondon et al., 2015). Clearly further studies, perhaps performed in knock-out animals (Min et al., 2010), are required to clarify these discrepancies.

2.4. Kynurenic acid and its biological targets

A substantial number of studies have assessed the role of kynurenic acid in systems endogenously expressing GPR35, with the broad assumption that effects were produced via agonism

of this receptor. Kynurenic acid is a neuroprotective, endogenous tryptophan metabolite produced by astrocytes via the kynurenine pathway (Figure 2). Kynurenic acid is appreciated to inhibit all three classes of ionotropic excitatory amino acid receptors as a broad-spectrum competitive antagonist. It acts at the glycine co-agonist site of the N-methyl-D-aspartate (NMDA) receptor with an IC_{50} of 8-15 μM in the absence of glycine and 239 μM in the presence of 10 μM glycine (Kessler et al., 1989; Hilmas et al., 2001). Moreover, it inhibits α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainite receptors (IC_{50} 10-500 μM) (Patel et al., 2001; Prescott et al., 2006; Bertolino et al., 1989; Alt et al., 2004; Mok et al., 2009). Kynurenic acid also acts as a non-competitive inhibitor of the α7-nicotinic acetylcholine receptor (IC_{50} ~ 7 μM) (Hilmas et al., 2001), and as an agonist of the aryl hydrocarbon receptor (EC_{25} 104 nM) (DiNatale et al., 2010) in addition to its action as an agonist of GPR35.

At GPR35 kynurenic acid displays a degree of species selectivity, producing EC_{50}s of between 39 – 217 μM at human, 7 – 66 μM at rat, and 11 μM at mouse (Wang et al., 2006; Zhao et al., 2010; Jenkins et al., 2011; Southern et al., 2013). Therefore, the use of kynurenic acid in CNS-derived preparations, within the concentration ranges assessed in many *in vivo* and *ex vivo* studies is likely to directly block the aforementioned glutamate receptors in addition to activating GPR35. For this reason, the role of kynurenic acid at a particular receptor tends to be dissected using antagonists of each of the glutamatergic receptor types. Since such studies have indicated that after treatment with various glutamatergic receptor antagonists there remains a response to kynurenic acid that is able to modulate glutamate release (Carpenedo et al., 2001; Alkondon et al., 2011; Banerjee et al., 2012), then GPR35 has been considered the likely target, given its expression pattern and association with $G\alpha_{i/o}$ signalling (Berlinguer-Palmini et al., 2013). The GPR35 antagonists described earlier have, therefore, also been employed to attempt to better define a role of GPR35 in such kynurenic acid-

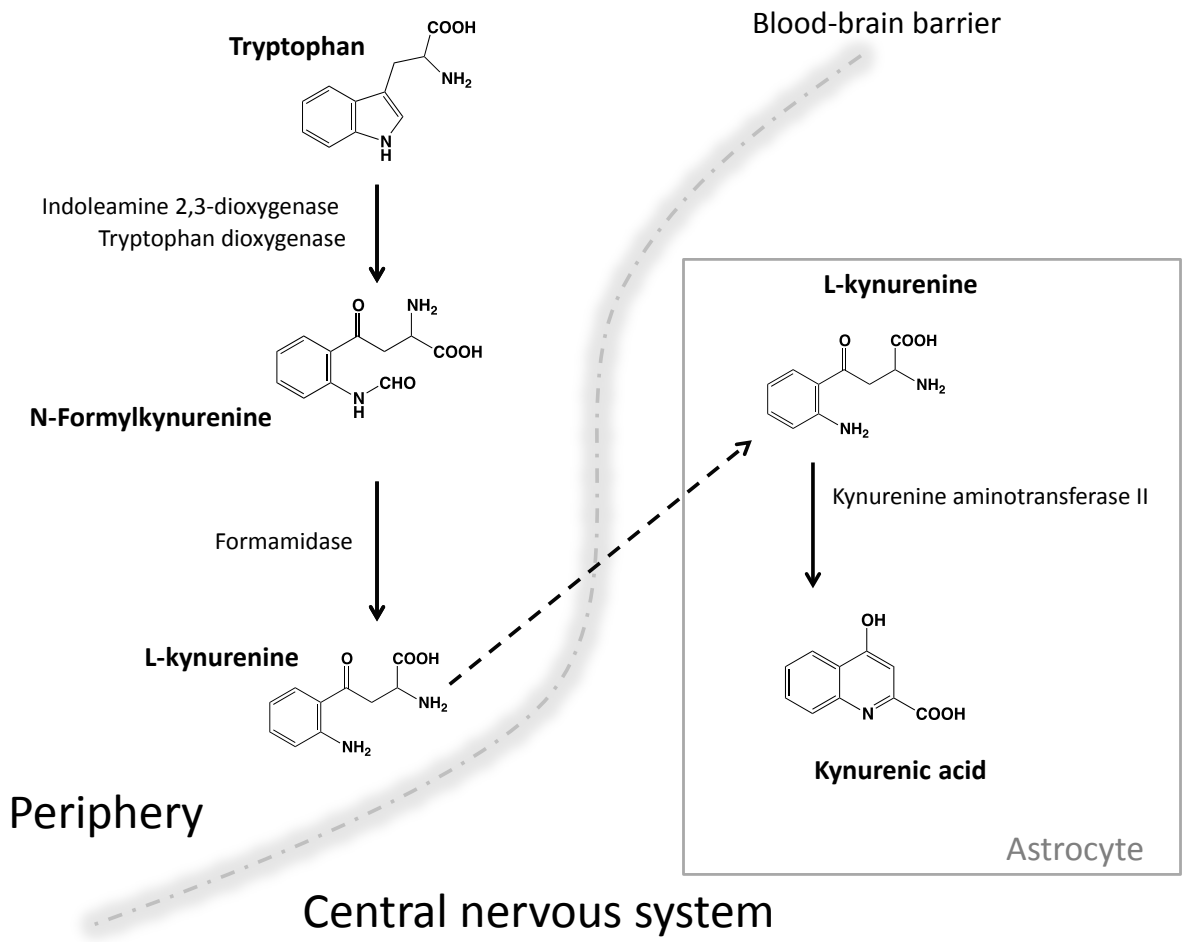

Figure 2. Kynurenic acid is synthesised from precursor L-kynurenine in astrocytes Kynurenic acid does not cross the blood-brain barrier but is synthesised by irreversible transamination from L-kynurenine, carried out by the action of kynurenine aminotransferase II in astrocytes. L-kynurenine, the major metabolite of tryptophan, crosses the blood-brain barrier through the large neutral amino acid carrier. Newly formed kynurenic acid is readily liberated from astrocytes and goes on to carry out its pharmacological functions in the extracellular milieu.

induced effects (Berlinguer-Palmini et al., 2013). However, as also noted earlier, careful consideration must be given to the use of either CID-2745687 or ML-145 and interpretation of the results, as there is strong evidence that these compounds block agonist-induced signalling at human GPR35 but not at the rat or mouse forms of the receptor (Jenkins et al., 2012). This leads to uncertainty in findings that have employed these compounds to implicate responses of GPR35 in *ex vivo* rodent models. Moreover, consideration must be given to the concentration of kynurenic acid employed in native expression systems to study the activation profile of GPR35 because significantly

lower concentrations of kynurenic acid have been employed in a number of functional assays than would be anticipated to be required based on studies using cloned receptors (Wang et al., 2006; Barth et al., 2009; Cosi et al., 2011; Jenkins et al., 2011; Moroni et al., 2012). Assigning function to a particular receptor or response is further complicated given that the AMPA and kainite receptor antagonists 6,7-dinitro-2,3-quinoxalinedione (DNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) were recently shown to act as moderately potent agonists of GPR35 (Southern et al., 2013). Therefore, application of these compounds in functional studies may abrogate glutamatergic release through inhibition of AMPA receptors *and* agonism of GPR35, confusing studies that have associated the use of these ligands only with blockade of the AMPA/kainite receptors (Carpenedo et al., 2001). As a result of this, it may be pertinent to also assess antagonists of the NMDA and α7 nicotinic receptors as agonists of GPR35. In a pharmacological context it is prudent, therefore, to employ a selection of structurally diverse, commercially available GPR35 agonists. This approach was taken recently in native rat hippocampal slices to strengthen the argument for a role of GPR35 in modulating neuronal activity (Alkondon et al., 2015). It is a significant disappointment that, currently, studies employing GPR35 knock out mice have been restricted to blood pressure regulation, and other cardiovascular, endpoints (Min et al., 2010) rather than in neurobiological studies.

2.6. Zaprinast and its biological targets

Beyond kynurenic acid, zaprinast is the most frequently employed GPR35 agonist in functional studies. Indeed, since its original description as an agonist of GPR35 (Taniguchi et al., 2006), zaprinast has, *de facto*, become the reference agonist for this receptor (Yang et al., 2010; Jenkins et al., 2010; Zhao et al., 2010; Deng et al., 2011a; 2012b; Berlinguer-Palmini et al., 2013; Neetoo-Isseljee et al., 2013; Divorty et al., 2015). Widespread use of zaprinast in this context reflects the routine demonstration of its capacity to activate GPR35 and that it shows reasonable potency at various species orthologues (Taniguchi et al., 2006; Jenkins et al., 2012). However, zaprinast is better known, and was originally described, as a relatively potent inhibitor of phosphodiesterases (PDEs) (Lugnier et al., 1986), and is most commonly reported as an inhibitor of subclasses of cGMP-specific

PDEs, including PDE5 (IC_{50} 0.4-0.8 μM) and PDE6 (IC_{50} 0.15 μM) (Beavo, 1995; Loughney et al. 1998). Zaprinast also modulates with reasonable potency PDE1 (IC_{50} 0.35 μM), and with moderate potency PDE9 (IC_{50} 35 μM), PDE10 (IC_{50} 22-33 μM), and PDE11 (IC_{50} 5-33 μM) (Taher et al., 1994; Fisher et al., 1998; Fujishige et al., 1999; Fawcett et al., 2000; Yuasa et al., 2000; Nakamizo et al., 2003), which, in addition to GPR35, are also expressed within specific regions of the brain (Fisher et al., 1998; Bischoff, 2004; Kruse et al., 2009). A number of studies assessing the function of zaprinast at GPR35 tend use the PDE5 inhibitor sildenafil as an independent PDE inhibitor that lacks agonism at GPR35 (Berlinguer-Palmini et al., 2013; Alkondon et al., 2015). However, potential contribution of zaprinast to inhibition of other PDEs is rarely accounted for, and considering the reported EC_{50} of zaprinast at human (2-8 μM), mouse (1 μM), and rat (0.1 μM) orthologues of GPR35 (Taniguchi et al., 2006; Jenkins et al., 2012), this remains a relevant concern.

3. Background to GPR35 function

3.1. GPR35 tissue expression profile

Interest in GPR35 in the context of neuropharmacology is relatively recent. In part, this may stem from initial studies suggesting that GPR35 was not expressed to a significant level in human or rat brain (O'Dowd et al., 1998; Wang et al., 2006). These studies employed RNA from broad brain regions and/or relatively insensitive detection methods. Subsequently, however, GPR35 expression has been identified within discrete regions of the CNS and peripheral nervous system, including the caudate nucleus in human, the medulla oblongata, hippocampus, spinal cord and dorsal root ganglia (DRG) of mouse (Wang et al., 2006; Cosi et al., 2011), and the DRG, spinal cord, hippocampus and cerebrum of rat (Taniguchi et al., 2006; Ohshiro et al., 2008; Berlinguer-Palmini et al., 2013; Alkondon et al., 2015). Moreover, substantial levels of GPR35 mRNA recorded in other tissues, including high levels in immune and gastrointestinal tissue (Wang et al., 2006; Yang et al., 2010), and lower levels in heart, lung and skeletal muscle (Horikawa et al., 2000; Taniguchi et al., 2006; Leonard et al., 2007; Min et al., 2010) had focussed attention on these tissues, and diseases associated with their dysregulation, as being more appropriate to explore the function and therapeutic potential of

GPR35 (Milligan, 2011). Studies that detect mRNA corresponding to GPR35 are consistent with localised CNS expression, with a number of reports expanding this to immunodetection (Ohshiro et al., 2008; Cosi et al., 2011; Franck et al., 2011; Deng et al., 2011a). Although such studies are of vital importance to confirm expression, concerns about the specificity of staining with many anti-GPCR antisera remain widespread (Michel et al., 2009) and controls employing tissue from knock-out animals are currently lacking to adequately validate antibody/antisera specificity.

3.2. Identification of GPR35 and chromosomal location

GPR35 was first identified in genomic DNA through homology cloning using degenerate oligonucleotide primer sequences based on the transmembrane sequence of GPR1 (O'Dowd et al., 1998). Human GPR35, situated at chromosomal locus 2q37.3, encodes two alternatively spliced protein sequences. GPR35a, which encodes a 309 amino acid, 7-transmembrane domain polypeptide, and GPR35b, which is identical in sequence apart from containing a 31 amino acid N-terminal extension (Okumura et al., 2004). Although the majority of literature on human GPR35 focusses on the short (GPR35a) isoform, it is important to note that there is limited information currently available on whether GPR35a and GPR35b are differentially regulated and/or provide different functionalities *in vivo*. In human gastric cancer, there does appear to be some differences between the two transcripts with GPR35b being identified from tumours and non-tumorous surrounding regions whilst GPR35a was found in the tumorous regions only, at a level lower than GPR35b, but with a higher transforming capability (Okumura et al., 2004). Such differences were not observed in primary cardiomyocytes where GPR35a and GPR35b mRNA transcripts were found to be expressed to similar levels and to respond in a similar manner to stimuli (Ronkainen et al., 2014). Moreover, *in vitro* pharmacological efforts using both the long and short isoforms of GPR35 indicated that they respond with similar potency to GPR35 agonists (Guo et al., 2008; Zhao et al., 2010; Mackenzie et al., 2014). As such, any distinctiveness in function between the splice variants is not likely to reflect pharmacological variation but it is possible that the extended N-terminal domain of GPR35b may provide protein-protein interactions in a native setting that would not be evident in transfected cell systems. This, however, has yet to be explored directly. Production of these two transcript variants has only been

reported for human, with transcripts akin only to the short version of human GPR35 identified in mouse and rat. Mouse GPR35, which shares 73.4 % overall protein sequence identity with human GPR35a, translates into a protein of 307 amino acids from chromosomal locus 1D (Taniguchi et al., 2006), whilst rat GPR35, which shares 72 % overall homology with human and 85 % overall homology with mouse, encodes a protein of 306 amino acids from a chromosome location of 9q36 (Taniguchi et al., 2006).

3.3. Genetic disorders associated with chromosomal locus 2q37.3

Chromosome 2q terminal deletions are among the most frequently reported cytogenetic abnormalities in individuals with autism spectrum disorders. The occurrence of autism or autistic features in children with deletion of chromosome 2q37.3 has been reported in a number of cases (Smith et al., 2001; Ghaziuddin and Burmeister, 1999; Falk and Casas, 2007; Devillard et al., 2010; Chong et al., 2014). The genomic region lacking *GPR35*, *GPC1* (glypican 1), and *STK25* (serine threonine protein kinase 25) has been associated with tracheomalacia, short phalanges and eczema (Cassas et al., 2004; van Karnebeek, 2002; Falk and Casas, 2007). However, for the majority of studies focusing on 2q37.3, the role of GPR35 has not been considered due to a lack of information regarding its expression and functionality in the nervous system. Terminal, *de novo*, microdeletions of the subtelomeric long arm of chromosome 2 have also been associated with Albright's hereditary osteodystrophy (AHO)-like syndrome and brachydactyly-mental retardation. Patients with these disorders present with developmental delay (with mild to severe mental retardation), behavioral abnormalities, autism, obesity, short stature, brachydactyly type E, craniofacial dysmorphism, along with cardiac, tracheal, gastrointestinal, genitourinary tract and CNS abnormalities (Jensen and Hoo, 2004; Chassaing et al., 2004; Fernandez-Rebollo et al., 2009).

Although AHO syndrome is characterised by an inactivating mutation in the *GNAS* gene encoding the stimulatory $G\alpha_s$ subunit (Ringel et al., 1996), functionality of this G protein is normal in AHO-like syndrome, which is often associated with *de novo* microdeletions at chromosome 2q37.3. Terminal deletions of chromosome 2 affect the genomic DNA sequences of at least thirty genes including *GPR35*, *GPC1*, and *STK25* (Shrimpton et al., 2004). GPR35 haploinsufficiency, arising as

a result of a *de novo* deletion of the maternal copy of *GPR35*, was put forward as a plausible candidate for AHO-like and brachydactyly-mental retardation syndromes (Shrimpton et al., 2004) but this has not been extended subsequently.

Additionally, increased copy number variation of *GPR35* was observed in a female foetus that upon autopsy presented with cloacal malformation with anal atresia, bilateral renal dysplasia, urethral agenesis, a secondary bell shaped thorax, and Potter syndrome, as a result of a *de novo* microduplication at the 2q37.3 locus (Hilger et al., 2013). This region, spanning 25 kb, contained exons 3-4 of a *CAPN10* splice variant and exons 3-6 of *GPR35*, of which the latter was reasoned (based on disease-association and available gene expression analysis) to be wholly or at least partly responsible for the described phenotype (Hilger et al., 2013). These birth defects qualify for the group of "VACTERL association" disorders, which are characterised by vertebral defects (including hemivertebrae and abnormal spinal curvature), anorectal malformations, cardiac defects, tracheoesophageal fistula with or without esophageal atresia, rental malformations, and limb defects (Solomon, 2011; Siebel and Solomon, 2013). In line with GPR35's association with $G\alpha_{13}$ signalling (Jenkins et al., 2010), RhoA activation and the potential role of lysophosphaditic acid species as ligands of GPR35 (Oka et al., 2010), it is interesting to speculate that GPR35 could play a role in cell polarity, which is an important feature of development critical for organ function. However, once again, further studies, perhaps based on knock out models, are required to clarify the role of this receptor and its function under normal physiological conditions in order to ascribe these disorders to GPR35. The suggestion that species of lysophosphaditic acid may activate GPR35 is intriguing given the relatively close relatedness of GPR35 to the lysophosphaditic acid receptors LPA4, LPA5 and LPA6 (Im, 2013).

4. The emerging function of GPR35 in the nervous system

4.1. Modulation of synaptic transmission

In an early study investigating coupling of human GPR35a and GPR35b to native neuronal signalling pathways and effectors, human GPR35 isoforms were transiently transfected into cultured rat superior cervical ganglion neurones and the effect on whole cell calcium channel currents (I_{Ca}) monitored using the patch-camp technique (Guo et al., 2008). Using a double pulse voltage protocol it was observed that the major component of current originated from É-conotoxin GVIA-sensitive N-type calcium channels ($Ca_v2.2$). The N-type calcium channel is widely expressed in the CNS and controls neurotransmitter release, alongside P/Q- and R-type channels. These channels are localised to presynaptic terminals, where their voltage-dependent activation leads to an influx of calcium ions, which in turn initiates exocytosis of synaptic vesicles containing various neurotransmitters (Wheeler et al., 1994). Under native conditions there was no reduction in I_{Ca} following application of kynurenic acid (300 µM) or zaprinast (10 µM) to cultured rat superior cervical ganglion neurones. However, when human GPR35 was transiently expressed in these cells, kynurenic acid and zaprinast inhibited I_{Ca} currents by 38 % and 59 %, respectively (Guo et al., 2008). This led the authors to suggest that endogenous expression of rat GPR35 was absent from superior cervical ganglion neurons. However, gene expression or immunocytochemistry were lacking in this report. Despite this, an interesting finding from this paper was the observation that GPR35 appeared to modulate I_{Ca} through $G^{2\,3}$ subunit activity, as voltage-dependent inhibition presented with slowed prepulse activation and partially relieved the inhibition of the depolarizing conditioning pulse following application of GPR35 agonists (Guo et al., 2008). This profile was abolished following application of *Pertussis* toxin, suggesting the involvement of a GPR35-$G\alpha_{i/o}$ coupled pathway.

Fast synaptic transmission is mediated synergistically by multiple types of high-voltage-activated Ca^{2+} channels, including N-type calcium channels, in the mammalian CNS. Therefore, it is interesting that application of GPR35 agonists to rat hippocampal slices endogenously expressing GPR35 generated a concentration- and time-dependent reduction in the frequency of spontaneous action potentials in *cornu Ammon* (CA)1 *stratum radiatum* interneurons (Alkondon et al., 2015). Responses to zaprinast, dicumarol, amlexanox, and pamoic acid were monitored using a standard patch-clamping technique and acted to reduce the frequency of fast current transients (Alkondon et

al., 2015). The GPR35 antagonist/inverse agonist ML-145 (1 μM), meanwhile, displayed the reverse effect and significantly increased the mean frequency of fast current transients. Moreover, co-application of ML-145 (1 μM) with zaprinast (10 μM) significantly reduced the inhibitory effect of zaprinast (Alkondon et al., 2015). Despite the issues with the reported lack of affinity of ML-145 at rat GPR35 (Jenkins et al., 2012), in these studies the rank-order of potency of a range of agonists was similar to that observed when employing *in vitro* pharmacological methods using fluorescently-tagged and overexpressing cell systems to define ligand structure-activity relationships at GPR35 (Divorty et al., 2015). This is a strong example of employing the breadth of available GPR35 pharmacology to build an argument for the direct contribution of this receptor (Alkondon et al., 2015).

Agonism of GPR35 has also been demonstrated to be involved in the reduction of evoked excitatory post synaptic currents at the CA1 pyramidal neurones of the rat hippocampus, through application of both zaprinast and kynurenic acid (Berlinguer-Palmini et al., 2013). To attempt to eliminate effects independent of GPR35 (e.g. being through kynurenic acid inhibition of NMDA and α-7 nicotinic receptors, or zaprinast inhibition of PDE5 and/or PKG), specific inhibitors were employed, and these did not affect the evoked excitatory post synaptic current in the same manner as kynurenic acid and zaprinast. Furthermore, the effect of these ligands was ablated following pre-incubation with the GPR35 antagonist/inverse agonist, CID-2745687 (Berlinguer-Palmini et al., 2013). Although the same issues of reported species specificity of CID-2745687 (Jenkins et al., 2012) clouds interpretation, in conjunction with the previously described findings it seems possible that GPR35 agonists may act to reduce the frequency of action potentials through inhibition of N-type calcium channels, leading to a smaller Ca^{2+} influx, a reduction in neurotransmitter release and a reduction in the evoked post synaptic current at the post-synaptic cell.

4.2. Nociception, neuropathic and inflammatory pain

GPR35 has been shown to be highly expressed in the DRG of both rats and mice (Ohshiro et al., 2008; Cosi et al., 2011). The DRG contains neurones that convey sensory information from the

periphery to the CNS, and become an important source of increased nociceptive signalling as a result of increased neuronal excitability (Sapunar et al., 2012). After neurogenesis of the DRG, signalling via the Ret (rearranged during transfection) tyrosine kinase receptor within the innervation targets is one of the mechanisms that shape the development of sensory neurones. In the early stages of DRG formation, two subpopulations of large-size neurones emerge, one that contains Ret (early Ret neurones) and further differentiate into low-threshold mechanoreceptors (Bourane et al., 2009; Luo et al., 2009). Another subpopulation of Ret containing neurones diverge later in the development of the DRG from smaller unmyelinated neurones, including nociceptors (late Ret neurones) (Franck et al., 2011). Conditional (Cre) knock out C57/BL6 mice lacking expression of Ret in nociceptive neurones were associated with a 66 % reduction in GPR35 as compared with wild type mice (Franck et al., 2011). These mice also presented with decreased sensitivity to mustard oil, had an increased sensitivity to cold in the acetone test, displayed a significant increase to cold hyperalgesia in the ischaemic nerve injury model of neuropathic pain, and displayed hypersensitivity to mechanical stimuli as indicated by a lower threshold to paw withdrawal using von Frey filaments (Franck et al., 2011). Since the Ret knock out mice did not display altered expression of typical antinociceptive receptors, and based on the altered expression of GPR35 at both a protein and mRNA level as well as the literature surrounding GPR35, the authors suggested that an inhibitory function of GPR35 on synaptic transmission by nociceptive neurones may be dysfunctional in Ret conditional knock out mice, leading to the observed behaviours (Franck et al., 2011).

GPR35 has also been observed in a subpopulation of small-to-medium diameter sensory neurones that contained TRPV1 (transient receptor potential cation channel subfamily V member 1) and larger sized neurones that convey non-nociceptive information (such as touch and light pressure), which contained GPR35 but not TRPV1 (Ohshiro et al., 2008). This led the authors to suggest that GPR35 could play a role in the conversion of mechanical stimuli into nerve impulses. TRPV1 is a non-selective cation channel that mediates Ca^{2+} release activity to mediate hyperalgesia, neurogenic inflammation and neuropathic pain. The function of TRPV1 can be modulated by $G\alpha_{i/o}$-coupled GPCRs that inhibit its activity through modulation of cAMP levels. Linked to this, application of

kynurenic acid and zaprinast resulted in a reduction of cAMP in cultured DRG that was abolished by pre-treatment with the $G\alpha_{i/o}$ inhibitor *Pertussis* toxin. As such, GPR35 could mediate visceral pain perception through modulating the action of TRPV1. This has not yet been demonstrated for GPR35 in any follow-up studies, although, interestingly, both the α7 nicotinic and NMDA receptors have been shown to specifically modulate the activity of TRPV1 to mediate mechanical hyperalgesia (Lee et al., 2012; Shelukhina et al., 2014).

Functional studies demonstrating activation of GPR35 and a reduction in pain perception have employed the acetic acid writhing test (Cosi et al., 2010). Mice were pre-treated by subcutaneous injection with suitable doses of either zaprinast or kynurenic acid and, subsequently acetic acid (0.6 %) was applied through intraperitoneal injection. Writhing behaviour was then monitored. Five mg/kg zaprinast and 300 mg/kg kynurenic acid significantly reduced writhing behaviour by 54 % and 58 % relative to phosphate buffered saline-injected control mice (Cosi et al., 2010). Following confirmation of *GPR35* expression in the DRG and spinal cord of mice, functional analysis using zaprinast and kynurenic acid on isolated, cultured, glial cells revealed a $G\alpha_{i/o}$-coupled reduction of cAMP levels following pre-stimulation with forskolin (Cosi et al., 2010).

In vivo studies employing the formalin test in rats to investigate the role of zaprinast in visceral pain modulation demonstrated that pre-treatment with zaprinast (10, 30 or 100 μg) by intrathecal injection, followed by subcutaneous injection of formalin (5 %) into the planar surface of the hind paw significantly reduced the sum of flinches compared with wild type mice (Yoon et al., 2005). Specifically, zaprinast reduced flinching behaviour during phase one and two of the formalin test. The acute phase of the formalin test predominately represents C-fibre activation as a result of peripheral stimulation (Martindale et al., 2001; McCall et al., 1996), whereas the tonic phase typically represents the inflammatory response emanating from the initial stimulus, suggested to be a result of NMDA receptor activation (Davidson et al., 1997; Vaccarino et al., 1993). The PDE5 inhibitor sildenafil and nonsteroidal anti-inflammatory drugs reduce writhing behaviour only at the late phase of the formalin test (Malmberg and Yaksh, 1992; 1993; Mixcoatl-Zecuatl et al., 2000), while systemic morphine reduces both phases (Oluyomi et al., 1992; Capuano et al., 2009; Sevostianova et al., 2003).

462 Interestingly morphine acts in a synergistic manner with zaprinast (Heo et al., 2005), indicating that
463 zaprinast and morphine act through distinct processes to modulate nociceptive behaviour. However,
464 since many of these key studies are now rather dated and were performed before a broader
465 pharmacological tool-kit of GPR35 ligands became available, it would be of considerable interest to
466 see a number of these studies repeated with better and more selective tool compounds.

467 The GPR35 agonist pamoic acid has also been associated with the modulation of visceral pain
468 in mice using the acetic acid abdominal constriction test (Zhao et al., 2010). Pre-incubation with
469 subcutaneous injection of pamoic acid (25, 50, and 100 mg/kg) for twenty minutes dose-dependently
470 reduced the pain associated with intraperitoneal administration of acetic acid (0.6 %), causing 50 %
471 antinociception at 40 mg/kg. This was indicated to be a similar effective dose to that of acetyl
472 salicylate (aspirin). However, as aspirin did not promote ² -arrestin-2 translocation in cells expressing
473 GPR35, the authors suggested different mechanisms of action for these two compounds (Zhao et al.,
474 2010). Subsequent drug screening efforts have also reported aspirin to be inactive at GPR35.
475 However, 2,3,5-trihydroxybenzoic acid, salicyluric acid and gentisuric acid, which are metabolites of
476 aspirin, were reported to activate human GPR35 in both dynamic mass redistribution and ² -arrestin-2
477 recruitment assays (Deng and Fang, 2012a). Recent literature has suggested that some of the benefits
478 exerted by aspirin could be mediated by GPR35 (Dodd et al., 2013). This is based in part on studies
479 designed to understand how aspirin application inhibits acute inflammation, irrespective of its effects
480 on the inhibition of prostaglandin synthesis. Aspirin was found to acetylate cyclooxygenase-2
481 (COX2) within the endothelium, resulting in the synthesis of 15-(R)-hydroxyeicosatetraenoic acid.
482 This in turn was rapidly metabolised by leukocyte 5-lipoxygenase to 15-epi-lipoxin A_4; 15-epi-lipoxin
483 A_4 then elicited nitric oxide synthesis from constitutive nitric oxide (eNOS) and inducible nitric oxide
484 (iNOS) synthase (Paul-Clark et al., 2004). Oral administration of aspirin (200 mg/kg), one hour prior
485 to assessment was found to reduce IL-1² -stimulated leucocyte flux, rolling velocity, adherence and
486 extravasation through the endothelium (Paul-Clark et al., 2004). This provided evidence
487 demonstrating that the mechanism of aspirin's inhibition of acute inflammation involved modulating
488 leukocyte function via the nitric oxide pathway. Since these studies were carried out *in vivo* it is

interesting to speculate upon an involvement of aspirin metabolites and agonism of GPR35 in this process, as previous studies indicate that application of kynurenic acid increases the adhesion of leukocytes to vascular endothelial cells and shedding of neutrophil L-selectin from human peripheral monocytes in a manner that was significantly reduced by short hairpin mediated silencing of GPR35 (Barth et al., 2009).

4.3. Neuroinflammation, mast cells and inflammatory disease

Inflammatory bowel disease and primary sclerosing cholangitis are two interlinked chronic inflammatory conditions of which the etiology is incompletely understood. Recently, single nucleotide polymorphisms of GPR35 were found to be risk factors for early-onset inflammatory bowel disease (Imielinski et al., 2009), ulcerative colitis (Yang et al., 2015) and both ulcerative colitis and primary sclerosing cholangitis (Ellinghaus et al., 2013) through genome wide association studies. Both of these conditions are associated with significant abdominal discomfort and pain, and have been linked with changes to neurally-controlled functions (Bernstein et al., 2002; Lakhan and Kirchgessner, 2010; Strack et al., 2011; Vermeulen et al., 2014).

Mast cell numbers are increased in both inflammatory bowel disease and primary sclerosing cholangitis (Sasaki et al., 2002; Ishii et al., 2005), and have been suggested to contribute a direct role to the pathogenesis of inflammatory bowel disease (De Winter et al., 2011). Mast cells are located with close apposition to afferent nerve endings and enteric neurones in the gastrointestinal tract (Stead et al., 2006; Buhner and Schemann, 2011), acting to relay information to the central nervous system (Rijnierse et al., 2007; De Winter et al., 2011). Recent GPR35 literature indicates that a substantial number of compounds with mast cell stabilising activity are also agonists of GPR35; these include luteolin, quercetin, ellagic acid, gallic acid, dicumarol, furosemide, nedrocromil, nivimedone, cromolyn, lodoxamide, bufrolin, amlexanox, pemirolast and doxantrazole (Jenkins et al., 2010; Yang et al., 2010; Deng et al., 2012b; Deng and Fang 2012b; Yang et al., 2012; Neetoo-Isseljee et al., 2013; Southern et al., 2013; Mackenzie et al., 2014). Moreover, cromolyn was shown to be effective in the treatment of cholangiopathy associated with primary sclerosing cholangitis as a result of reducing mast cell numbers and histamine release (Kennedy et al., 2014) whilst nedrocromil reduced

inflammation and fibrosis in a rat model of colitis (Xu et al., 2002). Although a functional link between GPR35 agonism and mast cell stabilisation remains to be demonstrated, *GPR35* is expressed in mast cells and is upregulated in response to IgE stimulation (Yang et al., 2010). *GPR35* is also highly expressed in basophils and eosinophils (Yang et al., 2010), natural killer cells (Fallarini et al., 2010), CD14+ monocytes, dendric cells, peripheral blood lymphocytes (Wang et al., 2006), and neutrophils (Barth et al., 2009; Wang et al., 2006), suggesting an involvement for this receptor in the immune system and potentially at the neuro-inflammatory axis.

5. Conclusions

The role of GPR35 in the modulation of synaptic transmission, neurogenic and inflammatory pain, and potential signalling pathways involved in these processes are beginning to emerge. There has previously been a disconnect between gene knockout, single nucleotide polymorphisms, and pathophysiological conditions associated with GPR35 versus the basic signal transduction pathways that emanate from this receptor under normal physiological conditions (Mackenzie et al., 2011). With the most recent findings suggesting a GPR35-$G\alpha_{i/o}$-linked mechanism of inhibition of synaptic transmission, and possible regulation of GPR35 by Ret tyrosine kinase (Franck et al., 2011) and hypoxia (Ronkainen et al., 2014) we are beginning to discern the basic signalling pathways of GPR35 and processes regulating it's expression. This exciting new avenue of research expands the potential therapeutic value of GPR35 beyond that as a target for the treatment of heart failure and hypertension (Min et al., 2010; Sun et al., 2008). However, close attention to the pharmacological differences between species orthologues of GPR35 is required to better validate conclusions and the use of both cells and tissues from knock out animals will be vital to overcome concerns about effects of GPR35 active ligands reflecting non-GPR35 mediated mechanisms.

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Conflict of interest statement

There is no conflict of interest

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