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NOVEL INSIGHTS WHICH MAY TRANSLATE INTO TREATMENTS FOR IRRITABLE BOWEL SYNDROME

Topic Editor
Angelo A. Izzo

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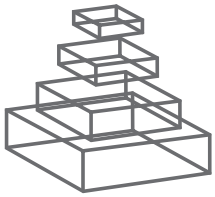

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NOVEL INSIGHTS WHICH MAY TRANSLATE INTO TREATMENTS FOR IRRITABLE BOWEL SYNDROME

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Irritable bowel syndrome (IBS) is a chronic disorder characterized by abdominal pain and altered bowel habit, with symptoms not explained by obvious biochemical structural causes. IBS is a very common disease - with prevalence estimated for western countries being 10-15% - and adversely affects the quality of life, with considerable direct and indirect costs. Despite the prevalence and the social impact of IBS, the exact etiopathogenesis is incomplete and the pharmacological treatment is unfortunately largely unsatisfactory. We invite researchers to submit papers (original papers or review articles) dealing with – but not limited to – the experimental and clinical pharmacology of IBS, including novel insights into the role and the pharmacological modulation of 5-hydroxytryptamine receptors, GABA receptors, corticotrophin-releasing factor, transient receptor potential channels, cannabinoid receptors, κ-opioid receptors, beta adrenoreceptors, protease-activated receptors, tackykinin receptors, cholecystokinin receptors, bile acid modulators and chloride channels in IBS motor disturbances, visceral hypersensitivity, intestinal secretion and permeability. Papers dealing with the experimental or clinical pharmacology of probiotics/prebiotics, nutraceuticals or plant products as well as systematic reviews/meta-analyses of the clinical data are also welcomed.

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Novel insights which may translate into treatments for irritable bowel syndrome

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Keywords: 5-hydroxytryptamine, brain-gut axis, cannabinoid receptors, enkephalinase inhibitors, irritable bowel syndrome, kynurenine pathway, pharmacotherapy, toll-like receptors

Irritable bowel syndrome (IBS) is a very common functional disorder of the digestive tract. Despite the prevalence and the social impact of IBS, the exact etiopathogenesis is incomplete and the pharmacological treatment is unsatisfactory. In this research topic, *Frontiers in Pharmacology* brings together a group of review and original articles focusing on IBS.

Fabrizio De Ponti brilliantly outlined the drugs currently used in—or in development for—IBS, within a scenario in which the specific armamentarium of medications is unsatisfactory (De Ponti, 2013). Jakub Fichna and Martin Storr focused their review article on the disturbances in the brain-gut axis as possible cause of IBS (Fichna and Storr, 2012). The Authors illustrated the pathophysiological mechanisms which contribute to the generation of IBS symptoms, with a special emphasis to stress, emotion and psychological factors. The future of anti-IBS drugs targeting the brain-gut axis were also highlighted. The bidirectional communication between the brain and the gut opens up new treatment possibility for IBS patients with mood disturbances that are refractory to first-and second-lines therapies.

Jeremy Gale and Lesley Houghton reviewed the preclinical and clinical data which support the potential use of gabapentin and pregabalin in disorders characterized by visceral hypersensitivity, such as IBS (Gale and Houghton, 2011). Both gabapentin and pregabalin bind with high affinity to alpha 2 delta subunits of voltage-gated calcium channels and inhibit both visceral nociception and motility. Although limited, clinical studies on visceral pain are in agreement with animal results and support a continued research and development of the alpha 2 delta ligands in IBS.

Eberlin et al. reviewed the pharmacokinetics, pharmacodynamics and clinical data of racecadotril, a powerful and selective enkephalinase inhibitor, which has emerged as a promising drug in the antisecretory therapy (Eberlin et al., 2012). In multiple direct comparative studies, racecadotril was at least as effective as loperamide in the treatment of acute diarrhoea, and exhibited significantly better tolerability. Although the results are robust and encouraging, the potential of racecadotril in D-IBS patients remains to be established.

A pan-Irish study investigated the consequences of toll-like receptors (TRLs) activation on the production of kynurenine (i.e., one of the metabolites of the kynurenine pathways derived from tryptophan) in IBS (Clarke et al., 2012). Whole blood from IBS patients and healthy controls was cultured with a number of TLR agonists. Tryptophan and kynurenine were assayed, by HPLC, both in the plasma and in cell culture supernatants. IBS subjects had an elevated plasma kynurenine:tryptophan ratio compared to healthy controls, which is suggestive of tryptophan metabolism via the kynurenine pathway. Furthermore, a differential downstream profile of kynurenine production subsequent to TLR activation in IBS patients - compared to healthy controls – was demonstrated. Collectively, such results suggest (1) that a pharmacological modulation of TLRs, by controlling the abnormal kynurenine pathway, be a novel potential strategy for IBS and (2) the use of plasma tryptophan metabolites assay as a biomarker for IBS diagnosis.

Beattie et al. investigated the *in vitro* and *in vivo* pharmacodynamic properties of a novel 5-HT_4 receptor agonist, namely TD-8954 (Beattie et al., 2011). TD-8954 was found to be a potent ($pKi = 9.4$) and selective (>2000-fold over the all other 5-HT receptors and over a plethora of receptors, enzymes, and ion channels) 5-HT_4 receptor agonist *in vitro* with strong *in vivo* gastrointestinal activity in guinea pigs, rats, dogs, and in healthy humans. TD-8954 may have value in the treatment in C-IBS sufferers.

Interleukin-6 (IL-6) is elevated in the plasma of D-IBS patients (Rana et al., 2012) and IL-6 gene polymorphisms may change individual susceptibility to IBS (Barkhordari et al., 2010). O'Malley et al. evaluated the effect of IL-6 on colonic ion transport (a measure of intestinal absorption and secretion) in the distal colon of the Wistar Kyoto rat (WKY) (O'Malley et al., 2012), a pre-clinical model for IBS. In colonic sheets mounted in Using chambers, IL-6 evoked secretion preferentially in WKY colons (as compared to control rats) and this effect was believed to be due to increased sensitivity of submucosal neurons to the pro-inflammatory cytokine. Such results bolsters our knowledge of IL-6 as a contributory factor in the pathophysiology of IBS.

Cannabinoids—via CB_1 and/or CB_2 receptor activation—exert pharmacological actions which are potentially beneficial in IBS (Izzo and Coutts, 2005; Storr et al., 2008). Kimball et al. evaluated the effect of selective cannabinoid receptor agonists in a mouse model of accelerated upper gastrointestinal transit resembling post-inflammatory IBS (PI-IBS) (Kimball

Abbreviations: 5-HT, 5-hydroxytryptamine; C-IBS, constipation predominant irritable bowel syndrome; D-IBS, diarrhoea predominant irritable bowel syndrome; hInEpCs, human intestinal epithelial cells; IBS, irritable bowel syndrome; IL-6, interleukin-6; iPSC induced pluripotent stem cell; PI-IBS, post-inflammatory irritable bowel syndrome; TLRs, toll-like receptors; WKY, Wistar Kyoto rat.

et al., 2010). The experimental model is generated by intracolonic administration of mustard oil, which induce transient colitis and, in the longer term (i.e., 28 days after mustard oil) a functional increase in gastrointestinal transit that is observed when there is no inflammation (Kimball et al., 2005). It was found that both cannabinoid receptor subtypes were up-regulated in the small intestine, an effect closely associated to increased efficacy of both CB_1 and CB_2 receptor agonists in normalizing the accelerated transit (Kimball et al., 2010). These results suggest that the altered cannabinoid CB_1 and CB_2 responsiveness is maintained long after an initial inflammatory period and suggest a role of cannabinoid receptors in the underlying pathophysiology of PI-IBS.

The differentiated Caco-2 cells intestinal cell line, derived from a human colon carcinoma has been used for drug absorption studies as well as to evaluate the epithelial barrier integrity in relation to IBS as well as to investigate the intestinal permeability of IBS drugs (Catalioto et al., 2008; Piche et al., 2009). Within our research topic on IBS, Kauffman et al. compared two alternative sources of human intestinal cells, i.e., commercially available primary human intestinal epithelial cells (hInEpCs) and induced pluripotent stem cell (iPSC)-derived intestinal cells, to Caco-2, for possible use in intestinal transport assays (Kauffman et al., 2013). Measurements of intestinal marker expression, formation of monolayers with tight junction formation and functional molecule transport and binding revealed that primary hInEpCs and iPSC-derived intestinal cells could offer an alternative source of human intestinal cells for understanding intestinal epithelial patho(physiology) and drug transport.

In summary, this research topic should provide a useful resource for IBS researchers, both basic and clinical. The pharmacological studies, together with new strategies for drug discovery, highlight that more research is urgently required to translate novel and innovative basic concepts into prescribing options for healthcare professionals. It is hoped that this collection of articles will inspire further research into IBS.

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Drug development for the irritable bowel syndrome: current challenges and future perspectives

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Medications are frequently used for the treatment of patients with the irritable bowel syndrome (IBS), although their actual benefit is often debated. In fact, the recent progress in our understanding of the pathophysiology of IBS, accompanied by a large number of preclinical and clinical studies of new drugs, has not been matched by a significant improvement of the armamentarium of medications available to treat IBS. The aim of this review is to outline the current challenges in drug development for IBS, taking advantage of what we have learnt through the Rome process (Rome I, Rome II, and Rome III). The key questions that will be addressed are: (a) do we still believe in the "magic bullet," i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms? (b) IBS is a "functional disorder" where complex neuroimmune and brain-gut interactions occur and minimal inflammation is often documented: do we need to target gut motility, visceral sensitivity, or minimal inflammation? (c) are there validated biomarkers (accepted by regulatory agencies) for studies of sensation and motility with experimental medications in humans? (d) do animal models have predictive and translational value? (e) in the era of personalized medicine, does pharmacogenomics applied to these medications already play a role? Finally, this review will briefly outline medications currently used or in development for IBS. It is anticipated that a more focused interaction between basic science investigators, pharmacologists, and clinicians will lead to better treatment of IBS.

Keywords: drug targets, translational medical research, brain-gut interactions, drug selectivity, biomarkers, 5-hydroxytryptamine, transient receptor potential channels, neuroimmune intestinal interactions

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder, characterized by recurrent abdominal pain or discomfort in combination with disturbed bowel habits in the absence of identifiable organic cause. Many medications are used for the treatment of patients with IBS, although their actual benefit is often a matter of debate. In particular, only a few are specifically labeled for IBS. In fact, notwithstanding great progress in our understanding of the pathophysiology of IBS thanks to a large number of preclinical and clinical studies of new drugs, the specific armamentarium of medications available is scant. The aim of this review is to outline the current challenges in drug development for IBS, taking advantage of what we have learned through the Rome process (from Rome I in the 1980s to Rome III published in 2006; Drossman, 2006).

The key questions that will be addressed are: (a) do we still believe in the "magic bullet," i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms? (b) IBS is a "functional disorder" where complex neuroimmune and brain-gut interactions occur and minimal inflammation is often documented: do we need to target gut motility, visceral sensitivity, or minimal inflammation? (c) are there validated biomarkers (accepted by regulatory agencies) for studies of sensation and motility with experimental medications in humans? (d) do animal models have predictive and translational value? (e) in the era of personalized medicine, does pharmacogenomics applied to these medications already play a role? Finally, this review will briefly outline medications currently used or in development for IBS. It is anticipated that a more focused interaction between basic science investigators, pharmacologists, and clinicians will lead to better treatment of IBS.

Abbreviations: EMA, European Medicines Agency; ENS, enteric nervous system; FDA, food and drug administration; 5-HT, 5-hydroxytryptamine; IBS, irritable bowel syndrome.

THE "MAGIC BULLET": A CONCEPT THAT NEEDS RETHINKING

The key pharmacodynamics, pharmacokinetic, and safety features for drugs to be used in the treatment of IBS are outlined in **Table 1**.

A selective drug is defined as a compound interacting only with one receptor subtype and leaving other receptors unaffected at concentrations achieved at therapeutic doses. The literature on the treatment of IBS has often resorted to the concept of the "magic bullet," i.e., a very selective drug displaying a single receptor mechanism capable of controlling IBS symptoms (Camilleri et al., 2006a). This was often considered the key to efficacy avoiding side-effects. This approach is no longer ideal because of several important pitfalls.

First, drug selectivity is always a relative concept, which ignores the basic fact that most molecules, even at therapeutic doses,

Table 1 | Key features for drugs to be used in the treatment of IBS.

	Key features
Pharmacodynamics	The drug should target a whole pathophysiological mechanism rather than a single receptor
	Possible targets: motility, secretion, visceral sensitivity, neuroimmune interactions/minimal inflammation, brain-gut axis
	The effect should be maintained over time during treatment
Pharmacokinetics	Good oral bioavailability (unless local action in the gut is specifically wanted)
	Half-life allows once daily dosing
	No metabolites with different or unwanted pharmacological actions
	Avoid CYP substrates with high likelihood of drug interactions
	Consider interactions with food or herbal products
Safety	Specificity cannot always avoid *off-target* effects because the same receptor/system also mediates other effects
	A drug can also hit *antitargets* (i.e., unwanted targets), another source of side-effects

may have several, sometimes disparate biological effects (i.e., hit a large number of targets in the pharmacological space; Garcia-Serna et al., 2010; Kawasaki and Freire, 2011). These effects may depend on the fact that a single receptor/effector pathway plays a role in different systems, so that even selective compounds have *off-target* effects (**Table 1**). In addition, there are many instances when the compound is endowed with additional pharmacological properties that hit the so-called *antitargets* (i.e., unwanted targets), responsible for side-effects, which are clarified only after the compound has undergone clinical trials. The classical example is provided by the cardiac side-effects due to hERG K^+ channel blockade by the early 5-HT_4 receptor agonists (Tonini et al., 1999).

The second issue is that the multifactorial pathophysiology of IBS (with multiple brain-gut and neuroimmune interactions) makes it unrealistic to expect that drugs acting on a single receptor may achieve substantial therapeutic gain over placebo in an area where the placebo response rate is substantial (approaching 40% across all randomized controlled trials; Ford and Moayyedi, 2010). As in other fields (Morphy et al., 2004), evidence suggests that a balanced modulation of multiple targets can provide a superior therapeutic effect and side effect profile compared to the action of a selective ligand. *Designed* multiple ligands that hit a large variety of targets have been produced through rational approaches in which structural features from selective ligands are combined (Morphy et al., 2004). A key challenge in the design of multiple ligands is attaining a balanced activity at each target of interest with a suitable pharmacokinetic profile.

The third issue is that mechanisms underlying symptoms in IBS may differ among patients, hence the need to consider using multiple therapies. With selective drugs, primary clinical endpoints were achieved in less than 70% of patients with the approved agents such as tegaserod or alosetron (Camilleri et al., 2000; Muller-Lissner et al., 2001; Cremonini et al., 2003). On the other hand, it seems reasonable to propose treatment with combination therapy, which is the rule when treating medical conditions such as hypertension or asthma, when monotherapy is no longer adequate. Because of the redundancy of mechanisms controlling neurosensory, neuromuscular, and neuroimmune functions in the gut, it is conceivable that effective treatment of functional gut disorders may require combination therapy.

One example is provided by tachykinin receptor antagonists, which have so far given disappointing results because of inherent differences among animal models and humans: it has been suggested that the analgesic efficacy of multi- or pan-tachykinin receptor antagonists is superior to that of mono-receptor antagonists (Holzer, 2004a).

When drugs address a specific target (e.g., a symptom such as visceral hypersensitivity or motility), heterogeneity in the pathophysiology impacts negatively on the therapeutic gain, if patients are not carefully selected in a clinical trial. Indeed, some of the disappointing results of the past can be ascribed to the lack of understanding of pathophysiology: the same symptom (e.g., diarrhea) does not necessarily depend on the same pathways in all patients.

Thus, new drugs should target a pathophysiological mechanism (provided that it is known!), rather than a specific receptor; on the other hand, recruiting carefully selected patient subgroups may significantly reduce the generalizability of the results of the trial.

Pharmacokinetics may help to achieve gut selectivity and reduce side-effects. This approach is particularly relevant when there are potential actions outside the gut, as it is indeed the case with peripherally restricted opioid receptor antagonists (such as methylnaltrexone and alvimopan), which do not cross the blood brain barrier and, in addition, have very low oral bioavailability (De Ponti, 2002; Holzer, 2004b). An example of minimally absorbed compound is also the guanylate cyclase-C agonist linaclotide (Wensel and Luthin, 2011; Busby et al., 2013), which is now FDA- and European Medicines Agency (EMA)-approved for IBS with constipation.

Another important pharmacokinetic property is the lack of interactions with food or other drugs. Significant interactions with CYP2D6 and CYP3A4 should be predicted in early drug discovery because of their involvement in drug metabolism with important pharmacogenetic aspects.

Finally, as regards safety aspects, apart from the standard safety evaluations, two issues deserve special attention following the experience with cisapride (torsades de pointes associated with QT prolongation; De Ponti et al., 2001) and alosetron (ischemic colitis; Moynihan, 2002). It is clear that even very rare events may negatively impact the risk/benefit balance of drugs that are used to provide symptom improvement of non-serious (though troublesome) diseases such as IBS (De Ponti et al., 2002; Tack et al., 2012). It is remarkable that in IBS with diarrhea, Shah et al. (2012) found that one adverse event resulting in study

discontinuation occurred for every 2.3 and 2.6 patients who benefited, respectively, from tricyclic antidepressants and alosetron, i.e., the number needed to harm was approximately 3. This is quite low, considering the numbers needed to treat reported in the literature for drugs in IBS (Brandt et al., 2009; Camilleri and Mayer, 2009; Menees et al., 2012). Shah et al. (2012) conclude that, rather than simply focusing on the number needed to treat, clinicians should be aware of harm when using pharmacotherapy for IBS.

IBS AS A "FUNCTIONAL DISORDER": NEW PERSPECTIVES AND GLOBAL REGULATORY FRAMEWORK

The classical concept of IBS as a functional disorder derives from the fact that no organic cause can be identified and the diagnosis of IBS is one of exclusion after other disorders have been ruled out. In addition, the precise mechanisms underlying symptom generation are unknown.

However, research of the past 20 years has provided significant advances in the understanding of the pathophysiology of IBS, with an emerging consensus that the various clinical manifestations (including non-gastrointestinal comorbid symptoms) of chronic abdominal pain can best be viewed as a dysregulation in the complex interplay between events occurring in the gut lumen (including microbiota), the mucosa, the enteric nervous system (ENS), and the central nervous system (Mayer and Tillisch, 2011; Matricon et al., 2012). This dysregulaton leads to alterations in sensation, motility, brain-gut interactions, and neuroimmune interactions. Considerable evidence documents that sensitizing proinflammatory mediators, mast cells and their products, tryptases, are increased in tissues of patients with colorectal hypersensitivity (Cenac et al., 2007; Balestra et al., 2012; Buhner et al., 2012).

It has been shown that colonic mast cell infiltration and mediator release in proximity to mucosal innervation may contribute to abdominal pain perception in IBS patients (Barbara et al., 2004). Indeed, mucosal mast cell mediators from IBS patients excite rat nociceptive visceral sensory nerves (Barbara et al., 2007). In a recent study (Balestra et al., 2012), mucosal biopsies were obtained from the descending colon of patients with IBS and controls. Mucosal mast cells were identified immunohistochemically. The impact of spontaneously released mucosal mediators on guinea pig electrically stimulated longitudinal muscle myenteric plexus (LMMP) preparations was assessed *in vitro* by means of selective receptor antagonists and inhibitors. Patients with IBS showed an increased mast cell count compared with controls. Application of mucosal mediators of IBS to LMMPs potentiated cholinergic twitch contractions, an effect directly correlated with mast cell counts and mediated by activation of prostanoid receptors, TRPV1, and P2X receptors. These results support the role of mucosal inflammatory mediators and mast cell activation in altered motor function of IBS.

It is also intriguing that, in patients with IBS, 5-HT spontaneous release was significantly increased irrespective of bowel habit and correlated with mast cell counts and the severity of abdominal pain. This suggests that increased 5-HT release contributes to development of abdominal pain in IBS, probably through mucosal immune activation (Cremon et al., 2011).

Several studies have reported the onset of IBS-like symptoms following established bacterial or viral infections of the GI tract (Barbara et al., 2009). This so-called "postinfectious" IBS occurs in approximately 10% of patients undergoing a documented infectious gastroenteritis, and risk factors to develop symptom persistence are longer duration of the gastroenteritis, female sex, psychosocial stressors at the time of the infection, and psychological factors such as anxiety or depression. Although a causal relationship between abdominal pain and acute or chronic infections cannot be established most of the times, it is tempting to speculate that host-microbial interactions in vulnerable individuals during the early phase of the disorder may lead to permanently altered immune response, which then continues to play a role when symptoms persist in the absence of the infectious organism.

The participation of the gut microenvironment in the pathophysiology of IBS is suggested by studies indicating an interplay between luminal factors including the microbiome, the epithelial barrier, and the mucosal immune system (Stanghellini et al., 2010; Camilleri et al., 2012). In an animal model (McVey Neufeld et al., 2013), microbiota were shown to be necessary for normal excitability of gut sensory neurons and this provides a potential mechanism for the transfer of information between the microbiota and the nervous system.

In postinfectious IBS and in IBS with diarrhea, decreased expression and structural rearrangement of tight junction proteins in the small bowel and colon may lead to increased intestinal permeability. These abnormalities might contribute to the outflow of antigens through the epithelium, causing overstimulation of the mucosal immune system. Accordingly, subgroups of patients with IBS show higher numbers and activation of mucosal immunocytes, especially mast cells. Immune factors, released by these cells, including proteases, histamine, and prostanoids, might also participate in maintaining the permeability dysfunction and contribute to the activation of abnormal neural responses, which, in turn, are involved in abdominal pain perception and changes in bowel habits.

All these mechanisms represent new therapeutic targets in IBS. Here, it is important to remember that probiotics are also currently viewed as an attractive therapeutic option in IBS because of their recognized safety and of their documented biological effects on the host. Preclinical studies have shown that some probiotic strains exhibit potentially useful properties including anti-inflammatory effects, improvement of mucosal barrier homeostasis, beneficial effects on intestinal microbiota, and a reduction of visceral hypersensitivity. However, it remains to be determined to what extent a beneficial effect on these parameter translates to a significant effect on clinical outcomes: although the effect of probiotics on IBS is positive in some randomized, controlled studies, the gain over placebo is small and identification of a tailored probiotic approach for subgroups of patients represents a future challenge.

The complex neuroimmune and brain-gut interactions sometimes associated with minimal mucosal inflammation (Ford and Talley, 2011) and neuroplastic changes in the ENS (Giaroni et al., 1999) pose several questions as regards potential targets for pharmacological intervention: should the therapeutic focus be primarily gut motility, visceral sensitivity, or minimal inflammation? Assessment of these parameters in humans can be undertaken by

using a variety of invasive and non-invasive techniques, some well established and others requiring further validation. By using these techniques, alterations in both sensory and motor function have been reported in IBS and our understanding of sensorimotor dysfunction has indeed increased. Thus, inflammatory, immunologic, and other processes, as well as psychosocial factors such as stress, can alter the normal patterns of sensitivity and motility through alterations in local reflex activity or via altered neural processing along the brain-gut axis. A firm relationship between sensorimotor dysfunction and the production of symptoms, however, has been difficult to show. Thus, the clinical relevance of the former requires further research.

In this context, it is important to remember that in 2003 the EMA adopted a document produced by the Efficacy Working Party on the "*Points to consider on the evaluation of medicinal products for the treatment of the irritable bowel syndrome*" (EMA, 2003). Although the document now needs to be updated (it still refers to Rome II criteria), a key statement is that "*The patient's global assessment of symptoms and abdominal discomfort/pain should be used as the two primary endpoints. Statistically significant changes must be found in both parameters.*" Thus, clinical efficacy must rely on clinical endpoints in the patient's perspective, for instance through the global assessment of multiple symptoms. Mechanistic (pathophysiological) studies provide a rationale for drug development, but do not generally predict symptomatic success and do not necessarily identify the most appropriate dose for clinical trials. An important goal is to develop non-invasive tests that identify important pathophysiological mechanisms and assess symptom pattern in short-term (4 weeks) therapeutic trials that pave the way to longer trials. Notably, the EMA document carefully considers the duration of efficacy trials, stating that they must be long enough to determine whether the response is sustained and to determine the effects of treatment withdrawal. A duration of 6 months of active treatment is considered necessary considering the cyclic and non-life threatening nature of the disease.

For inclusion and exclusion criteria for IBS, the current EMA "*Points to consider*" document refers to Rome II criteria (current Rome III criteria differ from Rome II and, notably, the Rome IV process is expected to start in 2013). A revised EMA guideline is awaited soon and should come into force by the end of 2013. A key issue that needs to be addressed, apart from the update to Rome III, is the discrepancy with the FDA guidance issued in 2012 (FDA, 2012). Indeed, the EMA document recommends the two co-primary endpoints indicated above, whereas the FDA guidance recommends a primary endpoint that measures the effect of treatment on two major IBS signs and symptoms: abdominal pain and abnormal defecation (stool frequency or stool consistency, depending on subtype of IBS). The FDA guidance also acknowledges that patient-reported outcome (PRO) measures of the signs and symptoms of the condition are the only currently available measures that can adequately define a treatment effect in a clinical trial. In addition, because of the limitations of using a single-item patient-reported rating of overall change as a primary endpoint, the FDA document recommends the development of a multi-item PRO instrument. The PRO measure(s) should capture all the clinically important signs and symptoms of the target population. The ongoing regulatory discussion will certainly help all those involved in clinical trials to plan future research.

Linaclotide may serve as an example of the current regulatory situation, with differences in endpoints recommended by the FDA and the EMA: the efficacy and safety of this agent in patients with IBS-C was evaluated in two randomized, placebo-controlled Phase 3 trials. These trials were designed according to both FDA and EMA guidelines and findings based on FDA-recommended endpoints were reported in two recent studies (Chey et al., 2012; Rao et al., 2012), whereas the findings of a planned, separate analysis of both trials based on the distinct efficacy parameters prespecified for EMA submission were published separately (Quigley et al., 2013).

In closing this section, it should be remembered that, over the past 25 years, the Rome process has insisted on clinical features to diagnose IBS on the assumption that grouping of patients with similar features facilitates identification of patients most likely to respond to a given pharmacological agent, but this is not necessarily so because the same symptom (pain) may have several underlying pathophysiological mechanisms. This explains the criticism raised by some investigators against the Rome criteria (Dang et al., 2012). On the other hand, it must be acknowledged that there are several reasons to establish an accurate diagnosis of IBS: to relieve patient uncertainty and initiate the most appropriate treatment, avoiding the burden of unnecessary medications or diagnostic procedures and surgeries (Mearin and Lacy, 2012). In other words, the Rome criteria try to transform the diagnosis of IBS from one of exclusion into a positive diagnosis based on history, physical examination, use of precise diagnostic criteria in the absence of specific alarm features.

BIOMARKERS FOR IBS

Biomarkers are objectively measurable indicators of normal or pathological processes or pharmacological responses to a therapeutic intervention (Anonymous, 2001). In order to improve development and usage of biomarkers, a score system of different types of biomarkers has been proposed, depending on their impact on drug development (Wehling, 2009).

Positive modifications of biomarkers which imply improvement of the disease can be taken as endpoints during drug development. To provide a more complete picture, a therapeutic target can coincide with the biomarker (e.g., TNFα) or, as a component of the disease mechanism, modulate it (e.g., NF-κB).

Unfortunately, as stated in the 2012 FDA document, no validated and accepted biomarker exists in IBS. In addition, the limited repertoire of clinical manifestations of sensorimotor disorders of the gut such as IBS can actually derive from multiple mechanisms, leading in turn to similar symptoms. In many clinical programs of new drugs for IBS, the emphasis was primarily on symptom assessment of broad groups of patients identified by the Rome criteria. As already discussed above, this approach was not ideal and it not surprising that drugs of potential value have been abandoned.

Certain biomarkers can, in a limited fashion, be used to predict the success of a drug in IBS or to understand its mode of action. These studies may be incorporated in the recommended steps for drug development, but should be viewed only as preliminary/complementary steps of the development program, which

must comply with the regulatory guidance quoted in the previous section.

Currently established tests that can be used as potential biomarkers for clinically relevant endpoints in IBS include the following.

INTRALUMINAL MEASUREMENTS OF COLONIC OR RECTAL MOTILITY AND SENSATION

Intraluminal measurements may serve as biomarkers for motor or sensory modulation in IBS. Manometry has long been used for pathophysiological investigations (Camilleri et al., 2008) and can be used as a useful technique to study the effects of drugs on colonic motility (De Schryver et al., 2002; Dinning and Scott, 2011). Another possible test is intracolonic measurement of postprandial tone using a barostat, which, in healthy subjects (von der Ohe et al., 1994), showed the potential of 5-HT_3 receptor antagonists to prevent diarrhea and other postprandial symptoms in diseases including IBS and carcinoid diarrhea (von der Ohe et al., 1993). This indicates that measuring tone intraluminally may be a useful biomarker for preliminary tests before subsequent trials for efficacy. A recent study in healthy subjects (Sweetser et al., 2009) investigated the effects of lubiprostone on colonic sensation and motility with the following endpoints: colonic compliance, fasting and postprandial tone and motility indexes, pain thresholds, and sensory ratings to distensions. This investigation well exemplifies the potential of pharmacodynamic studies in drug development.

Although testing visceral sensitivity may provide useful mechanistic insights when developing new medications, results always require careful interpretation and are sometimes disappointing. For instance, alosetron was shown to alter colonic compliance, but not colonic sensitivity to isobaric distension (Delvaux et al., 1998). Previously, the κ opioid receptor agonist fedotozine was shown to decrease sensitivity to colonic distension, but the therapeutic gain in placebo-controlled studies in IBS was found to be of insufficient magnitude for further development (Dapoigny et al., 1995; Delvaux et al., 1999; Ness, 1999). Asimadoline is another example of drug tested for its effect on visceral sensitivity in humans (Delgado-Aros et al., 2003).

One disadvantage of sensation biomarkers is that the sample size required to avoid a type 2 error while assessing clinically meaningful effect sizes is higher than with transit endpoints in healthy volunteers and probably even higher in patients. Nevertheless, these sample sizes (12–20 per treatment arm) are still more practical than testing symptom endpoints, which require much larger samples. Thus, a 25–30% effect size can be demonstrated with ∼20 subjects per treatment arm in sensation-based studies and ∼12 per treatment arm in studies of transit, on the basis of the variability reported in published studies (Camilleri et al., 2006b).

RADIOPAQUE MARKERS FOR COLONIC TRANSIT

The radiopaque marker test for colonic transit is a widely available test, as shown by early studies with loperamide for diarrhea and fiber for constipation, where radiopaque markers were used to assess whole gut transit time (Cann et al., 1984a,b). The overall effects of drugs for IBS can be predicted by the marker transit test, although transit times are not characteristic of IBS (Horikawa et al., 1999) and other studies addressing more specific endpoints suggest that the colonic marker transit time (<15 or >60 h) accurately predicts the extremes of stool consistency, with significant overlap for transit times between those extremes (Degen et al., 2001).

RADIOSCINTIGRAPHIC MARKERS FOR COLONIC TRANSIT

Scintigraphic transit measurements are sufficiently well characterized to allow meaningful pharmacodynamic conclusions on the effect of therapeutic agents (Cremonini et al., 2002; Camilleri, 2010a; Vazquez-Roque et al., 2012). Namely, several examples support the use of detailed colonic transit measurement in the development of medications for IBS-associated changes in bowel function. First, alosetron (a 5-HT_3 receptor antagonist) slows overall colonic transit and, on average, increases the time for emptying the ascending colon by 50% (Viramontes et al., 2001a). Second, tegaserod (a 5-HT_4 receptor agonist) accelerates overall colonic transit and, on average, halves the time for emptying the ascending colon (Prather et al., 2000), and several studies showed that this medication was effective in the treatment of IBS with constipation (Muller-Lissner et al., 2001; Novick et al., 2002; Kellow et al., 2003). However, tegaserod was withdrawn by the manufacturer for safety issues in 2007 and was made available only under a restricted access program (Al-Judaibi et al., 2010). Finally, a more recent example of use of scintigraphy to assess transit includes linaclotide (Andresen et al., 2007).

URINE SUGARS FOR *IN VIVO* GUT PERMEABILITY

Because of the possible role of disruption of intestinal mucosal barrier function in the pathophysiology of IBS, recently a urine sugar (lactulose and mannitol) excretion test was validated in patients with IBS and diarrhea (Rao et al., 2011). Urine sugars at 0–2 and 8–24 h reflect small bowel and colonic permeability, respectively and are increased in patients with IBS and diarrhea vs. controls. This method can be applied to study the effects of agents directed at mucosal pathophysiology, such as mast cell stabilizers or modulators of microbial flora.

ANIMAL MODELS: PREDICTIVITY ISSUES AND CURRENT ROLE

Translational medicine defines conditions and prerequisites to transfer more reliable results obtained from preclinical biomedical research to clinical applications, thus improving patient care through timely and efficient promotion of clinical innovation.

A disease model should mimic the clinical disease condition as much as possible and allow the investigation of unclear pathophysiological mechanisms toward the development of new potential therapeutic options. In spite of undeniable differences among species, animal models still represent the major source of information about biological system and provide an invaluable means to study complex physiological and biochemical interactions.

The predictivity of the disease model itself will substantially depend on the efforts toward optimization and scientific validation of the model, both in terms of resemblance and transferability to humans. Pharmacological targets and biomarkers must be reproduced and be susceptible to pharmacological modulation in the animal model, to ensure that the model is predictive of a therapeutic effect in humans. The real translational value of a biomarker is

represented by its capacity of being thoroughly informative after its validation in clinical studies which provide the bedside to bench approach.

An animal model used as a screening tool in drug development should ideally reproduce different biomarkers providing multiple endpoints to fully assess the spectrum of activity of a test compound. Accurate selection of endpoints plays a pivotal role to assure the translational value of disease models and should take into account the sensitivity and specificity of each endpoint toward prediction of activity.

Although development of new drugs for treatment of IBS can be facilitated by preclinical animal models (Bulmer and Grundy, 2011; Holschneider et al., 2011), it must be acknowledged that a single sufficiently predictive model of efficacy still does not exist and investigators have the option to use multiple models to assess different aspects of the pathophysiology of IBS. This section is only intended as a brief outline of the most commonly used animal models of visceral pain and disturbed gastrointestinal motility, which are reviewed elsewhere (Camilleri et al., 2006a).

MOTILITY

The techniques used to record motility or measure transit in animals may differ from techniques used in humans but the endpoints are identical to those mentioned in the previous section.

VISCERAL PAIN

There are several forms of stimulation and endpoints to measure visceral pain: (a) *mechanical stimuli*: experiments can be performed in awake or anesthetized rats, and the most frequently used stimulus of pain in animals is distension of a gut segment with a balloon connected to a barostat to measure simultaneously compliance and the response to the painful stimulus (Rouzade et al., 1998). Poor standardization of methods across laboratories is a drawback of these investigations. (b) *Chemical stimuli:* infusing glycerol into the rat colon through a chronically implanted catheter induces abdominal cramps. This model is considered relevant because intracolonic glycerol induces abdominal pain in humans and mimics pain reported by patients with IBS (Louvel et al., 1996). (c) *Other stimuli:* the significance of other models of visceral pain, such as the "writhing test" (consisting of an intraperitoneal injection of an irritant compound such as acetic acid), is questionable.

In the aforementioned models, it is important to distinguish between evaluation of *allodynia* (decrease in the threshold of sensitivity to distension) and *hyperalgesia* (enhanced response to painful stimulus). A commonly used endpoint is the contraction of abdominal muscles induced by rectal or colorectal distension in the rat; the contractions are recorded by electromyography, where the number of spike bursts correlates with the intensity of the stimulus applied (Morteau et al., 1994).

Visceral distension also induces *viscero-visceral reflexes*, such as relaxation of anal sphincters during rectal distension or change in *blood pressure*, which is a pseudoaffective response used to assess visceral pain.

In summary, selection of one or more definitive animal models of visceral hyperalgesia is not possible and using results from more than one animal model may enhance the probability of selecting effective drugs for further development. Since pain, though a major problem, is not the only symptom affecting quality of life, animal models detailing the effects of drugs on motility and visceral sensitivity may add a further dimension to the assessment of new compounds.

THE ROLE OF PHARMACOGENOMICS

Pharmacogenomics refers to the variability of the expression of individual genes relevant to disease susceptibility as well as drug response at cellular, tissue, individual, or population level. Pharmacogenetics refers more specifically to the study of individual variations in DNA sequence related to drug response. The growing interest in this field is due to the fact that risk/benefit evaluations of drugs are not fully appreciated if one does not fully consider individual variations that may significantly affect pharmacokinetics and pharmacodynamics.

POLYMORPHISMS AS MARKERS OF DISEASE

As an example, patients with IBS had significantly reduced frequencies of the high producer genotype for interleukin 10 than controls (21 vs. 32%; $p = 0.003$): this suggests a genetic predisposition in at least some patients with IBS to produce lower amounts of the anti-inflammatory cytokine interleukin 10 (Gonsalkorale et al., 2003) and lends support to the hypothesis that there may be an inflammatory or genetic component in some cases of IBS (Bashashati et al., 2012).

POLYMORPHISMS AFFECTING DRUG RESPONSE

Genetic variations can affect drug response in at least three different ways (Camilleri and Katzka, 2012): (a) changes in drug metabolism, e.g., through functional CYP450 2D6 genes, which determine the pharmacokinetics and plasma levels of many commonly used agents such as antidepressants; (b) changes in drug transporters, which may affect the response to medications: for instance, polymorphisms in the promoter for synthesis of serotonin transporter (SERT-P) influence response to serotonergic medications in depressed individuals. SERT polymorphisms were associated with a greater colonic transit response in those with long homozygous than those with heterozygous polymorphisms in D-IBS (Camilleri et al., 2002); (c) genetic polymorphism in drug targets. Several examples are provided by recent studies (Camilleri and Katzka, 2012; Vazquez-Roque et al., 2012).

In summary, pharmacogenetics is a rapidly growing field which may provide important pieces of information to fully understand the variability of drug action in patients and cannot be ignored in drug development programs, although its exploitation probably still needs some time.

CLASSES OF DRUGS USED OR UNDER DEVELOPMENT IN IBS

A detailed discussion of all the classes of drugs proposed as potential therapeutic agents in IBS is beyond the scope of this review. The main pharmacological approaches to IBS are summarized in **Table 2**, which does not include agents traditionally used in IBS, such as laxatives and antidiarrheal agents, respectively for constipation and diarrhea, and probiotics. The gut microbiome as a therapeutic target is covered elsewhere (Floch et al., 2011; Simren

Table 2 | Main pharmacological approaches in IBS.

Drug class	Examples	Pharmacodynamics
5-HT$_4$ receptor agonists	Prucalopride Naronapride Velusetrag TD-8954	Enteric neurons, smooth muscle cells Increased motility/bowel frequency
5-HT$_3$-receptor antagonists	Ramosetron	Inhibition of secretion, motility, nociception
TPH$_1$ blocker	LX-1031	Inhibits 5-HT synthesis by blocking tryptophane hydroxylase 1
Cl-C2 channel activator	Lubiprostone	Increased intestinal water and electrolyte secretion Accelerates transit
Guanylate cyclase-C agonist	Linaclotide	Increased intestinal water and electrolyte secretion Accelerates transit
PAR2 blockers	GB88	Inflammation
TRPV1 blockers	Capsazepine	Inflammation
TRPV4 blockers	RN1734	
Mast cell stabilizers	Ketotifen	Inflammation
μ-Opioid receptor agonists	Loperamide	Enterocyte, enteric neurons, afferent neurons, and inflammation
μ-Opioid receptor antagonists	Naloxone Methylnaltrexone alvimopan	Enteric neurons, afferent neurons, and inflammation
κ-Opioid receptor agonists	Asimadoline	Enteric neurons and afferent neurons Increase in sensory threshold
β$_3$-Adrenoceptor agonists	Solabegron	Smooth muscle
α$_2$-Adrenoceptor agonists	Clonidine	Enteric neurons and enterocytes
NK$_1$ receptor antagonists	Ezlopitant	Enteric neurons, ICC, smooth muscle, immune cells
NK$_2$ receptor antagonists	Nepadutant	Reduced smooth muscle contractility
NK$_3$ receptor antagonists	Talnetant	Role in nociception not confirmed in clinical trials in patients with IBS
CCK$_1$ receptor antagonists	Loxiglumide	Afferent vagal nerves and enteric neurons
Antibiotics	Rifaximin	Poorly absorbed with virtually no systemic effects

et al., 2013). The reader is also referred to a recent detailed review of current and potential pharmacological approaches in IBS (Camilleri, 2012). Herbal preparations used in IBS are covered by another review (Rahimi and Abdollahi, 2012). Brain-gut interactions and possible sites/mechanisms of pharmacological intervention along the brain-gut axis are discussed in a recent review in this Journal (Fichna and Storr, 2012).

Several meta-analyzes of pharmacological treatments for IBS have been published in recent years and there is ongoing debate on their interpretation (Lesbros-Pantoflickova et al., 2004; Brandt et al., 2009; Camilleri and Mayer, 2009).

SEROTONERGIC AGENTS

5-Hydroxytryptamine (5-HT) plays a key role in the control of gastrointestinal motility, sensitivity, and secretion (De Ponti, 2004). Several 5-HT receptor types are present on both nerves and smooth muscle and mediate a number of different actions (De Ponti, 2004). Actions of 5-HT are terminated by a reuptake system, which is inhibited by antidepressants (Gershon and Jonakait, 1979). *Selective serotonin reuptake inhibitors* (SSRIs) alter motility in the stomach, small bowel, and colon (Gorard et al., 1994), but no convincing beneficial therapeutic effects have been reported in IBS. Interestingly, the tryptophane hydroxylase inhibitor LX-1031 was recently reported to have therapeutic potential in IBS (Tack et al., 2011).

5-HT$_3$ receptor antagonists include alosetron and ramosetron (Camilleri et al., 2000; Hirata et al., 2007; Lee et al., 2011); alosetron delays orocecal and colonic transit times, and reduce colonic compliance but not sensitivity to isobaric distension (Gore et al., 1990; Talley et al., 1990; Scolapio et al., 1995). Shortly after its introduction, alosetron was withdrawn due to suspected side-effects of colonic ischemia (Moynihan, 2002).

5-HT_4 receptor agonists, after withdrawal of tegaserod, now include prucalopride, naronapride, velusetrag (Camilleri, 2010b), and TD-8954 (Beattie et al., 2011). These agents are thought to act on intrinsic neurons to stimulate gastric, small bowel, and colonic transit in health, in constipation and in IBS with constipation (Bouras et al., 1999; Poen et al., 1999; Degen et al., 2001). In the stomach, 5-HT_4 receptor agonists enhance (postprandial) proximal gastric volumes in health, but have no effects on sensation (Tack et al., 2003). Prucalopride was also shown to be effective in the treatment of constipation (Emmanuel et al., 2002).

LINACLOTIDE

This is an example of guanylate cyclase-C agonist (Busby et al., 2013), which has been shown to reduce visceral hypersensitivity in preclinical studies and to improve abdominal pain and constipation symptoms in phase 2 and 3 clinical trials of patients with IBS and constipation (Johnston et al., 2013).

LUBIPROSTONE

This is an oral bicyclic fatty acid selectively activating type 2 chloride channels in the apical membrane of the intestinal epithelial cells, hence stimulating chloride secretion, along with passive secretion of sodium and water, inducing peristalsis and laxation, without stimulating gastrointestinal smooth muscle (Schey and Rao, 2011). It is indicated in IBS with constipation. Considering the importance of epithelial barrier function and cell integrity and the known impact of stressors, the observation that lubiprostone exhibits the additional distinct property of effective protection or repair of the epithelial barrier and cell function after stress suggests potential clinical importance for patients with impaired barrier function, which might occur in IBS (Cuppoletti et al., 2012).

TACHYKININ RECEPTOR ANTAGONISTS

Three distinct receptors, NK_1, NK_2, and NK_3, mediate the biological effects of endogenous tachykinins SP, NKA, and NKB, in the gastrointestinal tract. Through the locations of NK receptors on intrinsic nerves, extrinsic nerves, inflammatory cells, and smooth muscle, inhibition of tachykinin receptors has the potential to inhibit motility, sensitivity, secretion, and inflammation in the gastrointestinal tract (Holzer, 2004a; Lecci et al., 2004). NK_1 receptor antagonists also have antiemetic properties (Holzer, 2004a). Several tachykinin receptor antagonists have been developed so far, but the results, in general, have been disappointing. Recently, chronic treatment with AV608 (NK_1 receptor antagonist) in IBS has been reported to be associated with improved mood and pain ratings and activity of emotional arousal related brain regions (Tillisch et al., 2012).

ADRENOCEPTOR AGONIST

The α_2-adrenoceptor agonist clonidine was shown to reduce colonic tone and pain sensation in response to distension (Bharucha et al., 1997; Malcolm et al., 2000; Viramontes et al., 2001b). A preliminary study of clonidine in IBS with diarrhea suggested therapeutic potential for clonidine, but clinical application is hampered by dose-limiting side-effects like somnolence or hypotension. Among β_3-adrenoceptor agonists, solabegron is an example (Grudell et al., 2008).

OPIOID RECEPTOR LIGANDS

Three types of opioid receptors, μ-, δ-, and κ-receptors, located in the ENS as well as on nociceptive pathways, have effects on human gastrointestinal function. Opioid receptor activation reduces visceral pain through peripheral (spinal afferents) and central mechanisms, and inhibits motility through decreased acetylcholine release. κ-Opioid receptor agonists have been proposed as a pharmacological approach to the treatment of altered visceral sensitivity. Acute studies with fedotozine and asimadoline showed decreased sensitivity to gastric or colonic distension (Coffin et al., 1996; Delvaux et al., 1999, 2002; Delgado-Aros et al., 2003). However, therapeutic studies in IBS and FD with fedotozine have been disappointing (Dapoigny et al., 1995; Read et al., 1997). The μ-opioid receptor agonist loperamide, used in the treatment of diarrhea, inhibits secretion, reduces colonic transit, and increases resting anal sphincter tone (Corazziari, 1999). Peripherally acting μ-opioid receptor antagonists (e.g., N-methylnaltrexone and alvimopan) normalize bowel function in opiate-treated patients without compromising central opioid analgesia (Holzer, 2004b). Racecadotril, a neutral endopeptidase (NEP) inhibitor, increases the exposure to NEP substrates including enkephalins: it was found consistently effective in animal models and patients with various forms of acute diarrhea by inhibiting secretion from the gut without changing gastrointestinal transit time or motility (Eberlin et al., 2012). In direct comparative studies with loperamide, racecadotril was at least as effective, but exhibited fewer adverse events in most studies, particularly less rebound constipation (Eberlin et al., 2012). However, its potential in IBS remains to be established.

MISCELLANEOUS AGENTS

CCK has a large number of effects on gastrointestinal contractility and secretion (Walsh, 1994). CCK1 receptor antagonists like loxiglumide and dexloxiglumide enhance gastric emptying in health and in IBS with constipation, though effects on colonic motility are unclear (De Ponti and Malagelada, 1998; Scarpignato and Pelosini, 1999) and clinical usefulness has not been established.

The transient receptor potential ion channel of the *vanilloid type 1 (TRPV1)*, expressed by primary afferent neurons, is viewed as a trigger for chemonociception and may be upregulated in some functional gut disorders (Chan et al., 2003). TRPV1 and TRPV4 blockade are areas of current investigation (Holzer, 2011; Fichna et al., 2012).

Muscarinic receptor antagonists and *smooth muscle relaxants* are used in some countries for the treatment of IBS. Meta-analysis suggests they are superior to placebo in IBS-related pain (Poynard et al., 2001), though the quality of trials is often questionable.

Cannabinoid CB_1 receptors are expressed on nociceptive afferents and ENS neurons while CB_2 receptors are expressed on immune cells (Schicho and Storr, 2011). Activation of CB_1 receptors slows gastrointestinal transit in animals through inhibition of acetylcholine release. The non-specific agonist delta-9-tetrahydrocannabinol has strong antiemetic properties and delays gastric emptying in humans (Frytak et al., 1979; McCallum et al., 1999). The observation that, in an animal model of intestinal inflammation, CB_1 and CB_2 receptor subtypes are upregulated opens a new perspective on the possible use of CB_1 or CB_2 receptor

agonists in postinfectious IBS with diarrhea (Kimball et al., 2010). Indeed, dronabinol, a non-selective CB agonist, reduces fasting colonic motility in patients with IBS with diarrhea or alternating (Wong et al., 2011).

Finally, α2δ ligands such as gabapentin and pregabalin (Gale and Houghton, 2011) have undergone a number of preclinical and clinical tests for their potential in disorders with visceral hypersensitivity. In fact, voltage-sensitive Ca^{2+} channels are involved in neural function and have an α2δ binding site to which the aforementioned ligands bind potently, reducing Ca^{2+} influx at the nerve terminals. Pregabalin was effective in several animal models of visceral pain (Gale and Houghton, 2011).

CONCLUSION

From the above overview, it is clear that research in the treatment of IBS is still very active. Although in the past decade some innovative pharmacological agents have not fulfilled their promise because of unexpected side-effects, it is likely that new pathophysiological concepts as well as the publication of new regulatory documents by the FDA and the EMA will be of great help for drug developers.

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Brain-gut interactions in IBS

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Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with an estimated prevalence of 10–20%. Current understanding of the pathophysiology of IBS is incomplete due to the lack of a clearly identified pathological abnormality and due to the lack of reliable biomarkers. Possible mechanisms believed to contribute to IBS development and IBS like symptoms include physical stressors, such as infection or inflammation, psychological, and environmental factors, like anxiety, depression, and significant negative life events. Some of these mechanisms may involve the brain-gut axis (BGA). In this article we review the current knowledge on the possible involvement of the BGA in IBS and discuss new directions for potential future therapies of IBS.

Keywords: irritable bowel syndrome, brain-gut axis, pathophysiology, autonomic nervous system, hypothalamo-pituitary-adrenal axis

INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder with an estimated prevalence of 10–20% (Philpott et al., 2011). According to Thompson et al. (2000) it accounts for about 3% of all general practice and up to 40% of all GI referrals. IBS causes considerable morbidity amongst its sufferers, who manifest with abdominal pain and altered stool consistency and frequency (Drossman and Dumitrascu, 2006; Lee et al., 2007; Adeyemo et al., 2010). Although not life-threatening, it is a heavy economic burden due to increased work absenteeism and impaired quality of life of its sufferers, as well as increased use of health care services (Sandler et al., 2002).

Current understanding of the pathogenesis of IBS is unsatisfactory due to the lack of demonstrable pathological abnormalities and reliable biomarkers. Traditionally, IBS has been considered a purely functional disorder. A hypothesis based on specimens obtained at endoscopy and in serological cytokine studies views IBS as a localized low grade inflammatory disorder with mast cells (MC) playing a particularly important role (Mayer and Collins, 2002; Philpott et al., 2011). An alternative hypothesis states that food allergy may be responsible (Atkinson et al., 2004). Most recently, the relationship between the neural and immunological networks within the gut and the bi-directional communication between the gut and the central nervous system (CNS), often related to as the brain-gut axis (BGA) attract most attention (Collins and Bercik, 2009).

In this review we focus on the disturbances in the BGA as a plausible cause of IBS. We overview the pathophysiological mechanisms contributing to symptom perception and generation and the endogenous systems involved. Particular attention is given to stress, emotion and psychological factors in the IBS pathogenesis. We also discuss new directions for potential future therapies of IBS based on discussed mechanisms.

Abbreviations: ACC, anterior cingulate cortex; ACTH, adrenocorticotropic hormone; ANS, autonomic nervous system; BDNF, brain-derived neurotrophic factor; BZD, benzodiazepine; CNS, central nervous system; CRD, colorectal distension; CRF, corticotrophin-releasing factor; DLPFC, dorsolateral prefrontal cortex; DRG, dorsal root ganglia; DRN, dorsal raphe nucleus; ENS, enteric nervous system; GI, gastrointestinal; HPA axis, hypothalamo-pituitary-adrenal axis; IBS, irritable bowel syndrome; MC, mast cells; MRI, magnetic resonance imaging; NE, norepinephrine; PAG, periaqueductal gray; PVN, paraventricular nucleus; SERT, serotonin transporter protein; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin re-uptake inhibitor; TCA, tricyclic antidepressant; UCN, urocortin.

THE BRAIN-GUT AXIS

The BGA constitutes the enteric nervous system (ENS) and the gut wall in the periphery, the CNS, and the hypothalamo-pituitary-adrenal (HPA) axis (Collins and Bercik, 2009). The bi-directional communication between the gut and the CNS is based on the neural, endocrine and neuroimmune pathways. Neuronal pathways include afferent fibers originating in the dorsal root of the ganglia of the thoracic spinal cord (T1–T10) projecting to integrative cortical areas, such as the cerebral, anterior and posterior cingulate, insular, and amygdala cortices and efferent fibers to smooth muscle and glands, originating in nuclei within the brainstem, as well as S2–S4 spinal levels (parasympathetic) and in the lateral horn of the thoraco-lumbar spinal cord (T1–L3; sympathetic; Mulak and Bonaz, 2004; Gaman and Kuo, 2008; O'Mahony et al., 2011). The main pain signaling pathways in the BGA are the spinothalamic tracts and dorsal columns with descending supraspinal afferents originating from the rostral ventral medulla (Gaman and Kuo, 2008).

In physiological conditions, signals from the GI tract influence the brain, which in turn can exert changes in motility, secretion, and immune function (Mayer et al., 2006). The axis is therefore an important communication system for healthy regulation of food

intake, digestion, gut sensations, and control of the bowel movements. Structural and functional disruptions in the BGA cause changes in perceptual and reflexive responses of the nervous system and may lead to GI disorders, including IBS, which often comorbid with chronic psychiatric diseases (Clarke et al., 2009; Gros et al., 2009).

STRUCTURAL AND FUNCTIONAL ABNORMALITIES IN THE CENTRAL NERVOUS SYSTEM

Visceral hypersensitivity is a key mechanism underlying abdominal pain, one of the main symptoms of IBS (Azpiroz et al., 2007; Barbara et al., 2011). Visceral hypersensitivity is thought to be determined by central and peripheral mechanisms, as it may result from altered transmission within the gut wall, the spinal cord, or the brain. However, the specific contribution of the BGA components to hypersensitive responses in IBS remains unclear.

Direct imaging techniques were recently employed to detect the abnormalities in the structure and functioning of the brain and their possible implications in the pathology of IBS. There is only one structural magnetic resonance imaging (MRI) study (Davis et al., 2008), in which the thinning in the anterior midcingulate and insular cortex, structures important for perception of internal body states were observed in the IBS patients. These results were later confirmed by functional MRI (Blankstein et al., 2010). Although the underlying cause of cortical thinning was not elucidated, factors like decreased cell size, apoptosis of neural cells, death of glia and astrocytes, fewer dendritic spines, reduced synaptic density, and excitotoxicity related to enhanced glutamate signaling were suggested as possible contributors. Seminowicz et al. (2010) reported morphometric brain differences between female IBS patients and controls in terms of regional increases and decreases in gray matter density. These alterations occurred primarily in brain areas involved in attention and emotion modulation, as well as cortico-limbic pontine pain modulatory systems and in networks processing interoceptive information. Further studies of Blankstein et al. (2010) evidenced increased gray matter density in the hypothalamus of the IBS patients. Currently it is not possible to discern whether these changes are a predisposing factor for IBS or a secondary change after sustained visceral signals (Fukudo and Kanazawa, 2011).

In their excellent paper on imaging techniques used in the studies of brain-gut interactions, Rapps et al. (2008) reviewed the possible central mechanisms implicated in IBS and found published reports somewhat contradictory. The region that attracted most attention was the anterior cingulate cortex (ACC), one of the six most commonly reported cortical areas that display pain-evoked activity during acute stimulation in humans (Chen et al., 2011). ACC showed altered activity during rectal stimulation in IBS patients in comparison to healthy controls (Rapps et al., 2008 and citations therein). Interestingly, although greater pain by rectal balloon distension was reported by the IBS patients with a history of sexual or physical abuse, changes in their ACC activity were less pronounced than in other IBS patients and the controls (Ringel, 2002). In line with these observation was the study of Mertz et al. (2000), who demonstrated differential activation of the brain between IBS patients and controls. The ACC, the insula, the prefrontal cortex, and the thalamus were more activated in the IBS patients as compared with healthy controls and the pattern was related to the experience of individuals.

Hall et al. (2010) revealed differences in the central responses in health and in IBS to a single ramp-tonic distension of the colon across a distributed network of regions, involving sensory, striatal, limbic, and frontal areas. The IBS participants showed heightened activation of the ACC, suggesting increased affective responses to painful visceral stimuli. However, it was also observed that the activation of the thalamic, striatal, and dorsolateral prefrontal cortex (DLPFC) regions was relatively greater in control subjects, as compared to IBS patients, which may reflect increased ascending input to the brain, in particular to the cortex and a heightened arousal reaction to distension. Greater recruitment of the DLPFC by controls than IBS patients is consistent with the notion of abnormal descending modulation in IBS.

To further explore the central mechanisms of visceral hypersensitivity in IBS, Lawal et al. (2006) examined total cortical recruitment in response to subliminal (sub-conscious) stepped changes in distension pressure and observed that visceral hypersensitivity in IBS patients is due to increased afferent signaling to the brain, rather than altered processing at the level of the brain. However, the results of the study were later questioned, among others by Lackner et al. (2006), who showed that cognitive behavioral therapy in IBS patients is associated with a reduction of baseline activity in the ACC and accompanied by improvements of GI symptoms. Dorn et al. (2007) showed a contributory part of neurosensitivity in the form of enhanced activity with central neural networks independent of cognitive function.

The most novel findings of Chen et al. (2011) showing that the patients with IBS have white matter abnormalities in the insula, ACC, and other brain areas associated with pain, interoception, and homeostasis indicate that functional gray matter abnormalities in IBS patients are accompanied by white matter aberrations. The white matter deficiencies of the descending modulation of pain and dysfunction of the medial pain system may be responsible for the emotional aspect of pain in IBS.

In conclusion, as evidenced by the results of the meta-analysis performed by Tillisch et al. (2011), a greater engagement of regions associated with emotional arousal and endogenous pain modulation, but similar activation of regions involved in processing of visceral afferent information was observed in patients with IBS compared to controls. These results support a role for structural and functional abnormalities in the CNS in IBS.

COGNITIVE–BEHAVIORAL MODEL OF IBS

IBS is often considered a bio-psychosocial disorder (Engel, 1977; Camilleri and Choi, 1997; Drossman, 1998), which suggests that psychological (e.g., emotions, cognitions, and behavior), social (e.g., modeling, support), and physiological (e.g., cramps, bloating) factors may induce and exacerbate its symptoms (Toner et al., 1998; Mach, 2004). Individual cognitive and emotional responses to recurrent GI symptoms and associated life events may also affect the therapeutical efficacy of anti-IBS treatments (Kennedy et al., 2012).

CENTRAL MECHANISMS

Abnormal activity within higher-order brain systems may alter cognitive and affective processes and contribute to both abnormal pain regulation and higher levels of anxiety and depression, typically reported in chronic pain conditions (Ribeiro et al., 2005) and IBS (Piche et al., 2011, and citations therein). The cognitive-behavioral model of IBS is focused particularly on emotional arousal and organism response to stress and the integrated network of structures, which include the hypothalamus, amygdala, and periaqueductal gray (PAG), as well as a number of neuromodulators and hormones.

Greenwood-Van et al. (2001) showed in animal models that there is a link between the central pathways mediating stress and anxiety and the mechanisms regulating the GI sensitivity. A key component of this link is the amygdala, which is known for its role in the regulation of emotional behavior and the expression of fear and anxiety. Further studies in rodents demonstrated that colonic sensitivity and motility are increased following fear conditioning (Gue et al., 1991; Tyler et al., 2007). In accordance, studies on IBS patients showed substantial activation of the hypothalamus and amygdala, as well as decreased activity of the antinociceptive PAG (Naliboff et al., 2001). More recent investigations employing rectosigmoid balloon distension in IBS patients have shown increased activity in the amygdala, insula, cingulate, and prefrontal cortex, which form a network of brain structures involved in regulating affective and sensory processes (Naliboff et al., 2003; Wilder-Smith et al., 2004; Myers and Greenwood-Van, 2009).

ROLE OF ANS AND HPA AXIS

The autonomic nervous system (ANS) and the hypothalamus-pituitary-adrenal (HPA) axis are commonly regarded as the major components of the stress response system in the vertebrates. Alterations of this complex system have been linked to a variety of anxiety-related psychiatric disorders and stress-sensitive pain syndromes. Stress and stress-related psychosocial factors have also been proposed to act in IBS, particularly its post-infectious variety (PI-IBS), by overarching inflammation and the BGA (Arborelius et al., 1999; Gwee et al., 1999; Fukudo, 2007; Spiller and Garsed, 2009).

The correct function of the ANS and its cross-talk with CNS are important factors preventing from IBS. Disturbances at the ANS level, indicated by decreased parasympathetic and increased sympathetic activity and altered autonomic reflexes often occur in the IBS patients and account for the level of perception to GI stimuli and extra-intestinal symptoms (Azpiroz, 2002; Jarrett et al., 2003; Spaziani et al., 2008).

The key activator of the HPA axis is corticotrophin-releasing factor (CRF), an endogenous 41-amino acid neuropeptide secreted from endocrine cells in the paraventricular nucleus (PVN) of the hypothalamus (Aguilera et al., 2008). The action of CRF is mediated by the CRF1 and CRF2 receptors, which belong to the G protein-coupled receptor family (Kostich et al., 1998). CRF receptor activity can also be modulated by other peptides, like urocortins (UCN; Bale and Vale, 2004; Tache and Brunnhuber, 2008). In the mammalian brain three urocortins have been identified: UCN I, which binds to both receptors, and UCN II and UCN III, selectively binding to CRF2 receptor (Morin et al., 1999; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Bale and Vale, 2004; Dautzenberg et al., 2004). However, the neuroendocrine, autonomic, and behavioral responses to fear and stress are mediated exclusively by CRF and UCN I, which are selective CRF1 receptor ligands (Vale et al., 1981; Bale and Vale, 2004; Tache et al., 2009; Chen et al., 2011).

Corticotrophin-releasing factor and UCN I initiate the signaling cascade in the HPA axis by stimulating the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which in turn induces synthesis and secretion of glucocorticoids from the adrenal cortex. Growing evidence suggests that also the extra-hypothalamic CRF system is poised to play a critical role in both psychiatric and the BGA disorders (Lowry and Moore, 2006; Bravo et al., 2011).

In rodents, stress-induced release or exogenous administration of CRF and UCN I increased anxiety-like behaviors and stimulated colonic secretion, intestinal motility, and visceral sensitivity (Moreau et al., 1997; Slawecki et al., 1999; Saunders et al., 2002; Vetter et al., 2002; Million et al., 2003; Martinez et al., 2004; Tache et al., 2004, 2009). Johnson et al. (2010) provided evidence that elevated corticosterone levels affected the amygdala and significantly increased brain activation in response to colorectal distension (CRD) compared to that seen in cholesterol-treated controls. Elevated CRF expression was found in the thalamus of the rats exposed to neonatal maternal separation (Tjong et al., 2010). Deletion of the CRF1 gene using transgenic models or intraventricularly administered CRF1 antagonists had anxiolytic effects and attenuated stress- and CRF-induced alterations in gastric and colonic motor function (Smith et al., 1998; Million et al., 2003; Martinez and Tache, 2006; Trimble et al., 2007).

Only a limited number of studies in IBS patients measured basal and stimulated HPA axis hormone levels in response to meal, hormone challenge, or mental stress (Chang et al., 2009, and citations therein) and some of them demonstrated increased HPA axis responses in IBS compared to controls. Fukudo et al. (1998) observed that the intravenous injection of CRF in IBS patients induced exaggerated motility of the colon and increased visceral pain sensitivity compared with healthy controls, whereas administration of a non-selective CRF receptor antagonist ameliorated these responses (Lembo et al., 1996; Sagami et al., 2004). The recent study by Chang et al. (2009) showed that basal levels of plasma ACTH were significantly decreased, while both 24 h basal plasma cortisol levels and stress-induced cortisol levels were mildly elevated upon visceral stimulation in female IBS patients compared to controls, suggesting a dysregulation of the HPA axis in IBS. However, the role of the observed dysregulation of HPA axis in modulating IBS severity or abdominal pain remained unclear.

A meta-analysis performed by Tillisch et al. (2011) revealed that the central nucleus of amygdala indirectly activates the HPA axis and increases ACTH and glucocorticoid secretion via subcortical regions, which relay on PVN (Redgate and Fahringer, 1973; Feldman and Weidenfeld, 1998; Herman et al., 2003; Shepard et al., 2003). The CRF-dependent involvement of the amygdala in the induction of anxiety-like behavior, visceral hypersensitivity, altered bowel habits and other common feature of IBS has been later confirmed in animal studies (Tache et al., 2002; Myers and Greenwood-Van, 2007, 2010; Venkova et al., 2010).

The hippocampus may also be involved in several aspects relevant to the IBS symptomatology, e.g., pain, anxiety, and stress (Prado and Roberts, 1985; Bannerman et al., 2004; Kwan et al., 2005; McEwen, 2007; Niddam et al., 2011). Saito et al. (2002) demonstrated that the induction of visceral pain by CRD increased the release of hippocampal noradrenaline in animal models. Niddam et al. (2011) observed abnormal hippocampal glutamatergic neurotransmission in IBS patients and inverse correlation between glutamate-glutamine concentrations and emotional stress indicators, which was not observed in healthy individuals. It remains possible that the observed hippocampal glutamatergic hypofunction could result from a generally impaired HPA axis tone or it could represent compensatory mechanisms of adaption to enhanced glucocorticoid feedback.

PSYCHOSOCIAL FACTORS AND IBS

According to the cognitive-behavioral model, a history of abuse and other psychosocial factors may induce and aggravate symptoms of IBS, influence illness experience, and affect treatment outcome.

Ringel et al. (2008) showed that patients with IBS and a history of abuse had a significantly lower pain and urge thresholds and a greater tendency to report pain in response to aversive rectal distentions compared with patients with IBS or abuse history alone. However, neuro-sensory sensitivity remained unchanged. These observations suggest that the abuse history in IBS patients may affect central mechanisms of pain amplification or regional brain activation at sites linked to affect and attention, resulting in heightened awareness to visceral and somatic symptoms, greater pain reports, and greater clinical behavioral responses to painful visceral stimuli. Nevertheless, changes in peripheral signaling by nociceptive DRG neurons, including those innervating the colon cannot be excluded, as suggested by several animal studies (Khasar et al., 2008; Winston et al., 2010).

It was also observed that there is a higher prevalence of psychological and psychiatric disorders observed in IBS patients: depression, somatization disorder, generalized anxiety disorder, panic, and phobic disorders and coping difficulties (for review see, Arebi et al., 2008). Drossman et al. (1999) estimated that up to 70% of the patients referred to tertiary centers with IBS meet diagnostic criteria for anxiety or depression. However, Elsenbruch et al. (2006) revealed that women with IBS were characterized by an exaggerated anticipatory anxiety response at baseline, but essentially unaltered anxiety and neuroendocrine responses to a public speaking stressor. These results would suggest that IBS patients show essentially normal emotional responses when faced with challenging psychosocial situations.

Although well-evidenced, the impact of psychosocial factors on the neurochemical responsiveness of visceral nociceptive pathways and the physiological function of the GI remains unclear. It is possible that the psychosocial stressors and/or stressful early life events modulate the immune response of the gut to infectious agents and cause low level inflammation and mast cell infiltration and degranulation in the bowel (Barbara et al., 2004; Ohman and Simren, 2010; Chen et al., 2011; Philpott et al., 2011). This is supported by questionnaire-based studies indicating an increased prevalence of atopic diseases among IBS patients (Philpott et al., 2011, and citations therein) and a report published by Barbara et al. (2004), demonstrating that there is an increased number of degranulating MC in patients with IBS compared to that in the healthy controls. Increased mucosal immune activation and elevated blood concentrations in pro-inflammatory cytokines are also believed to impact the CNS functioning (for review see; Kennedy et al., 2012). Although these large molecules do not freely pass the blood-brain barrier, a number of studies have provided substantial evidence for their central mechanisms of action, sympathetic arousal and the HPA axis activation (Dinan et al., 2006).

In rodents, early life stress in the form of separation of neonates from the mother results in permanent changes in the CNS, which include unrestrained secretion of CRF and increased expression of its receptors (Owens and Nemeroff, 1993), increased regional norepinephrine release (Southwick et al., 1999), downregulation of β-receptors, decreased benzodiazepine receptor, and γ-aminobutryic acid type A receptor (Caldji et al., 2000). A significant increase in 5-HT-positive cell number and 5-HT content after CRD stimulation was also observed in the colon of animals, which experienced maternal separation (Ren et al., 2007). Videlock et al. (2009) demonstrated that IBS patients and controls with a history of early adverse life events (EAL) have a greater cortisol response to a visceral stressor compared to individuals without EAL, suggesting the involvement of the HPA axis.

CURRENT AND FUTURE MOLECULAR TARGETS FOR IBS TREATMENT

Various classes of drugs, like 5-HT3 antagonists, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), gabapentinoids, CRF-1 antagonists, β3 adrenoceptor agonists, somatostatin, *N*-methyl D-aspartate receptor antagonists, or melatonin are currently in use for the treatment of visceral analgesia and other symptoms of IBS. However, new molecular targets for the future IBS therapeutics are also being investigated.

SEROTONIN RECEPTORS

Serotonin (5-HT) is a key neurotransmitter and a signaling molecule that plays an important role in sensation, secretion, and absorption (for review see, Gershon and Tack, 2007; Garvin and Wiley, 2008). A number of studies reported altered serotonergic signaling activity in the brain and gut in IBS, including increase in plasma 5-HT in IBS-D (diarrhea-predominant) and PI-IBS, reduced levels in IBS-C (constipation-predominant) and changes in plasma and tissue levels of serotonin transporter protein (SERT; Dunlop et al., 2005; Atkinson et al., 2006; Zou et al., 2007; Camilleri, 2011). Drugs aimed at selective modulation of the 5-HT activity (SSRIs, 5-HT3, and 5-HT4 receptor antagonists) or both 5-HT and norepinephrine (NE) systems (serotonin-norepinephrine reuptake inhibitors, SNRIs, and tricyclic antidepressants, TCAs) have been used in the treatment of functional GI disorders, as well as in other chronic pain conditions, and psychiatric syndromes. New generation drugs with similar pharmacological profile may soon become novel efficient therapeutics in the treatment of IBS.

Several large clinical trials have demonstrated that serotonin receptor 5-HT3R antagonists, like alosetron, cilansetron, and ramosetron are among the most effective therapeutic options

to date for both male and female IBS-D patients (Jarcho et al., 2008, and citations therein). The 5-HT3R antagonists alleviate specific IBS symptoms, such as frequent bowel movements, feelings of urgency, and chronic abdominal pain and discomfort, acting through central and peripheral mechanisms. Although the precise mechanisms underlying their effectiveness remain incompletely understood, symptom improvement associated with an interaction with dopamine, cholecystokinin, glutamate, acetylcholine, and GABA (for review see, Barnes et al., 2009) and a reduction in amygdala and emotional arousal circuit activity (Berman et al., 2002) have been suggested. Inhibition of the spinal cord c-fos expression by 5-HT3R antagonists in response to noxious CRD (Kozlowski et al., 2000) suggests that 5-HT3R plays a role in the transmission of noxious information within the spinal cord. Excess 5-HT released from enterochromaffin cells (EC) in the colonic mucosa of both unselected and PI-IBS patients (Spiller, 2007) and decreased expression of SERT (Coates et al., 2004) may also account to this phenomenon.

5-HT3 antagonist-based therapies require the implementation of a risk management plan, as ischemic colitis and complications of constipation may occur (Chang et al., 2010). Therefore, a novel class of compounds (of which the prototype is LX-1031) is being developed that directly inhibits 5-HT synthesis in EC cells, potentially reversing the underlying pathogenetic factor in conditions like IBS-D. Such compounds could become an alternative to the application of classical 5-HT3 receptor antagonists in the treatment of IBS.

Recently, partial 5-HT1 receptor agonists, like buspirone, and antagonists, like robalzotan tartrate monohydrate (AZD7371), attracted much attention as they displayed a potent analgesic effect in the CRD-induced visceral pain model in rats (Sivarao et al., 2004; Lindstrom et al., 2009). However, the clinical development of AZD7371 has been discontinued due to severe adverse events, including hallucinations and the inability to demonstrate significant efficacy in IBS patients compared with placebo (Drossman et al., 2008).

The 5-HT4 receptors in the GI tract are found on enteric neurons and smooth muscle cells. Stimulation of 5-HT4 receptors leads to acetylcholine release and prokinetic effects (Gershon and Tack, 2007). The early generation 5-HT4 receptor agonists, such as cisapride and tegaserod, reversed slow motility and relieved constipation, but they have been withdrawn because of cardiac or vascular adverse effects (Gershon and Tack, 2007). A number of novel 5-HT4 agonists have recently been obtained as potential treatments for patients with IBS-C and appear to be safer than earlier generation agents in these classes (Camilleri et al., 2008; Manini et al., 2010).

The 5-HT7 and 5-HT2B receptors are yet another potential serotonergic target for future IBS treatment. The 5-HT7 receptors are present in humans and other animals and are linked with depression, circadian rhythm, neuroendocrine function, affective behavior and body temperature regulation (for review see, Vanhoenacker et al., 2000). They play an important role in regulating smooth muscle relaxation in the GI and nociceptive pathways (Carter et al., 1995; Meuser et al., 2002) and may thus be involved in the pathological mechanisms of GI dyskinesia, abdominal pain, and visceral paresthesia in IBS. It was demonstrated that 5-HT7 receptors also mediate stress and glucocorticoid-induced effects on hippocampal neurogenesis, which have been implicated in mood. Meanwhile, 5-HT2B receptor blockade was shown to reduce significantly pain behaviors in response to CRD (O'Mahony et al., 2010a).

Recent studies demonstrated that serotonergic neurotransmission can be markedly affected by CRF acting in a CRF receptor-dependent manner (Cryan et al., 2005; Valentino and Commons, 2005). The injection of low doses of CRF in the dorsal raphe nucleus (DRN) reduced the discharge rate of serotonergic neurons in the striatum (Kirby et al., 2000) and the nucleus accumbens (Lukkes et al., 2008) and at a higher dose increased striatal 5-HT release (Price et al., 1998). Additionally, 5-HT levels in the hippocampus were increased by i.c.v. administration of low and high doses of CRF (Penalva et al., 2002). These data suggest a close correlation between the serotonergic system and CRF, which may be taken into consideration when novel anti-IBS therapies are designed.

BENZODIAZEPINE RECEPTORS

One of the newly targeted classes of drugs for the treatment of visceral pain are benzodiazepine (BZD) receptor modulators. BZD receptors are located in subcortical and hypothalamic regions and appear important in controlling autonomic function, such as motor and sensory activity of the gut (for review see, Salari and Abdollahi, 2011). In addition, activation of the central BZD receptors affects GABA interaction with central GABA-A receptors and may influence the ANS, dorsal vagal nuclei, and the ENS. Peripheral BZD receptors were identified on immune cells and other peripheral tissues and may be involved in cell proliferation and immunomodulation (for review see, Zisterer and Williams, 1997).

The BZD receptors and their ligands, which belong to an important regulatory network between the CNS, behavior, and immune response, may thus become an attractive target for future IBS treatments. Recently, a novel BZD receptor ligand dextofisopam was developed for the management of IBS-D (Grundmann et al., 2010) and is currently under investigation.

NEUROKININ RECEPTORS

Substance P (SP) and the neurokinin-1 receptors (NK1R) are located throughout the BGA, including peripheral, spinal, supraspinal, and cortical sites of visceral afferent pathways, as well as brain regions involved in emotional arousal and autonomic function (Tillisch et al., 2012, and citations therein). It was observed that SP and NK1R signaling play an important role in nociceptive responses (hyperalgesia) and the autonomic and behavioral responses to stress in animals and humans.

Recent study by Tillisch et al. (2012) revealed that a 3-week treatment with a novel NK1R antagonist reduced activation of key regions of both the interoceptive afferent and emotional arousal network in response to noxious and non-noxious visceral stimulus in female IBS patients, causing a large decrease in pain-induced negative affect and decreased anxiety and pain ratings. This positive correlation suggests a potential for use of NK1R antagonists in IBS patients to decrease pain related distress.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Neurotrophins promote neuronal survival along with the growth and differentiation of new neurons and synapses. Brain-derived neurotrophic factor (BDNF) may be involved in the integration of excitatory and inhibitory neurotransmission and emerging evidence suggests that amygdaloid BDNF can regulate anxiety-like behaviors (Slack et al., 2004; Pandey et al., 2006).

Yu et al. (2012) recently observed a significant upregulation of BDNF in the colonic mucosa and structural alterations of mucosal innervation in biopsies from patients with IBS, as compared with controls. The enhanced expression of BDNF was closely correlated with the degree of abdominal pain in IBS. These results suggest that endogenous BDNF released in response to inflammation contributes to the development of central sensitization and thus plays a pathophysiological role in the altered gut sensation in IBS. Furthermore, the upregulation of BDNF may also play a role in the structural alterations of mucosal nerve fibers in patients with IBS. Inhibition of the BDNF system could therefore be beneficial for the alleviation of symptoms in the IBS patients.

SEX STEROID RECEPTORS

Because of the sex differences in perceptual responses and a female predominance of the disorder, attention has been drawn to the role of sex steroids, in particular ovarian hormones, in the development of IBS. Previous reports revealed that women with IBS often report exacerbation of symptoms, including visceral and somatic sensitivity during menses (Kane et al., 1998; Mayer et al., 1999; Houghton et al., 2002; Chang et al., 2006; Gustafsson and Greenwood-Van, 2011) and show greater, compared to men, activation of brain areas associated with affective responses including the amygdala and cingulate cortex (Berman et al., 2000; Naliboff et al., 2003). In contrast, male IBS patients show less visceral hypersensitivity than female patients, but have greater sympathetic nervous system responses measured by skin conductance, and decreased cardiovagal activity measured by heart rate variability compared to female IBS patients (Tillisch et al., 2005) and male controls.

Although ovarian steroid receptor levels are higher in some regions of the female brain (Greco et al., 2001; Milner et al., 2008), progesterone and estradiol-induced visceral hypersensitivity does not appear to be sex specific, as males also showed increased visceral sensitivity following hormone implantation on the amygdala (Myers et al., 2011). However, the amygdala may still represent the key supraspinal site mediating the actions of ovarian hormones on visceral pain in both males and females and account for differences in symptom generation in male and female IBS patients (Naliboff et al., 2003; Labus et al., 2008; Kilpatrick et al., 2010). The amygdala may thus become an interesting target for the IBS treatment and alleviation of pain.

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) have been localized on mucosal surfaces, including the colonic epithelial cells, and their expression is increased in the colonic mucosa of rat models of visceral hypersensitivity and mucosal biopsies from IBS patients (McKernan et al., 2009; Brint et al., 2011). TLRs are activated by various bacterial and viral cell components (Takeuchi and Akira, 2010), which stimulate transcription of inflammatory cytokines, like IL-1b, IL-6, and TNFa and affect transmission in the spinal cord, resulting in central sensitization and hyperalgesia (for review seel, Akira and Takeda, 2004; Arebi et al., 2008). Cytokines are also known to cross the blood-brain barrier, to affect the HPA axis and stress response and to stimulate secretion of CRH in rat, as well as in humans (for review see, John and Buckingham, 2003; Dantzer et al., 2008).

Recently, McKernan et al. (2011) demonstrated that TLR agonist-induced cytokine and cortisol release was markedly enhanced in stimulated whole blood from IBS patients compared with healthy controls. These results point out at the TLR as possible targets in the treatment of IBS.

RECEPTORS FOR ACETYLCHOLINE AND CATECHOLAMINES

There is an increasing evidence for the beneficiary role of cholinergic, dopaminergic, and noradrenergic pathways in regulating immunity and cytokine production in IBS, suggesting a positive influence of acetylcholine and catecholamines on the IBS symptoms (Dinan et al., 2008; Rosas-Ballina and Tracey, 2009). However, adrenaline was shown to act directly through adrenergic receptors on DRG neurons or indirectly by increasing levels of pronociceptive mediators following immune activation in the colon or repeated stress, thus increasing the excitability of the neurons and exacerbating pain sensation (Khasar et al., 2008; Winston et al., 2010; Ibeakanma et al., 2011). In contrast, no significant differences in NE responses to sigmoidoscopy were observed in women with IBS-D compared to healthy women (Chang et al., 2009).

These conflicting results point at the necessity of further studies on the involvement of cholinergic, dopaminergic, and adrenergic receptors and their ligands in development of IBS and their possible therapeutical application.

PAST, PRESENT, AND FUTURE OF ANTI-IBS DRUGS TARGETING THE BRAIN-GUT AXIS

For most IBS patients with mild symptoms, lifestyle, and dietary changes may be sufficient; for more moderate symptoms, medications that act on the gut (e.g., anticholinergics, peripheral 5-HT agents) can be considered. However, patients who suffer from severe IBS, characterized by increased levels of pain, poorer quality of life, psychosocial difficulties, or co-morbidity with mood disturbances are usually refractory to first- and second-line therapies (Drossman et al., 2000; Grover and Drossman, 2011). The bi-directional communication between the brain and the gut opens up new treatment possibilities for these patients and directs us to novel pharmacological targets for the anti-IBS drugs.

Almost all IBS patients could benefit from centrally acting treatments, like therapies focused on teaching better stress coping strategies, both at a cognitive and behavioral level (for review see, Larauche et al., 2012), or psychotropic agents. Some of the TCAs, SSRIs, SNRIs, or BZDs have already been employed in the treatment of IBS and proved effective in symptom relief via mood stabilization, modulation of pain perception and amelioration of GI motility and secretion (Ford et al., 2009; Grover and Drossman (2011) estimate that at least every one in eight patients with IBS is offered an antidepressant). However, the effects of psychotropic agents on bowel symptoms and visceral hypersensitivity in IBS

patients have been less robust and less consistent than the benefits reported for global symptoms and abdominal pain/discomfort (Chey et al., 2011). Furthermore, psychotropic agents are not free from undesired side effects. TCAs display anticholinergic properties, including constipation, tachycardia, urinary retention, and xerostomia; patients may also encounter central side effects including sedation, insomnia, agitation, and nightmares (Chey et al., 2011). Compared to TCAs, SSRIs have fewer side effects, but do not improve bloating or visceral pain (Tack et al., 2006). BZDs are used routinely in anxiety disorders, but their efficacy in symptom relief of IBS is under debate (Drossman et al., 2002). New generation of psychotropic agents is therefore anticipated.

Efficacious and safe serotonergic agents may also become future drugs in the treatment of IBS. Recently, novel mixed 5-HT1A agonists/5-HT3 antagonists, 5-HT1B/D agonists, and 5-HT2B antagonists have been proposed as new therapeutics for IBS (Tack et al., 2000; Mulak and Paradowski, 2006; Vera-Portocarrero et al., 2008; Asagarasu et al., 2009; O'Mahony et al., 2010b).

Other endogenous systems, which may become possible new targets in the IBS therapy, include GABA-B, CRF, NK, cannabinoid, and opioid receptors and their ligands. Preliminary data suggest that anxiolytic activity of GABA-ergic agent, gabapentin may be efficient in reducing central sensitization in hyperalgesia (for review see, Camilleri and Andresen, 2009). CRF receptor antagonists have also been proposed as a potential treatment of IBS (Martinez and Tache, 2006; Tache et al., 2009). However, due the failure of treatment with a CRFR1 antagonists to alter colonic transit and the global improvement scale in IBS patients (Sweetser et al., 2009), further studies are required.

The potential use of cannabinoid and opioid receptor ligands as anti-IBS agents has also been considered and has been reviewed in detail elsewhere (Fichna et al., 2009; Izzo and Sharkey, 2010).

CONCLUSION

In summary, there is striking evidence of a crucial involvement of the BGA in the development of IBS and IBS like symptoms. Though the role of the BGA is not fully understood, some concepts are at an advanced stage and allow speculation on possible future treatment options. Future research needs to identify the exact involvement of the discussed neurotransmitter systems and to identify at which level pharmacological treatment may be beneficial to patients with IBS.

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Alpha 2 delta ($\alpha_2\delta$) ligands, gabapentin and pregabalin: what is the evidence for potential use of these ligands in irritable bowel syndrome

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Irritable bowel syndrome (IBS) is a complex disorder that is characterized by abdominal pain and altered bowel habit, and often associates with other gastrointestinal symptoms such as feelings of incomplete bowel movement and abdominal bloating, and extra-intestinal symptoms such as headache, dyspareunia, heartburn, muscle pain, and back pain. It also frequently coexists with conditions that may also involve central sensitization processes, such as fibromyalgia, irritable bladder disorder, and chronic cough. This review examines the evidence to date on gabapentin and pregabalin which may support further and continued research and development of the $\alpha_2\delta$ ligands in disorders characterized by visceral hypersensitivity, such as IBS. The distribution of the $\alpha_2\delta$ subunit of the voltage-gated calcium channel, possible mechanisms of action, pre-clinical data which supports an effect on motor–sensory mechanisms and clinical evidence that points to potential benefits in patients with IBS will be discussed.

Keywords: $\alpha_2\delta$ ligands, gabapentin, pregabalin, irritable bowel syndrome, visceral sensitivity, central sensitization, peripheral sensitization, motility

INTRODUCTION

ABDOMINAL PAIN AND VISCERAL HYPERSENSITIVITY

Abdominal pain and discomfort along with altered bowel habit are integral to the diagnosis of irritable bowel syndrome (IBS). Although disordered bowel habit can often be improved in these patients, efficacious treatment of pain, abdominal discomfort, and associated symptoms, such as bloating, remains challenging. The identification and development of new drugs to treat these symptoms has been largely unsuccessful and remains problematic, probably linked to the complexity of the functional gastrointestinal disorders (FGIDs) in which multiple factors appear to contribute to their equally multifarious pathophysiology. For example, genetic predisposition, infection, and traumatic events in early life may all predispose individuals to developing IBS, whilst chronic stress, psychological symptoms, and maladaptive coping mechanisms can increase the frequency and severity of symptoms (Levy et al., 2006; Chitkara et al., 2008; Saito and Talley, 2008; Spiller and Garsed, 2009). Pathophysiologies identified to date include gastrointestinal dysmotility, abnormalities in the inflammatory/immune system, increased intestinal permeability, unstable or altered enteric flora, psychopathology, visceral and somatic hypersensitivity, and abnormal CNS processing (Longstreth et al., 2006; Spiller et al., 2007). Although not all abnormalities are present in all patients, visceral hypersensitivity which often associates with the symptoms of pain (Posserud et al., 2007) and bloating (Posserud et al., 2007; Agrawal et al., 2008) and which is often exacerbated by stress, is thought by many to be a determinant or biological measure of IBS (Mertz et al., 1995; Bouin et al., 2002). For example, Mertz et al. (1995) showed altered rectal perception in almost all IBS patients in the form of either lowered sensory thresholds, increased sensation intensity or altered viscerosomatic referral, whilst Bouin et al. (2002) suggested that a pain threshold of less than 40 mmHg in the rectum correctly identified IBS from non-IBS subjects. In addition, whilst only approximately half of IBS patients appear to exhibit lowered rectal sensory thresholds to balloon distension (Whitehead and Palsson, 1998; Posserud et al., 2007), almost all patients (70%) show hypersensitivity elsewhere in the gastrointestinal tract, especially in the jejunum (Francis et al., 1995; Hammonds et al., 1998). Moreover, in the latter studies (Francis et al., 1995; Hammonds et al., 1998) within the group as a whole, and especially in those subjects with diarrhea, lower pain thresholds were observed throughout the entire GI tract compared with healthy controls.

CENTRAL SENSITIZATION

These observations of pan-gastrointestinal visceral hypersensitivity and increased viscerosomatic referral, along with reported increases in expression of extra-intestinal symptoms such as headache, dyspareunia, heartburn, muscle pain and back pain (Whorwell et al., 1986; Mayer and Gebhart, 1994), and presence of fibromyalgia in some patients (Whitehead et al., 2002; Almansa et al., 2009) are consistent with a widespread aberrant central processing of pain (central sensitization) in these patients. Further support is provided by the observations that whilst healthy volunteers exhibit an inhibition of the somatic nociceptor flexion reflex (R-III) to slow ramp distension of the rectum, IBS

patients exhibit a facilitation of this reflex, suggesting enhanced spinal processing in IBS (Coffin et al., 2004). In addition, there are an increasing number of studies suggesting that IBS patients may also be hypersensitive to somatic stimuli. One such study, showed hypersensitivity to rectal balloon distension and cutaneous thermal stimulation of the hand and foot in IBS compared with control subjects (Verne et al., 2001). Interestingly foot hypersensitivity was greater than hand hypersensitivity, suggesting greater convergence and overlap of rectal and foot afferents at common lumbosacral levels (greater central hyperalgesia) than rectal and hand afferents at the levels of the cervical spinal (Verne et al., 2001).

POSSIBLE SYNERGISTIC MECHANISMS

Along with the spinal (central) sensitization, other possible synergistic mechanisms of visceral hypersensitivity include disturbances in the cognitive and emotional aspects of pain (e.g., hypervigilance, somatization, catastrophizing, depression), alterations in descending excitatory and inhibitory pathways (e.g., diffuse noxious inhibitory control, DNIC), and sensitization of afferent nerves (e.g., peripheral sensitization due to for example mucosal insult). A quantitative meta-analysis of functional neuroimaging studies in IBS patients during rectal distension showed greater recruitment of attentional (lateral prefrontal cortex), affective (ventral anterior cingulated cortex [ACC], amygdala, dorsal pons), and homeostatic afferent circuits (insula, dorsal ACC, thalamus) compared with controls, with increased regional activity in the insula (INS) and anterior midcingulate cortex (aMCC) being most commonly reported (Labus et al., 2009). More recent studies investigating anatomical differences in the brain between IBS and control subjects have shown morphometric changes in gray matter density predominantly in areas involved in cognition and evaluation, with changes in other areas of the brain being generally explained by anxiety and depression levels in the IBS patients (Seminowicz et al., 2010). In another study by Heymen et al. (2010), in which DNIC was assessed in IBS compared with healthy subjects by measuring the reduction in left hand thermal pain intensity during counter irritation by submersion of the right hand in 12°C water (conditioning stimulus, CS), and controlling for the non-specific effects on pain perception, such as distraction from the CS, psychological symptoms, and cardiovascular reactivity, it was shown that IBS patients demonstrate deficient DNIC probably attributed to disordered central analgesic mechanisms. This deficit has subsequently been shown to directly correlate with visceral hypersensitivity (Piche et al., 2010). Indeed in the morphological study described above significant reductions in gray matter density were observed in the periaqueductal gray, an area known to play a major role in descending pain modulation, which was independent of anxiety and depression (Seminowicz et al., 2010). Other studies have shown that peripheral mucosal insults, such as the presence of inflammation, injury or excess acid do not only increase pain sensitivity at the site of injury (primary hyperalgesia/peripheral sensitization) but also at more remote sites in the gastrointestinal tract (secondary hyperalgesia), via the process of central sensitization (Anand et al., 2007; Knowles and Aziz, 2009). One example in FGIDs, is the observation that pain thresholds to electrical stimulation were not just reduced at the distal end of the esophagus where acid was infused but also in the unexposed proximal esophagus of patients with non-cardiac chest pain, with this sensitization process being significantly magnified and prolonged compared with healthy volunteers (Sarkar et al., 2000). Another possible example is the onset of IBS following GI infection (post-infectious IBS) where persistent sensitization of the primary afferents due to for example increased mast cells numbers, T lymphocytes, and expression interleukin (IL)-1β (peripheral sensitization), especially in the presence of risk factors such as depression, hypochondriasis, and adverse life events (Spiller and Garsed, 2009), could lead to central sensitization and the persistence of symptoms, allodynia (pain to a stimulus that does not normally provoke pain), hyperalgesia (increase in intensity of pain to a stimulus that normally provokes pain), and dysmotility long after the resolution of illness.

Thus there appears to be a dyssynergy between the interaction of peripheral and central pain mechanisms, along with influences from cognitive and emotional factors, and abnormalities in descending inhibitory pathways that may all lead to the sensation of abdominal pain and hypersensitivity in IBS. Central mechanisms perhaps play a pivotal role integrating between these processes and thus may represent a promising target for the development of drugs for the treatment of IBS. For a more detailed discussion of central, peripheral, and psychological processes in IBS see the reviews Van Oudenhove and Aziz (2009), Knowles and Aziz (2009), and Anand et al. (2007).

$\alpha_2\delta$ BINDING SITES

Gabapentin (Neurontin) was first introduced as an antiepileptic drug but has more recently been used in the treatment of postherpetic neuralgia, diabetic neuropathy, migraine prophylaxis, and chronic pain conditions (Taylor, 2009; Tzellos et al., 2010). Pregabalin (Lyrica) is a second-generation compound structurally related to gabapentin and approved in the US for the management of neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia, fibromyalgia, and as adjunctive therapy for adults with partial onset seizures and for generalized anxiety disorder (GAD). In Europe pregabalin is approved for the treatment of neuropathic pain, epilepsy, and generalized anxiety disorder (Taylor, 2009; Tzellos et al., 2010). It has been, or is being, assessed in many clinical trials for disorders such as IBS and neuropathic pain in acute spinal cord injury (see US National Institute of Health, www.ClinicalTrials.gov). Pregabalin has been shown to be 2–10 times more potent than gabapentin and to possess more linear pharmacokinetics (Ben-Menachem, 2004; Huckle, 2004; Taylor, 2009; Tzellos et al., 2010).

Although structurally related to γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the CNS, gabapentin, and pregabalin are functionally inactive at $GABA_A$, $GABA_B$, or benzodiazepine receptors, and are not converted metabolically into GABA or a GABA receptor agonist (Ben-Menachem, 2004; Huckle, 2004; Taylor, 2009; Tzellos et al., 2010). In addition, clinically effective concentrations of gabapentin and pregabalin have been shown to have no effect on GABA synthesis, uptake or degradation (Ben-Menachem, 2004; Huckle, 2004; Field et al., 2006; Taylor, 2009; Tzellos et al., 2010).

Both gabapentin and pregabalin bind with high affinity to $\alpha_2\delta$ subunits of voltage-gated calcium channels and this has been

proposed as a likely site of their action. Further, in mice with mutations of the $\alpha_2\delta$ subunits that prevent drug binding, pregabalin and gabapentin are devoid of analgesic and anticonvulsant activity (Field et al., 2006). Voltage-gated calcium channels are ubiquitous in the body and are made up of an α_1 subunit, which makes up the ion-conducting pore, coupled together with other subunits including β, γ, and $\alpha_2\delta$. There is great heterogeneity within the family of α_1 subunits, of which 10 members have been described in mammals (for review; Catterall et al., 2005). The $\alpha_2\delta$ subunit appears to play a role not only in the operational characteristics of individual channels, but also to enhance trafficking of the α_1 subunits to the cell membrane, so influencing the number of functional calcium channels (Hendrich et al., 2008; Mich and Horne, 2008). The $\alpha_2\delta$ subunit exists as four distinct subtypes and these are encoded by four distinct genes (Klugbauer et al., 1999; Qin et al., 2002). Only subtypes 1 and 2 have been shown to exhibit binding for gabapentin and pregabalin and therefore might be expected to underwrite the analgesic, anticonvulsant, and anxiolytic activity of these drugs (Gong et al., 2001; Qin et al., 2002).

Despite the widespread localization of voltage-gated calcium channels, the focus of studies to map the distribution of the $\alpha_2\delta$ subunit has largely been restricted to tissues of the central nervous system, with few studies exploring the potential for wider distribution. At both the mRNA and protein level, $\alpha_2\delta$-subtype 1 is widely distributed throughout human brain (Gong et al., 2001). This widespread distribution of mRNA for the $\alpha_2\delta$-subtype 1 has been confirmed in the central nervous system of the rat and was reported in regions of the CNS involved in cortical processing, learning and memory, defensive behavior, neuroendocrine secretion, autonomic activation, primary sensory transmission, and general arousal (Cole et al., 2005). These observations have been confirmed at the protein level using immunostaining with an antibody specific for $\alpha_2\delta$-subtype 1 (Taylor and Garrido, 2008). In this study, the most prominently stained regions of the CNS included those areas involved in pain signaling, including the amygdala, entorhinal cortex, hippocampus, ACC, and insula (Taylor and Garrido, 2008). In addition, a population of small diameter peripheral sensory neurones in the dorsal root ganglia, together with their projections to the spinal cord, stained prominently (Taylor and Garrido, 2008). Immunostaining in the GI tract was also investigated, although only data from the small intestine was reported showing moderate staining in the smooth muscle (Taylor and Garrido, 2008). To date, there has been only one report of neuronal $\alpha_2\delta$ subunit expression in the intestine. In the guinea-pig, *in situ* hybridization has revealed the $\alpha_2\delta$-subtype 1 localized on the intrinsic primary afferent neurones of the intestine, where they appear to be associated with N-type calcium channels (Needham et al., 2010). In these neurones, pregabalin had inhibitory effects on both the action potential and the after hyperpolarization, raising the possibility that pregabalin may be able to reduce the excitability of these sensory neurones and so potentially inhibit GI hypersensitivity by an effect at these sites. The distribution of $\alpha_2\delta$-subtype 2 has been less extensively characterized. However, in the rat, mRNA encoding $\alpha_2\delta$-subtype 2 has been shown to be widely distributed within the CNS, with particularly dense staining in the brainstem, the periaqueductal gray matter, the spinal cord, and dorsal root ganglia (Cole et al., 2005). These are regions known to play an important role in autonomic function and pain processing. In tissues from human, mRNA for $\alpha_2\delta$-subtype 2 was detected in several brain regions but not in colon or small intestine (Gong et al., 2001). The absence of $\alpha_2\delta$-subtype 2 in human jejunum was also confirmed at the protein level.

The functional consequences of the binding of gabapentin/pregabalin with the $\alpha_2\delta$ protein remain controversial. Data from recombinant systems suggests that the function of the $\alpha_2\delta$ subunit is heavily dependent on the subtype of α_1 protein with which the $\alpha_2\delta$ subunit is co-expressed and the cell system into which the proteins are engineered. However, it is widely accepted that the mechanism of action of these agents involves a modulation of calcium conductance, but the precise mechanism for this remains to be elucidated. The modulation of calcium currents by gabapentin has been demonstrated in several studies of isolated neurones (Stefani et al., 1998; Sutton et al., 2002; van Hooft et al., 2002), although other studies have struggled to demonstrate such an effect (Schlicker et al., 1985). More recently, it has been proposed that gabapentin may exert an action through binding to the $\alpha_2\delta$ subunit within the cytosol, rather than at the cell surface, and that this interaction can over time reduce the trafficking of $\alpha_1/\alpha_2\delta$ complexes to functional sites within the cell membrane (Hendrich et al., 2008; Mich and Horne, 2008). Thus gabapentin and pregabalin may exert a range of effects, either acute or chronic, mediated through diverse mechanisms, to modulate calcium flux in nerve terminals. The consequences of this disruption of calcium-mediated membrane depolarization have been investigated extensively. Both gabapentin and pregabalin have been shown to inhibit the release of a wide range of neurotransmitters including noradrenaline, dopamine, 5-HT, acetylcholine, glutamate, substance P, and CGRP from isolated slices of brain and spinal cord from several species following stimulation with either potassium or capsaicin (Dooley et al., 2000; Patel et al., 2000; Fehrenbacher et al., 2003; Brawek et al., 2008). However, the inhibitory effect of gabapentin and pregabalin may be stimulus-dependant, as illustrated in the neocortex, where the magnitude of inhibition of the release of noradrenaline was reduced when neurotransmitter release was evoked by electrical stimulation rather than potassium (Dooley et al., 2000). It has been suggested that $\alpha_2\delta$ ligands may only exert their inhibitory effects on neurotransmitter release in "sensitized" situations and may exert only limited effects in situations of normal physiology. For example, in the spinal cord, gabapentin was only able to exert its presynaptic inhibitory influence on postsynaptic currents in animals in which experimental diabetic neuropathy had been established with streptozotocin and not in unsensitized animals (Patel et al., 2000). Similarly, the pregabalin-mediated reductions in substance P and CGRP release in the spinal cord of the rat are manifest only in animals in which inflammation had been induced following pre-treatment with intraplantar Freund's adjuvant and are absent in untreated animals (Fehrenbacher et al., 2003).

More recently, an additional mechanism of action has been suggested for gabapentin and pregabalin. In cell or neuronal cultures gabapentin and pregabalin were shown to inhibit the activation of the transcription factor NF-κB evoked by substance P (Park et al., 2008). If confirmed in additional studies, these observations might help explain the increased efficacy of gabapentin and pregabalin

in circumstances of prior inflammation or sensitization, which might be expected to lead to up-regulation of the NF-κB signaling pathway.

PRE-CLINICAL MODELS OF NON-GI NEUROPATHIC PAIN

The anti-allodynic and anti-hyperalgesic properties of gabapentin and pregabalin have been established in a wide range of animal models and the literature is too extensive to review here. In summary, both of these $\alpha_2\delta$ ligands have been shown to manifest these properties in animal models of inflammatory, surgical, and neuropathic pain, including the inhibition of both the static and dynamic components of allodynia (for example; Field et al., 1997a,b, 1999). Interestingly, in a study of both sympathetically-maintained and sympathetically-independent neuropathic pain, pregabalin was particularly potent at inhibiting both tactile and cold allodynia when given by the intrathecal route, suggesting a predominantly spinal site of action, although the involvement of supraspinal centers cannot be ruled out (Han et al., 2007). Pregabalin was unable to inhibit cold allodynia in the model of sympathetically-independent neuropathic pain when given via the intraperitoneal route (Han et al., 2007). Experiments involving direct recording from spinal neurons have demonstrated the ability of pregabalin to inhibit the C-fiber mediated response of spinal nociceptive-specific neurons, without any effect on the responsiveness of A-δ fibers (You et al., 2009). Further, pregabalin was also able to inhibit central sensitization of the spinal neurons induced by application of bee venom. Spinal transection confirmed that the effect of pregabalin in this study was likely to involve supraspinal centers, mediated through descending inhibitory controls (You et al., 2009).

PRE-CLINICAL MODELS OF GI SENSATION AND MOTILITY

Following the demonstration of the efficacy of gabapentin and pregabalin in animal models of neuropathic pain, investigations of the profile of these agents in small animal models of visceral pain followed. Initial focus was on understanding whether gabapentin and pregabalin might have utility in the treatment of IBS and so studies focused on models of rectal or colonic hypersensitivity. Caution needs to be applied when interpreting these data given the recent history of notable failure of these models to predict the effects of other new drugs on human pain and discomfort (e.g., those acting at neurokinin-1, NK_1, or corticotrophin releasing factor-1, CRF_1, receptors). Despite this in the rat, the intraperitoneal administration of lipopolysaccharide (LPS) results, some 9–12 h later, in hypersensitivity of the rectum. The hypersensitivity can be demonstrated following rectal distension with a small balloon and is manifest as both allodynia as well as hyperalgesia. In this model, pregabalin, following either oral or intraperitoneal administration, suppressed the rectal hypersensitivity response to LPS (Eutamene et al., 2000). Pregabalin (1–30 mg/kg p.o.) dose-dependently inhibited the allodynia observed following distension with the lowest volume of 0.4 mL. However, only the 10 mg/kg dose was able to reduce the nociceptive effect of the larger volumes of distension. These observations were then extended in a more chronic model of colonic allodynia (Diop et al., 2002). Seven days after the administration of trinitrobenzene sulfonic acid (TNBS) into the proximal colon of the rat, an allodynia to distension was demonstrated in the distal colon of the animal. Histological analysis revealed an inflammatory response in the proximal colon, characterized by the presence of inflammatory cells, necrosis, and hyperemia, 3 days after dosing with TNBS. This response had substantially diminished by day 7 and in the distal colon, no increase in inflammatory cells was observed at any time point. Hyperemia appeared to persist to day 7, the study day for these experiments. Pregabalin (30–200 mg/kg s.c.), given 30 min prior to the balloon distension of the distal colon, dose-dependently reversed the allodynia observed 7 days after TNBS administration. Similar effects were seen following oral administration, 1 h prior to balloon distension. In control animals not pre-treated with TNBS, the highest dose of pregabalin (200 mg/kg s.c.), which fully reversed TNBS-induced allodynia, had no effect on colonic pain thresholds, in contrast to morphine (0.3 and 1.0 mg/kg) which significantly raised thresholds (Diop et al., 2002). In this model, the inflammatory stimulus in the proximal colon establishes a secondary hyperalgesia and allodynia in the distal colon, presumably through central sensitization at the level of the lumbar spinal cord, although possibly also involving higher centers. These central nervous system structures are the likely site of action of pregabalin in this model and in the absence of sensitization, pregabalin had no effect on sensation.

A series of studies with the prototype $\alpha_2\delta$ ligand gabapentin, confirmed that the effects on visceral pain were shared across the class and not restricted to pregabalin. In both mice and rats, gabapentin was shown to reduce the response evoked by intraperitoneal administration of acetic acid, a model of acute visceral pain. Gabapentin (50–200 mg/kg i.p.), dose-dependently inhibited the number of abdominal contractions evoked by intraperitoneal acetic acid when given 40 min ahead of the stimulus (Feng et al., 2003). The maximum effect was seen at 200 mg/kg and inhibited the abdominal response by approximately 75%. This dose of gabapentin impaired the performance of rats on the beam-balance test, suggestive of sedation, which can interfere with the interpretation of tests of analgesia, raising the possibility that the antinociceptive effect of gabapentin was secondary to sedative effects. In this study, the investigators also attempted to develop some mechanistic understanding of these observations and measured acute changes (over a 90-min observation) in the intrathecal levels of several amino acids. Intraperitoneal administration of acetic acid evoked large rises in both aspartate and glutamate. These increases were inhibited completely by prior administration of gabapentin (100 mg/kg i.p.). In addition, acetic acid also increased the intrathecal levels of the inhibitory amino acid serine, an increase that was also inhibited by gabapentin pre-treatment. Intrathecal levels of the inhibitory amino acid, glycine was reduced to below baseline levels by gabapentin. In a broadly similar study, this time conducted in mice, Stepanovic-Petrovic et al. (2008) confirmed the activity of gabapentin to inhibit the nociceptive effects of peritoneal irritation evoked by acetic acid. In this study, intraperitoneal administration evoked a writhing response, characterized by abdominal contractions coupled with elongation of the body and extension of the hindlimbs. Intraperitoneal administration of gabapentin (10–70 mg/kg), 1 h before the acetic acid, dose-dependently inhibited the writhing response. Once again, this group compared the potency of gabapentin to inhibit

acetic-acid-induced writhing with its ability to impair motor function, assessed using the rotarod test (a test of performance in which the rodent is placed on a suspended horizontal rotating rod [not high enough to injure the animal but high enough that the animal avoids falling off] to measure balance, coordination and motor planning). In this study, gabapentin was devoid of activity in the rotarod test, even at doses of 2000 mg/kg, thus making it unlikely that the observed antinociceptive effects were occurring secondary to sedation. In an interesting study from Meymandi and Sepehri (2008), the antinociceptive effect of gabapentin (1–100 mg/kg i.p.) was confirmed in the acetic acid-induced writhing model. In the same study, the dose-response curve to morphine was also constructed and then a low effective dose of gabapentin was given as a combination with a sub-therapeutic dose (0.25 mg/kg i.p.) of morphine. This combination produced a synergistic effect, as writhing was inhibited by over 90% compared to control levels. Similarly, when the sub-therapeutic dose of morphine was combined with a sub-therapeutic dose of gabapentin (10 mg/kg i.p.), a synergistic response was observed, with writhing inhibited by approximately 70%. Interestingly, these synergistic effects were not inhibited by treatment with naloxone. Similar observations, of a synergistic interaction between gabapentin and morphine in models of visceral pain have been made previously in a rat model of experimental pancreatitis induced by bradykinin infusion into the pancreas (Smiley et al., 2004). In this model, gabapentin (100–300 μg intrathecally) only modestly inhibited the behavioral response to bradykinin. However, when the 300 μg intrathecal dose of gabapentin was combined with low intrathecal doses of morphine, shown previously to have modest if any inhibitory effects in this model, significant inhibition of all aspects of the behavioral response to bradykinin was observed. These observations taken together illustrate the inhibitory effect of gabapentin on visceral pain, strongly support the concept that this effect is not underwritten or confounded by inhibiting arousal and point to potential synergy with other antinociceptive mechanisms. These data also provide *in vivo* evidence to support the hypothesis that gabapentin reduces the release of excitatory and inhibitory neurotransmitters in the spinal cord.

Recently, a study has been published comparing the effect of pregabalin in the TNBS model of acute hypersensitivity with a model of acute hypersensitivity induced by restraint stress (Ohashi-Doi et al., 2010). As one might predict from previous data, pregabalin (10–100 mg/kg p.o.) reduced colonic nociceptive thresholds dose-dependently in animals sensitized previously with TNBS. In the stress restraint model, increased fecal output in terms of number of pellets and fecal weight was observed during the period of restraint stress. Pregabalin, over the same dose range as examined in the TNBS model, also dose-dependently inhibited the stress-induced increases in fecal output, but had no effect on naive, unstressed rats. This is the only demonstration to date that pregabalin can modulate stress-induced defecation in rats. A comparison of the effects in the two models suggests that pregabalin may be more potent at inhibiting the stress-induced increases in defecation than at inhibiting TNBS-induced colonic hypersensitivity.

The majority of studies of the effects of $\alpha_2\delta$ ligands on visceral pain have been restricted to acute models and largely to those evoked by chemical irritants or pro-inflammatory stimuli. More recently investigators have studied the effects of gabapentin and pregabalin in models where less invasive and possibly more physiologically relevant stimuli have been used to evoke an acute or chronic phenotype. In a rat model, where repeated tonic colorectal distension induces hypersensitivity, oral pregabalin (10 and 30 mg/kg) inhibited the development of hyperalgesia (Million et al., 2007). Moreover, using Fos staining to indicate neuronal activation, a single oral dose of pregabalin (30 mg/kg) blunted the activation of lumbosacral spinal neurones. These data raise the possibility that in this model, pregabalin inhibits spinal sensitization and so inhibits the development of hyperalgesia. It has been demonstrated that maternal separation of rats in the early neonatal period, a presumably highly stressful stimulus, can give rise to hyperalgesia, revealed by colorectal distension, that is sustained for many weeks after the original stress (Coutinho et al., 2002) In an elegant study, to date only published as an abstract, Coelho et al. (2008) confirmed that a dose of gabapentin (30 mg/kg s.c.) that inhibited acute visceral pain evoked by intraperitoneal acetic acid, was also able to inhibit the pain behaviors evoked by colorectal distension in rats that had undergone maternal separation 9–11 weeks earlier.

New $\alpha_2\delta$ ligands are starting to appear in literature, but to date, only one of these, PD-217014, which has similar binding affinity at the $\alpha_2\delta$ binding site as pregabalin, has been investigated in an animal model of visceral hypersensitivity (Ohashi et al., 2008). In the TNBS model described previously, oral dosing of PD-217014 (3–100 mg/kg) dose-dependently inhibited the reduction in colonic nociception threshold observed 7 days after TNBS administration. Maximum inhibition was reached at 60 mg/kg and the inhibition at this dose was long lasting, reaching a peak at 2 h post-dose and lasting for between 6 and 8 h. Pharmacokinetic/pharmacodynamic (PK/PD) analysis clearly demonstrated that maximum anti-hyperalgesia coincided with peak plasma exposures. Interestingly, whilst the anti-hyperalgesic effect persisted at 6 h post-dose, plasma levels had at this time diminished to low levels. These observations suggest that the persistence of the pharmacological effect of PD-217014 is not simply related to plasma exposure and this phenomenon requires further investigation.

In a recent study, Ravnefjord et al. (2008) demonstrated in normal, unsensitized rats that pregabalin (10–200 μmol/kg p.o.) inhibited the viscerosomatic response to phasic, noxious colorectal distension at 80 mmHg as well as the viscerosomatic response to ascending (10–80 mmHg), phasic distension . In this study, the highest dose of 200 μmol/kg p.o. also inhibited the increases in cardiovascular parameters (blood pressure and heart rate) seen in response to noxious distension at 80 mmHg. However, one of the most interesting observations in this study, and one that reveals another potential mechanism of action of pregabalin to reduce pain thresholds in these distension models, was an apparent leftward shift in the colonic pressure-volume relationship. These observations suggest that pregabalin may increase the compliance of the colon in response to distension and by this mechanism could effectively reduce the intensity of the nociceptive stimulus. Similarly, this could be a mechanistic explanation for the observations of antinociceptive activity in models employing colorectal distension as a nociceptive stimulus.

CLINICAL EVIDENCE AND POTENTIAL UTILITY IN IBS

To date only two clinical studies have been published (Lee et al., 2005; Houghton et al., 2007) assessing the effect of these compounds on visceral sensitivity in IBS and one abstract in healthy volunteers (Chua et al., 2009). No results from clinical trials examining the efficacy of $\alpha_2\delta$ ligands on symptoms in IBS patients have yet appeared in literature. However, there is one investigator-sponsored small placebo-controlled trial of pregabalin in IBS (NCT00977197), another investigator sponsored study looking at the effect of pregabalin on colonic sensorimotor function in healthy volunteers (NCT01094808) and a company-sponsored clinical trial assessing the effect of the new generation $\alpha_2\delta$ ligand, PD-217014 in IBS (NCT00139672) currently listed on the US National Institute of Health ClinicalTrial.gov website.

The first study published assessed the effect of gabapentin (300 mg/day for the first 3 days and the 600 mg/day for the subsequent 2 days) on rectal sensitivity to balloon distension in IBS patients with diarrhea diagnosed using Rome II (IBS-D; Lee et al., 2005). The authors reported that the threshold pressures for bloating, discomfort and pain, and rectal compliance all significantly increased after gabapentin but not following placebo. The increase in rectal tone seen after meal ingestion was unaffected. Unfortunately however, no direct comparison was made with placebo in this study, so the significance of their gabapentin findings needs to be viewed with caution. The second study published by the authors assessed the effect of pregabalin (titrated from 50 mg tid to 200 mg tid over 3 weeks) in IBS patients who exhibited rectal hypersensitivity to mechanical distension (Houghton et al., 2007). In comparison to placebo, pregabalin was shown to significantly increase or normalize the sensory thresholds for pain (anti-allodynic effect), along with first sensation and desire to defecate (anti-hyperalgesic effect), without desensitizing (i.e., make hyposensitive) the perception of distension. If confirmed by larger studies and the results from the study currently in progress assessing the effect of pregabalin on colonic sensorimotor function in healthy volunteers (NCT01094808) proves to be negative, then this would suggest that as shown in the animal models, desensitization only occurs in the presence of an hypersensitive state. Such a compound would be most desirable for treatment of IBS and confirms studies in healthy volunteers showing that gabapentin reduces signs of central sensitization induced by intradermal capsaicin (i.e., the area of brush and pinprick hyperalgesia) but not spontaneous or evoked pain induced by capsaicin (Gottrup et al., 2004). Similarly, a more recent study only published in abstract form to date, showed that pregabalin prevents the development of secondary hyperalgesia in the proximal esophagus after distal esophageal acidification but had no effect on the primary hyperalgesia induced in the distal esophagus (Chua et al., 2009), supporting a central mode of action for pregabalin in reduction of pain.

In addition to pregabalin's effect on visceral sensation, and as with the gabapentin rectal motor–sensory study (Lee et al., 2005) and in the animal models (Ravnefjord et al., 2008) described previously, rectal compliance increased following pregabalin, although there appeared to be no association with the observed reduction in visceral pain (Houghton et al., 2007). This suggests additional mechanisms of action, as yet to be explored. Similar observations have been seen before with both clonidine and nitroglycerine both increasing gastric compliance but only clonidine reducing pain perception (Thumshirn et al., 1999).

Generalized anxiety disorder, as with IBS, is a common disorder. Furthermore studies have shown that 32–58% of patients with IBS meet the diagnostic criteria for GAD (Lydiard, 2001), a condition which has recently been shown to improve following treatment with pregabalin (Stein et al., 2009). As well as improving overall anxiety levels in GAD patients, the study showed that treatment with pregabalin also led to an improvement in GI symptoms that very often coexist in these patients and similar to those seen in functional GI disorders, such as IBS (Stein et al., 2009). This raises the possibility that GI symptoms might improve as a consequence of the treatment of anxiety by pregabalin. Neither the presence of GAD nor levels of co-existing anxiety or depression were measured in the study of rectal hypersensitivity (Houghton et al., 2007), but the observation that pregabalin increased the sensory thresholds for first sensation and the desire to defecate, sensations not normally expected to be under significant psychological influence, might suggest that the anxiolytic properties of pregabalin were not playing a major role in modulating visceral sensation. In support of this hypothesis, other studies using anxiolytic agents, such as buspirone or antidepressants such as amitryptyline have shown no effect on colonic sensitivity to balloon distension (Mertz et al., 1998; Chial et al., 2003; Morgan et al., 2005). However, these observations do not exclude the possibility an anxiolytic effect for pregabalin in amelioration of IBS symptoms, especially in patients with anxiety-induced increased defecation (as implicated by the acute restraint stress animal model; Ohashi-Doi et al., 2010), but clinical trials are required in which psychological symptoms along with the cardinal IBS symptoms are measured to address the true efficacy of these agents in the treatment of IBS.

Other factors that might influence rectal sensation are the adverse effects associated with pregabalin, namely dizziness and/or somnolence. However, in the study of Houghton et al. (2007) these side effects had resolved in the majority of patients by the time sensitivity was assessed, and the change in sensory threshold in these patients was no different from that seen in those still retaining mild/moderate side effects, supporting data from animal studies (Ohashi-Doi et al., 2010).

Furthermore the improvement in sensory threshold tended to associate with a reduction in abdominal pain (Houghton et al., 2007), supporting the observations that pregabalin improves GI symptoms associated with GAD (Stein et al., 2009), and pain in patients with fibromyalgia (Hauser et al., 2009; Smith and Barkin, 2010; Straube et al., 2010). The results from the ongoing clinical trials are eagerly awaited, and whether the patients have been appropriately phenotyped to identify any sub-group improvement based on their hypersensitivity or anxiety status also remains to be revealed.

CONCLUSION

Gabapentin and pregabalin are valuable medicines being used for the treatment of a number of conditions, including neuropathic pain, epilepsy, anxiety, and fibromyalgia. A body of evidence implicates binding to the $\alpha_2\delta$ subunits of voltage-gated calcium channels on presynaptic neuronal membranes as their most likely

mechanism of action. However, recent data also points at potential additional mechanisms within the cell which may underwrite some of their chronic effects and also indicates potential modulation of pro-inflammatory pathways through inhibition of NF-κB signaling. The modulation of calcium fluxes evoked by gabapentin and pregabalin has been shown to reduce the release of a broad range of both excitatory and inhibitory neurotransmitters, primarily in the central nervous system and hence this mechanism has great potential to influence signaling pathways, including those involved in pain transmission. Emerging data supports a role for the $\alpha_2\delta$ subunit in neurotransmission in the enteric nervous system, but the functional importance of these observations has yet to be fully elucidated.

Data from animal models provides evidence for the inhibition of both visceral nociception and GI function by gabapentin and pregabalin in animals in which hypersensitivity has been induced by either an inflammatory stimulus or stress, but largely illustrates an absence of such activity on basal sensitivity or function.

When particularly strong noxious stimuli are used (acetic acid or distension to high pressures) effects on sensation in unsensitized animals can be observed. These observations are in concordance with earlier experiments performed using isolated *in vitro* preparations from animals in which hyperalgesia had been established. The precise mechanism through which the $\alpha_2\delta$ ligands inhibit intestinal allodynia and hyperalgesia has only been hinted at and much remains as supposition. The extensive literature that indicates the reduction in the release of neurotransmitters at the spinal and supraspinal level by $\alpha_2\delta$ ligands remains a valid hypothesis to explain the observations, with some supporting data obtained from animal models of visceral pain.

The limited number of clinical studies of visceral pain performed and reported to date support the observations in animals. In patients with IBS, both gabapentin and pregabalin have been shown to reduce rectal sensitivity to balloon distension and in the study with pregabalin, anti-allodynia, and anti-hyperalgesia was demonstrated in subjects with pre-characterized rectal hypersensitivity. Data is expected soon from a similar study in healthy volunteers which will illustrate whether these agents have, like in many animal models, little effect on sensory thresholds in subjects without hypersensitivity. Both animal and clinical data also suggest that $\alpha_2\delta$ ligands may alter intestinal compliance and the significance of this needs further investigation. These data, supported by observations from animal studies, support further investigation of $\alpha_2\delta$ ligands in disorders characterized by visceral hypersensitivity such as IBS. Carefully controlled, randomized clinical trials will be needed to fully understand the potential of these agents to treat these bothersome conditions.

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A comprehensive review of the pharmacodynamics, pharmacokinetics, and clinical effects of the neutral endopeptidase inhibitor racecadotril

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Racecadotril, via its active metabolite thiorphan, is an inhibitor of the enzyme neutral endopeptidase (NEP, EC 3.4.24.11), thereby increasing exposure to NEP substrates including enkephalins and atrial natriuretic peptide (ANP). Upon oral administration racecadotril is rapidly and effectively converted into the active metabolite thiorphan, which does not cross the blood–brain-barrier. Racecadotril has mainly been tested in animal models and patients of three therapeutic areas. As an analgesic the effects of racecadotril across animal models were inconsistent. In cardiovascular diseases such as hypertension or congestive heart failure results from animal studies were promising, probably related to increased exposure to ANP, but clinical results have not shown substantial therapeutic benefit over existing treatment options in cardiovascular disease. In contrast, racecadotril was consistently effective in animal models and patients with various forms of acute diarrhea by inhibiting pathologic (but not basal) secretion from the gut without changing gastro-intestinal transit time or motility. This included studies in both adults and children. In direct comparative studies with loperamide in adults and children, racecadotril was at least as effective but exhibited fewer adverse events in most studies, particularly less rebound constipation. Several guidelines recommend the use of racecadotril as addition to oral rehydration treatment in children with acute diarrhea.

Keywords: racecadotril, neutral endopeptidase, analgesia, hypertension, congestive heart failure, diarrhea, loperamide

BACKGROUND

Acute diarrhea is an alteration of normal bowel movements characterized by an increase in the water content, volume, or frequency of stools. The most common causes are bacterial and viral infections, particularly rotavirus infections, but the specific spectrum of infectious agents depends on the clinical setting (Farthing, 2000). Such infections cause intestinal hypersecretion leading to fluid loss and dehydration. Accordingly, oral rehydration is the cornerstone of treatment, and a standardized glucose-electrolyte solution has been developed under the auspices of the World Health Organization and is being used with great success. While this has significantly improved the prognosis of acute diarrhea, it remains a clinical problem in both the developing world and in industrialized countries and, particularly in developing countries, acute diarrhea is still responsible for the death of two to three million individuals per year worldwide (Farthing, 2006).

While the infection underlying acute diarrhea typically is self-limiting, the associated dehydration can be life-threatening, particularly in children or the elderly. Moreover, a shortening of the duration of acute diarrhea can also be an important medical aim. Therefore, drug treatment can also be a relevant part of the therapeutic approach, in most cases given on top of rehydration treatment. Among anti-diarrhea drugs antibiotics are typically limited to severe cases and other special situations. More frequently, μ-opioid receptor agonists such as codeine, loperamide, and morphine are being employed, among which loperamide has become most frequently used (Baldi et al., 2009). Their main mechanism of action is a reduction of gut motility and accordingly they can cause secondary constipation, abdominal pain, and abdominal distension.

Against this background, racecadotril has been developed as a possible alternative to the use of μ-opioid receptor agonists. Following its original registration as a prescription drug in France in 1992 it meanwhile is available in many countries around the globe, and since 2005 in some of them as a non-prescription drug. The present manuscript reviews the pharmacodynamic, pharmacokinetic, and clinical data for racecadotril and its active metabolite thiorphan. While the clinical focus of the manuscript is on the role of racecadotril in the treatment of diarrhea, we will also discuss other potential uses as they will aid the understanding of the overall clinical profile of the drug. Racecadotril has been reviewed in the past (Lecomte, 2000; Matheson and Noble, 2000; Schwartz, 2000) but those articles had a more limited scope and more than 40 new studies have been published since.

Abbreviations: ANP, atrial natriuretic peptide; i.c.v., intra-cerebroventricular; i.p., intra-peritoneal; i.v., intravenous; NEP, neutral endopeptidase.

MOLECULAR EFFECTS OF RACECADOTRIL

Racecadotril, formerly known as acetorphan, is a prodrug, which is converted to the active metabolite thiorphan (see below; **Figure 1**). Acetyl-thiorphan is another active metabolite of racecadotril but yields only low potency NEP inhibition (Lambert et al., 1993). Racecadotril has stereoisomers, and the *S*- and *R*-isomers of racecadotril are named ecadotril (also known as BP102 or as sinorphan) and retorphan, respectively (Lecomte et al., 1990). Thus, in the subsequent text racecadotril and thiorphan refer to the racemate, whereas ecadotril refers to the *S*-isomer of racecadotril.

At the molecular level racecadotril and thiorphan act by inhibiting the enzyme neutral endopeptidase (NEP, EC 3.4.24.11; see below), which is a membrane-metalloendopeptidase also known as enkephalinase. NEP has various substrates including enkephalins (hence the name enkephalinase) but also atrial natriuretic peptide (ANP), brain natriuretic peptide, substance P, neurotensins, and neuropeptide Y (van Kemmel et al., 1996; Turvill and Farthing, 1997). Therefore, NEP inhibition can potentially affect any of these mediators and observed *in vivo* effects in different organ systems may not always relate to the same enzyme substrate (see below).

The first report on thiorphan described an IC_{50} of 4.7 nM for NEP inhibition in striatal membranes (Roques et al., 1980). Inhibition of purified NEP activity from mouse brain yielded affinity estimates (K_i values) of 6.1 and 4500 nM for thiorphan and racecadotril, respectively; however, when racecadotril was pre-incubated with rat brain membranes for 15 min, an apparent K_i value of 8.6 nM was observed, probably reflecting rapid *in vitro* conversion to thiorphan (Lecomte et al., 1986). A similar study reported an IC_{50} of 1.8 nM for thiorphan with racecadotril being 1000 times less potent and acetyl-thiorphan having a value of 316 nM (Lambert et al., 1993, 1995). For *in vitro* inhibition of rat kidney NEP an IC_{50} of 5.4 nM was reported (Fink et al., 1995), apparently reflecting *in vitro* conversion to thiorphan as shown before in rat brain (Lecomte et al., 1986).

A second approach to assess thiorphan affinity for NEP has been radioligand binding studies. In saturation binding studies in various mouse tissues [^{3}H]-thiorphan exhibited an affinity (K_d value) of 0.46–0.77 nM, and the density of [^{3}H]-thiorphan binding sites was well correlated with measured NEP activity in a panel of 11 different mouse tissues (de la Baume et al., 1988). Similar saturation binding experiments using [^{3}H]-racecadotril as the ligand reported an affinity of 4–5 nM in rats (Fournet-Bourguignon et al., 1992), apparently reflecting conversion of racecadotril to thiorphan in the assay (Lecomte et al., 1986). A third approach has been to measure occurrence of enkephalin break-down products such as the tripeptide Tyr–Gly–Gly, and in isolated rat brain slices this has yielded an IC_{50} of 9 nM for thiorphan (Giros et al., 1986).

Correspondingly, it has repeatedly been observed that oral, intra-peritoneal (i.p.), or intravenous (i.v.) administration of racecadotril or ecadotril leads to a rapid reduction in NEP activity in plasma (Spillantini et al., 1986; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Stasch et al., 1996; Duncan et al., 1999; Lecomte, 2000), kidney (Gros et al., 1989), and brain (Lecomte et al., 1986; Spillantini et al., 1986). Such studies were performed with consistent results in rats (Lecomte et al., 1986; Stasch et al., 1996; Wegner et al., 1996; Duncan et al., 1999), mice (Lecomte et al., 1986), and humans (Spillantini et al., 1986; Gros et al., 1989; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Lecomte, 2000). *In vivo* inhibition of enkephaline metabolite formation was also observed in rat spinal cord after i.v. racecadotril (Llorens-Cortes et al., 1989) or in mouse striatum after intra-cerebro-ventricular (i.c.v.) thiorphan (Llorens-Cortes et al., 1986). Of note, assessment of NEP inhibition by measuring endogenous enkephalins can yield false negative results as enkephalines can also be metabolized by other aminopeptidases such as EC 3.4.11.2, and this can compensate for NEP inhibition (Bourgoin et al., 1986; Llorens-Cortes et al., 1986). As ANP also is a NEP substrate, NEP inhibition can also be assessed by changes of ANP concentrations in plasma and urine, which are described in detail in Section "Cardiovascular Studies."

The *R*- and *S*-stereoisomers of thiorphan inhibited purified NEP activity with similar potency 1.7 vs. 2.2 nM, respectively, and occurrence of the enkephalin metabolite Tyr–Gly–Gly with an IC_{50} of 10 nM (Giros et al., 1987). In the same study occurrence of Tyr–Gly–Gly in mouse striatum was also inhibited with similar potency by i.v. administration of ecadotril and retorphan, the stereoisomers of racecadotril (ED_{50} 0.4 and 0.8 mg/kg, respectively). On the other hand, with the same oral dose of ecadotril and retorphan inhibition of the *in vivo* binding of [^{3}H]-racecadotril in mouse kidney was somewhat stronger for the *S*-isomer (Lecomte et al., 1990). Similarly, a 30-mg oral dose of ecadotril produced somewhat greater inhibition of NEP activity and ANP levels in human plasma than the same dose of retorphan (Lecomte et al., 1990).

In conclusion, racecadotril and its metabolite acetyl-thiorphan are only low potency NEP inhibitors. However, racecadotril is rapidly converted to the active metabolite thiorphan *in vitro* (Lecomte et al., 1986) and *in vivo* (see Pharmacokinetic and

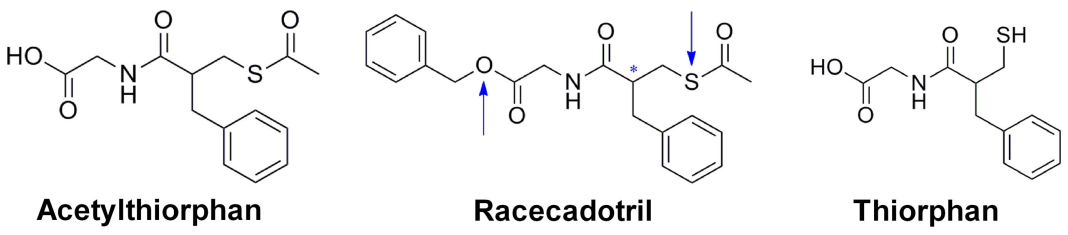

FIGURE 1 | Chemical structures of racecadotril and its two metabolites thiorphan and acetyl-thiorphan. The blue arrows indicate the sites of metabolization; the asterisk indicates the chiral center of the molecule.

Drug–Drug Interaction Studies), and thiorphan is an about 1000 times more potent NEP inhibitor than racecadotril with reported potencies of 0.4–9 nM. The *S*-isomers of racecadotril and thiorphan may be slightly more potent than the *R*-isomers.

PHARMACOKINETIC AND DRUG–DRUG INTERACTION STUDIES

ANIMAL PHARMACOKINETIC STUDIES

In mice, following i.v. administration, racecadotril was rapidly metabolized to thiorphan; thus, 30 min after the injection only thiorphan was recovered from the kidney whereas the parent compound racecadotril was not detected (de la Baume et al., 1988). In rats 92% of a single-dose (10 mg/kg) of radioactively labeled racecadotril was eliminated within 24 h (Matheson and Noble, 2000).

HUMAN PHARMACOKINETIC STUDIES

Racecadotril is rapidly absorbed following oral administration. For example, in a single-dose, placebo-controlled, double-blind cross-over study racecadotril doses of 30, 100, and 300 mg reached C_{max} within 60 min after oral administration; the latter two doses were associated with significant inhibition of plasma NEP activity seen as early as 30 min after ingestion, and that inhibition exhibited a $t_{1/2}$ of 3 h (Lecomte, 2000; Matheson and Noble, 2000). A single-dose study in healthy elderly people reported similar findings (Matheson and Noble, 2000). After ingestion of a single oral dose of 300 mg racecadotril plasma thiorphan levels peaked after 60 min reaching 805–1055 nM; after 240 min plasma levels were still at 92–204 nM (Hinterleitner et al., 1997). In a more recent study, a well validated liquid chromatography/tandem mass spectrometry method has been used to detect thiorphan in human plasma; 20 volunteers received a single oral dose of 200 mg racecadotril, which resulted in a thiorphan C_{max} of 520 ng/mL, a t_{max} of 1.35 h and a $t_{1/2}$ of 6.14 h (Xu et al., 2007). The pharmacokinetic properties of racecadotril are similar with chronic dosing as observed in a placebo-controlled, double-blind study with 30, 100, and 300 mg racecadotril being given orally thrice daily for 7 days, where pharmacokinetic parameters were similar on day 1 and day 7 and to the values observed in the single-dose studies (Matheson and Noble, 2000), indicating lack of accumulation upon chronic dosing. Concomitant food intake does not modify the bioavailability of racecadotril but peak NEP inhibition is delayed by about 90 min (data on file).

After oral administration racecadotril is rapidly and effectively metabolized to the active metabolite thiorphan which is the predominant species detected in plasma; the occurrence of thiorphan coincides in time with the inhibition of plasma NEP (Hinterleitner et al., 1997; Xu et al., 2007). Thiorphan has a plasma protein binding of approximately 90% (data on file).

An important question for any drug interfering with the endogenous opioid system is whether it crosses the blood–brain-barrier, i.e., whether upon oral racecadotril administration parent compound or active metabolite reaches relevant levels in the brain to cause NEP inhibition. Animal studies suggest that central nervous effects can occur after parenteral administration of racecadotril but not after oral racecadotril or parenteral thiorphan administration (see Studies on Central Nervous System Function); this is the apparent result of the combination of the rapid conversion of absorbed racecadotril to thiorphan and the lack of thiorphan passage through the blood–brain-barrier due to its less lipophilic chemical structure (**Figure 1**). In line with these animal data it has been reported that i.v. administration of racecadotril causes quantitatively similar NEP inhibition in plasma and cerebrospinal fluid in five healthy volunteers as compared to saline infusion (Spillantini et al., 1986), whereas a single high oral racecadotril dose (20 mg/kg) to two volunteers caused a marked reduction of plasma NEP activity within 30 min but did not affect liquor NEP activity (Lecomte, 2000). In accordance with the proposed lack of central nervous effects of orally administered racecadotril, a placebo-controlled cross-over study in 12 subjects being treated with 300 mg/kg racecadotril for 3 days did not detect any impairment of vigilance (Lecomte, 2000).

The active racecadotril metabolite thiorphan is converted to inactive metabolites, but the pathways mediating this conversion have not been characterized in great detail. While two studies using different methodological approaches have reported that racecadotril inhibits CYP 3A4-mediated formation of metabolites of the cancer chemotherapeutic drug irinotectan with an IC_{50} of 46 μM in human liver microsomes (Haaz et al., 1998a,b), it should be noted that this concentration is equivalent to about 10,000 times the potency of thiorphan for NEP inhibition. Thus, in concentrations which are achieved by therapeutic doses racecadotril is neither an inhibitor nor an inducer of cytochrome P450 enzymes and also not a substrate of the P-glycoprotein transporter (data on file). Accordingly, to date no interactions with other medicinal products have been identified and specifically concomitant treatment with loperamide or nifuroxazide does not affect the pharmacokinetics of racecadotril (data on file). Moreover, racecadotril does not modify protein binding of active substances strongly bound to proteins such as tolbutamide, warfarin, niflumic acid, digoxin, or phenytoin (data on file). The elimination of the inactive thiorphan metabolites occurs mainly via the renal route (data on file).

STUDIES ON CENTRAL NERVOUS SYSTEM FUNCTION

As discussed in Section "Pharmacokinetic and Drug–Drug Interaction Studies," parenteral administration of racecadotril can have central nervous effects but these are not apparent with oral racecadotril or parenteral thiorphan treatment. Most effects of racecadotril and its metabolites on brain function apparently are mediated by inhibition of enkephalin degradation, as opiate receptor antagonism in many cases abolishes them (see below).

ANALGESIA

Based on the role of morphine and other opiates in pain control, it was an obvious choice to test racecadotril in various pain models. The results have been rather inconsistent across models but much more consistent within models indicating that NEP inhibition selectively targets certain pain pathways. Thus, racecadotril was analgesic in the hot-plate jump test in unspecified mice with i.c.v. administration (Roques et al., 1980) and with i.v. administration in Swiss albino (Lecomte et al., 1986; Costentin et al., 1998), NMRI (Lambert et al., 1993, 1995) and DBA/2J mice but not C57BL/6J mice (Michael-Titus et al., 1989). Interestingly, in the latter study racecadotril increased locomotion in both strains, indicating that

the difference in analgesic effect does not reflect a pharmacokinetic strain difference. In NMRI mice i.v. acetyl-thiorphan and thiorphan were also effective in this model, although thiorphan less than the more lipophilic acetyl-thiorphan and racecadotril (Lambert et al., 1993, 1995). I.v. racecadotril was also analgesic in Swiss albino mice in the tail-withdrawal and the phenylbenzoquinone-induced writhing test (Lecomte et al., 1986). In an arthritis-based pain model in rats (vocalization induced by applying pressure to the left hind paw) racecadotril also exhibited analgesic activity (Kayser and Guilbaud, 1983), but this was weaker than that of the mixed peptidase inhibitor kelatorphan in the same model (Kayser et al., 1989). In the same test racecadotril was not analgesic in non-arthritic rats (Kayser and Guilbaud, 1983). In unspecified mice i.c.v. thiorphan was ineffective in the tail removal test but enhanced the analgesic effects of several enkephalines which are NEP substrates but not of others which are not NEP substrates (Roques et al., 1980). Moreover, racecadotril did not exhibit analgesic properties in the hot-plate licking test in Swiss albino (Lecomte et al., 1986) or NMRI mice (Lambert et al., 1993) or in albino mice in the tail immersion test, but enhanced the anti-nociceptive effect of an exogenously administered enkephalin in the latter model (Livingston et al., 1988). In GB1 mice in the acetic acid-induced abdominal constriction assay racecadotril was effective only at subcutaneous doses of 10 mg/kg and higher (Gray et al., 1998).

Upon chronic stimulation the opioid system can exhibit both desensitization and sensitization. Thus, the analgesic response to i.v. racecadotril was blunted after 14 days of i.c.v. treatment with thiorphan in rats (Bousselmame et al., 1991a). However, no cross-desensitization between racecadotril and morphine was observed in mice (Bousselmame et al., 1991b) or rats, and in the latter also no cross-sensitization for locomotive effects was observed (Khallouk-Bousselmame and Costentin, 1994).

It has also been tested whether racecadotril can enhance analgesic effects of other treatments. Thus, racecadotril or thiorphan enhanced naloxone-sensitive analgesia induced by transcranial electrostimulation in rats (Malin et al., 1989) or by nefopam in mice (Gray et al., 1999), and also enhanced analgesic effects of electroacupunture in rats (Zhou et al., 1990). In the acetic acid-induced abdominal constriction pain model in GB1 mice racecadotril enhanced the analgesic effect of morphine and of anti-depressants such as dothiepine and amitriptyline (Gray et al., 1998). On the other hand, racecadotril did not enhance naloxone–sensitive analgesic effects in four different pain models in mice (Michael-Titus and Costentin, 1987), and neither i.v. racecadotril nor i.c.v. thiorphan enhanced analgesic effects of dopamine receptor agonists in mice (Michael-Titus et al., 1990a). Another type of interaction between pain-related pathways was suggested by findings in mice in which nociceptin attenuated the analgesic response to racecadotril (Costentin et al., 1998).

In conclusion, racecadotril has direct analgesic effects and can enhance analgesic effects of some other types of drugs in some but not all pain models, but such studies were largely restricted to parenteral administration. Similar to direct opioid receptor agonists, racecadotril-induced analgesia can undergo desensitization upon long-term exposure but despite both morphine and racecadotril effects involving opioid receptors, they did not exhibit cross-desensitization. As the anti-nociceptive effects of racecadotril are restricted to some model systems, it can be expected that, if at all, racecadotril would be effective only in some forms of pain in patients and only upon parenteral administration; however, a clinical testing of potential analgesic effects of racecadotril in patients has not been reported to our knowledge.

OTHER NERVOUS SYSTEM STUDIES

The administration of natural or synthetic opioid receptor agonists elicits a locomotor response in rodents, which is considered to be an index of the activity of mesolimbic dopaminergic neurons. In both mice and rats i.v. racecadotril was reported to enhance locomotion in mice and rats in a naloxone–sensitive manner, and that response was blocked by a dopamine receptor antagonist and enhanced by a dopamine uptake inhibitor (Michael-Titus et al., 1987, 1990b). Similar to the analgesic racecadotril response (see Analgesia), the locomotor racecadotril response also was desensitized following a 14-day i.c.v. treatment with thiorphan (Bousselmame et al., 1991a). In line with the idea that racecadotril can affect dopaminergic transmission in the brain, it was found that i.v. racecadotril modulates dopaminergic transmission in rat olfactory tubercle but not striatum (Dourmap et al., 1990).

Both the opioid and the dopamine system in the brain are prone to addiction. Therefore, it was important to find that racecadotril did not exhibit abuse potential in rats or monkeys in doses up to 50 mg/kg (Knisely et al., 1989). Nevertheless, i.p. racecadotril prevented some but not all withdrawal symptoms in opioid-habituated mice and rats (Livingston et al., 1988; Dzolic et al., 1992). However, racecadotril alone was ineffective in inhibiting naloxone-induced morphine withdrawal symptoms in mice in another study, but a combination of subthreshold doses of racecadotril, CCK-4, and caerulein was effective (Bourin et al., 1999). In a double-blind, double-dummy, clinical proof-of-concept study in 19 heroin-addicted patients comparing 50 mg i.v. racecadotril and 75 μg oral clonidine; racecadotril appeared more effective than clonidine against objective withdrawal symptoms as quantified by the Himmelsbach scale, whereas both treatments were similarly effective against subjective withdrawal symptoms (Hartmann et al., 1991).

Some studies have explored potential metabolic effects of racecadotril. In sheep oral and i.v. administration of racecadotril increased food intake, whereas i.c.v. thiorphan reduced it; as oral racecadotril treatment does not lead to NEP inhibition in the brain and as i.c.v. thiorphan did not mimick the racecadotril effects, this appears to be a peripheral effect (Riviere and Bueno, 1987). Moreover, naltrexone blocked the former but not the latter effect, indicating an involvement of peripheral opioid receptors. A study with i.v. racecadotril in cats reported increases in sham food intake (Bado et al., 1989). In rats i.v. racecadotril caused naloxone-insensitive dose-dependent lowering of blood glucose which was accompanied by increased plasma insulin and C-peptide levels (Wu et al., 2010). While racecadotril did not affect insulin release from isolated pancreatic islets, the muscarinic receptor antagonist atropine blocked and the cholinesterase inhibitor physostigmine enhanced the racecadotril-induced insulin elevation, indicating that they may occur via modulation of parasympathetic nerve activity. In a follow-up study the same investigator group provided evidence that i.v. racecadotril and i.c.v. thiorphan directly inhibit

an insulin-degrading enzyme in the brain, and that brain insulin acts via the vagal nerve on plasma glucose (Lee et al., 2011). The clinical findings of these intriguing observations have not been explored in patients to our knowledge.

In a mouse behavioral dispair test, a model system for anti-depressant effects, immobility time was reduced by 10 mg/kg i.v. or 50 mg/kg i.p. in mice (Lecomte et al., 1986). Moreover, it was reported that thiorphan can provide neuroprotection in newborn mice (Medja et al., 2006).

CARDIOVASCULAR STUDIES

Most effects of racecadotril and its metabolites in the cardiovascular system apparently are largely mediated by inhibition of degradation of the natriuretic peptides. Thus, racecadotril-, ecadotril-, or thiorphan-induced inhibition of ANP break-down and/or elevation of ANP levels have been demonstrated in rats (Fink et al., 1996; Stasch et al., 1996), mice (Gros et al., 1989, 1990a,b; Lecomte et al., 1990; Stasch et al., 1996), and humans (Gros et al., 1989; Dussaule et al., 1991, 1993; Piquard et al., 2002) as assessed in plasma (Gros et al., 1989, 1990b; Lecomte et al., 1990; Dussaule et al., 1991, 1993; Schmitt et al., 1994; Fink et al., 1996; Stasch et al., 1996; Piquard et al., 2002) or in tissues such as kidney (Gros et al., 1989, 1990a). The increase in circulating ANP concentrations is typically associated with increased concentrations of the ANP-generated second messenger cyclic GMP in plasma (Dussaule et al., 1993; Stasch et al., 1995, 1996; Cleland and Swedberg, 1998; Piquard et al., 2002) or urine of animals and patients (Lecomte et al., 1990; Dussaule et al., 1991; Schmitt et al., 1994; Stasch et al., 1995, 1996; Cleland and Swedberg, 1998; Kimura et al., 1998; Duncan et al., 1999). The racecadotril and ecadotril effects on plasma ANP may be even more pronounced in patients (see below). Based on these findings, racecadotril has been studied in various animal models, in healthy volunteers, and in patient groups in which an increased exposure to ANP has been deemed beneficial.

An important physiological effect of ANP is promoting diuresis and natriuresis. Based on the consistent racecadotril effects on ANP, effects of racecadotril have been studied in various animal models and in humans. Thus, oral racecadotril treatment increased natriuresis in normotensive rats, which was accompanied by enhanced diuresis in some (Bralet et al., 1990) but not other studies (Stasch et al., 1995, 1996). Racecadotril-induced diuresis and natriuresis have also been reported in healthy volunteers (Gros et al., 1989; Lecomte et al., 1990). This was accompanied by an elevated glomerular filtration rate and lowered renal blood flow whereas plasma aldosterone concentration, renin activity, and mean arterial blood pressure were not altered (Schmitt et al., 1994).

TREATMENT OF ARTERIAL HYPERTENSION

Racecadotril has been tested in various animal models of hypertension, specifically for its ability to lower blood pressure, improve renal function, and to prevent or reverse organ hypertrophy. These models include spontaneously hypertensive rats (Bralet et al., 1990) and its stroke-prone substrain (Stasch et al., 1995), transgenic rats harboring a mouse renin gene (Stasch et al., 1996), hypertension induced by treatment with the immunosuppressant cyclosporine A in rats (Takeda et al., 2000), and the rat deoxycorticosterone acetate-salt model of mineralocorticoid-induced hypertension (Ito et al., 1999). Blood pressure lowering by oral racecadotril or ecadotril was consistently shown (Stasch et al., 1995, 1996; Ito et al., 1999; Takeda et al., 2000). In a first pilot study in 12 hypertensive patients receiving increasing sinorphan doses (25–200 mg bid) for a total of 6 weeks a dose-dependent blood pressure reduction was also observed (Lefrancois et al., 1990). In a subsequent randomized, double-blind clinical pilot study with a cross-over design in 16 hypertensive patients racecadotril was less effective than captopril in lowering blood pressure, but the combination of both drugs was more effective than either monotherapy (Favrat et al., 1995).

In line with the effects in normotensive animals and healthy human volunteers, racecadotril also increased diuresis and/or natriuresis in spontaneously hypertensive rats (Bralet et al., 1990), in transgenic rats (Stasch et al., 1996) and in deoxycorticosterone acetate-salt-treated rats (Ito et al., 1999) whereas numerical increases of diuresis and natriuresis did not yield statistical significance in stroke-prone spontaneously hypertensive rats (Stasch et al., 1995).

A prognostically relevant complication of hypertension is the development of hypertrophy of the heart and other cardiovascular organs. In this regard, chronic treatment with racecadotril reduced heart hypertrophy in stroke-prone spontaneously hypertensive rats (Stasch et al., 1995), in transgenic rats (Stasch et al., 1996) and in deoxycorticosterone acetate-salt-treated rats (Ito et al., 1999). A reduction of renal enlargements was observed less consistently in these studies.

Taken together these studies demonstrate beneficial effects of treatment with racecadotril on blood pressure, renal function, and cardiac hypertrophy in various animal models of hypertension, which is in line with the elevated ANP levels in such animals. While clinical pilot studies have confirmed blood pressure lowering effects in hypertensive patients, these were too modest in comparison to established anti-hypertensive treatments to warrant further clinical investigation of racecadotril in this indication; potential enhancement of blood pressure lowering by other drugs was also deemed insufficient to be of clinical relevance.

TREATMENT OF CONGESTIVE HEART FAILURE

Congestive heart failure leads to atrial dilatation which is the most important physiological stimulus for ANP secretion. This enhanced ANP secretion is generally seen as a counter-measure to increase diuresis and natriuresis and thereby lower cardiac after-load. Accordingly, racecadotril has been evaluated in animal models and in patients with heart failure. Animal models of heart failure in which racecadotril or ecadotril have been tested include rats with volume overload due to aortic valve insufficiency (Kimura et al., 1998) or to an atrio-ventricular fistula (Wegner et al., 1996), rats after a myocardial infarction (Duncan et al., 1999), dogs with coronary microembolization (Olivier et al., 2000; Mishima et al., 2002), and dogs with heart failure due to sino-atrial pacing (Solter et al., 2000).

In line with the fluid retention, an activation of the renin–angiotensin system is a hallmark of congestive heart failure. Such activation was mitigated by treatment with racecadotril in animal models (Wegner et al., 1996; Kimura et al., 1998; Duncan et al.,

1999). A similarly reduced activity of the renin–angiotensin system was observed in an early and short-term pilot study in heart failure patients (Kahn et al., 1990) but not in a larger chronic study in such patients (Cleland and Swedberg, 1998). Whether indirectly by reducing activity of the renin–angiotensin system or more directly by increasing ANP exposure, diuretic and/or natriuretic effects of racecadotril were observed in the volume overload rats (Wegner et al., 1996), coronary microembolization dogs (Olivier et al., 2000), and in dogs with pacing-induced heart failure (Solter et al., 2000) but not in post-myocardial infarction rats (Duncan et al., 1999). Accordingly, improvements of the cardiac pump function were observed in most of these models (Kimura et al., 1998; Olivier et al., 2000; Mishima et al., 2002), whereas blood pressure did not change (Mishima et al., 2002).

Some of these heart failure models, particularly those involving volume or pressure overload typically lead to cardiac hypertrophy. In line with the other findings it has been found that chronic racecadotril treatment ameliorates such cardiac hypertrophy in the volume overload rat models (Wegner et al., 1996; Kimura et al., 1998). Similarly, racecadotril treatment also reduced cardiac hypertrophy in the dog microembolization model (Mishima et al., 2002). However, in the post-myocardial infarction rat model neither racecadotril nor perindopril alone reduced development of cardiac hypertrophy whereas their combination did (Duncan et al., 1999).

In accordance with the observed increase in plasma ANF and/or urinary cGMP, three studies have explored whether racecadotril or ecadotril may have therapeutic benefit in heart failure patients. In a series of small pilot studies in patients with severe heart failure (left ventricular ejection fraction 20%), 2 days of ecadotril treatment doubled plasma ANP levels despite starting from a markedly elevated baseline (Kahn et al., 1990). This was accompanied by a reduced renin activity and pulmonary capillary wedge pressure. Based on those encouraging findings a clinical, placebo-controlled dose-ranging study (50–400 mg ecadotril twice daily for 6 months) was performed in 259 patients with moderate heart failure (left ventricular ejection fraction of <35%; Cleland and Swedberg, 1998). This confirmed a dose-dependent increase in plasma and urinary cGMP but patients did not show reduced activation of the renin–angiotensin system or, more importantly, clinical improvement. Another study in a similar population of 50 moderate heart failure patients with 10 weeks of treatment with increasing racecadotril doses (up-titration from 50 to 400 mg twice daily) also failed to demonstrate clinical improvement (O'Connor et al., 1999).

Taken together racecadotril has shown promising findings in animal models of congestive heart failure but patient studies have not confirmed a sufficient clinical potential to warrant further development in this indication.

TREATMENT OF OTHER CARDIOVASCULAR CONDITIONS

Racecadotril has also been tested in animal models and/or clinical pilot studies for various other indications related to cardiovascular function. Studies in rats have reported that i.v. racecadotril may protect the heart against adrenaline-induced arrhythmia (Lishmanov et al., 2001) or against arrhythmia induced by short ischemia–reperfusion episodes (Naryzhnaia et al., 2001), the latter effect being blocked by a δ-opioid receptor antagonist. In a mouse model of pulmonary hypertension ecadotril was found to have synergistic beneficial effects with sildenafil (Baliga et al., 2008).

In a single-dose pilot study in liver cirrhosis patients racecadotril 30 and 100 mg increased plasma ANP and cGMP and caused a transient diuresis and natriuresis response relative to placebo; the activity of the renin–angiotensin system apparently was not affected in these patients (Dussaule et al., 1991). In a single-dose cross-over pilot study in chronic renal failure patients 100 mg ecadotril inhibited enkephalinase, increased plasma cGMP and natriuresis; aldosterone, glomerular filtration rate, or blood pressure were not affected (Dussaule et al., 1993). Finally, a single dose of 200 mg ecadotril increased plasma endothelin-1, ANP, and cGMP and diuresis and natriuresis in a randomized placebo-controlled study in heart transplant recipients (Piquard et al., 2002).

STUDIES IN THE GASTRO-INTESTINAL TRACT

Most effects of racecadotril and its metabolites on gastro-intestinal function apparently are mediated by inhibition of enkephalin degradation, as opiate receptor antagonism in many cases abolishes them (see below). However, an inhibition of the degradation of neuropeptide Y and the closely related peptide YY by NEP may also be involved as both of these peptides have anti-secretory effects in the gut (Playford and Cox, 1996).

EXPERIMENTAL STUDIES RELATED TO DIARRHEA

It is well established that enkephalines have potent anti-secretory properties in the gut but do not affect gut motility (Turvill and Farthing, 1997). In an initial study in rats, i.v. racecadotril inhibited castor oil-induced diarrhea, an effect which was abolished by the opioid receptor antagonist naloxone (Lecomte et al., 1986). Such findings in the castor oil model of diarrhea were confirmed in rats with oral racecadotril and with i.v. thiorphan; they were blocked by subcutaneous but not by i.c.v. naloxone, indicating that the opioid receptors mediating this effects are located peripherally (Marcais-Collado et al., 1987). Racecadotril also reduced castor oil-induced diarrhea in human volunteers in a placebo-controlled study (Baumer et al., 1992).

To explore the underlying mechanism of anti-diarrhea effects of racecadotril, several studies have been performed. Thus, excessive fluid secretion from the gut is a pathophysiological hallmark of diarrhea. Racecadotril inhibited cholera toxin-induced but not basal secretion in canine jejunum (Primi et al., 1999). This was confirmed in a parallel group study in human volunteers, in which cholera toxin was administered by segmental perfusion directly into the proximal jejunum (Hinterleitner et al., 1997). In another study, racecadotril inhibited secretion induced by rotavirus infection in an *in vitro* model of intestinal secretion, Caco-2 cells (Guarino et al., 2009), a model which may be of value because rotavirus infection is a very frequent cause of childhood diarrhea.

A potential complication of diarrhea treatment is inhibition of intestinal motility as it can lead to secondary constipation and, perhaps even more important, intestinal retention of harmful infectious organisms. In rats oral 40 mg/kg racecadotril was reported not to affect gastro-intestinal transit time, whereas 2 mg/kg loperamide did (Marcais-Collado et al., 1987). Using the

same approach in mice, 20 mg/kg i.v. of racecadotril or thiorphan or 0.5 mg/kg oral loperamide also did not significantly affect transit time, whereas 10 mg/kg oral or 0.5 mg/kg i.v. loperamide significantly prolonged it (Marcais-Collado et al., 1987). A potential consequence of effects on gastro-intestinal transit time was explored in newborn piglets, in which a 4-day oral treatment with 20 mg/kg racecadotril twice daily did not significantly affect E. coli content of the proximal jejunum, whereas 1 mg/kg oral loperamide twice daily markedly increased it; accordingly, the *E. coli* content of the stool was significantly reduced by loperamide but not by racecadotril (Duval-Ilfah et al., 1999). In placebo-controlled studies in human volunteers racecadotril treatment for up to 1 week also did not modify oro-coecal, colonic or overall gastro-intestinal transit times (Baumer et al., 1989; Bergmann et al., 1992).

In conclusion, both racecadotril and direct μ-opioid receptor agonists have effects on the gut which lead to limitation of pathological fluid loss. While the receptor agonists do so primarily by prolonging transit time and hence providing more opportunity for fluid reabsorption, racecadotril does so by inhibiting fluid secretion; the latter may be preferable as it directly targets the primary pathophysiological mechanism underlying acute diarrhea and also reduces the chance of retention of infectious agents in the gut.

NON-DIARRHEA GASTRO-INTESTINAL STUDIES

I.v. administration of racecadotril was shown to inhibit gastric secretion in cats induced by pentagastrin, histamine or 2-deoxy-D-glucose in a naloxone-sensitive manner, whereas the meal-induced secretion was not affected (Bado et al., 1987). In rats i.v. racecadotril and i.c.v. thiorphan, but not i.v. thiorphan, inhibited gastric acid secretion; this was no longer observed following vagotomy, indicating a central nervous system-mediated effect (Chicau-Chovet et al., 1988). In mice i.p. racecadotril and thiorphan given prior to a fatty meal enhanced gastric emptying in a naloxone-sensitive manner; gastric emptying in response to a non-fat meal was enhanced by low and inhibited by a high thiorphan dose in a naloxone–resistant manner, and racecadotril was without significant effect under these conditions (Liberge et al., 1988).

Effects of racecadotril have also been investigated in the feline gall bladder. In a feline cholecystitis model racecadotril inhibited fluid secretion in a naloxone–sensitive manner, but did not block fluid transport in the normal gall bladder (Jivegard et al., 1989). In that study racecadotril also caused transient gall bladder contraction and increased bile outflow from the liver. In another study in the same species i.v. racecadotril but not i.v. thiorphan caused naloxone–sensitive contraction of the spincter Oddi, indicating a central nervous system-mediated effect (Thune et al., 1992).

In a controlled cross-over study in 10 healthy volunteers, 2.5 mg/kg i.v. racecadotril attenuated relaxation of the lower esophagus sphincter but did not affect contraction (Chaussade et al., 1988). Racecadotril enhanced the propagation of electrical signals in the distal colon in rats in the fasted and fed state in a naloxone–sensitive manner (Benouali et al., 1993). In a study in healthy volunteers racecadotril produced an atropine-resistant promotion of electrical activity in the rectum, which was absent in patients with Hirschsprung's disease (Grimaud et al., 1989).

PLACEBO-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA

The data of all controlled studies with racecadotril in the treatment of acute diarrhea in adults are summarized for efficacy in **Table 1** and for adverse events in **Figure 2**. The original registration of racecadotril as a treatment for acute diarrhea in adults in France in 1992 was based on three placebo-controlled studies. In a double-blind, placebo-controlled, randomized dose-ranging study 49–55 patients per group with acute diarrhea presumed to be due to food poisening or infection-related received 30, 100, or 300 mg racecadotril or placebo three times per day until recovery for up to 10 days (data on file). The primary outcome parameter was time to cure, which was 68.4, 69.6, 65.0, and 72.0 h with 30, 100, and 300 mg racecadotril and placebo, respectively (not significant). Secondary outcome parameters included number of diarrhoic stools in the first 10 h (2.0–2.2 with the three racecadotril doses as compared to 2.7 days with placebo; $p = 0.06$) and in first 3 days (6.8–7.7 with the three racecatodril doses as compared to 8.6 days with placebo; $p = 0.03$). The incidence of the adverse events anal burning, painful anal contractions, spontaneous abdominal pain, nausea, vomiting, loss of appetite, asthenia, and insomnia did not differ significantly between groups.

In a double-blind, randomized, placebo-controlled study adult out-patients with acute diarrhea of presumed infectious origin having started less than 5 days before (95 on racecadotril, 98 on placebo) were treated with an initial dose of 200 mg racecadotril followed by an additional 100 mg dose after each unformed bowel movement until recovery or for a maximum of 10 days (Baumer et al., 1992). Resolution of diarrhea occurred significantly faster in a Kaplan–Meier type analysis with racecadotril than with placebo, e.g., on day 4 the cumulative probability of recovery was 75% with racecadotril vs. 37% with placebo. Accordingly, mean duration of treatment was 3.0 ± 0.2 days with active treatment vs. 4.4 ± 0.3 days with placebo. Several secondary endpoints including anal burning, spontaneous abdominal pain, nausea, anorexia, pain on abdominal palpation, and abdominal distension were also significantly improved by racecadotril as compared to placebo. The percentage of patients reporting adverse effects with racecadotril and placebo was 16.8 vs. 18.4%, respectively.

In another double-blind, parallel group, placebo-controlled study 70 adult patients with acute diarrhea of presumed infectious origin were included and randomized to receive 100 mg racecadotril or placebo three times daily until recovery for a maximum of 6 days (Hamza et al., 1999). The primary outcome parameter was mean stool weight, which was significantly smaller in the racecadotril as compared to the placebo group (355 ± 35 vs. 499 ± 46 g, respectively). Secondary outcome parameters for which racecadotril was significantly superior to placebo included number of diarrhoic stools after 1 day of treatment 4.3 ± 0.4 vs. 5.4 ± 0.4, respectively) and percentage of patients passing at least one formed stool on the second day of treatment (15.6 vs. 5.3%, respectively). The incidence of reported adverse events was 3.1% with racecadotril vs. 5.3% with placebo; abdominal distension, not classified as an adverse event in this study, was 5.6% with racecadotril vs. 18.2% with placebo.

Following registration in 1992, one additional double-blind, randomized, placebo-controlled trial was performed in 110 men with cholera, in which 100 mg racecadotril was administered every

Table 1 | Efficacy of racecadotril in the treatment of acute diarrhea in adults.

Outcome parameter	Number of patients	Racecadotril	Comparator	Reference
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA				
Time to recovery, h	54–55 per group vs. 49[§]	65.0–69.9	72.0	data on file
% Probability for recovery on day 4	95 vs. 98	75*	37	Baumer et al. (1992)
Stool weight, g	32 vs. 38	355 ± 35*	499 ± 46	Hamza et al. (1999)
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN ADULTS WITH CHOLERA				
Total stool output, g	54 vs. 56	315 ± 31	280 ± 21	Alam et al. (2003)
STUDIES IN ADULTS WITH ACUTE DIARRHEA ASSOCIATED WITH CANCER CHEMOTHERAPY (5-FLUORO-URACIL)				
Number of stools per day	15 (sequential racecadotril vs. no treatment)	4.9*	6.3	Dorval et al. (1995)
STUDIES IN ADULTS WITH DELAYED DIARRHEA DUE CANCER CHEMOTHERAPY (IRINOTECAN)				
Treatment responder	11	36%	–	Saliba et al. (1998)
Prophylaxis of diarrhea	68 vs. 68 no treatment	55%	59%	Ychou et al. (2000)
OCTREOTIDE-CONTROLLED STUDIES IN ADULTS WITH TREATMENT-RESISTANT DIARRHEA IN AIDS PATIENTS				
Stools/day	13 (cross-over)	−2.4*	−1.4	Beaugerie et al. (1996)
DOUBLE-BLIND, LOPERAMIDE-CONTROLLED STUDIES IN ADULTS WITH ACUTE DIARRHEA				
Time to diarrhea resolution, days	37 vs. 32	2.2 ± 0.2	2.3 ± 0.2	Roge et al. (1993)
Number of stools	82 vs. 75	3.5 ± 0.5	2.9 ± 0.4	Vetel et al. (1999)
Duration of diarrhea, h	473 vs. 472	55.0	55.0	Prado (2002)
Duration of diarrhea, h	31 vs. 31	19.5	13.0	Wang et al. (2005)
Time recovery, h	30 vs. 31	36 ± 4*	63 ± 6	Gallelli et al. (2010)

$*p < 0.05$ vs. comparator; [§] dose-ranging study using 30, 100, and 300 mg racecadotril thrice daily. For details on individual studies see main text Section "Studies in the Gastro-Intestinal Tract."

4 h until recovery for a maximum of 72 h as an adjunct to standard treatment (Alam et al., 2003). Both treatments did not differ significantly with regard to total stool output, duration of diarrhea or patients with resolution of diarrhea within 72 h. Adverse events noted as per-protocol such as vomiting, reappearance of dehydration, abdominal pain, headache, or anorexia were not different between the treatment groups. Taken together these studies consistently demonstrate efficacy of racecadotril as compared to placebo with similar adverse event incidences with both treatments. However, it should be noted that treatment regimens and outcome parameter varied considerably between studies.

STUDIES IN OTHER FORMS OF ADULT DIARRHEA

Some studies have been performed with racecadotril in the context of diarrhea associated with cancer chemotherapy. In a pilot study in 15 cancer patients treated with 5-fluoro-uracil were given a daily dose of 300 mg/d racecadotril for 7 days for the treatment of acute diarrhea (Dorval et al., 1995). As compared to the control period, i.e., earlier cycle of chemotherapy, the number of stools per day was reduced in each patient with a statistically significant reduction of mean number from 6.3 to 4.9 and the number of days with liquid stools significantly dropped from 4.7 to 2.4.

Delayed onset diarrhea is a dose-limiting side-effect of the second-line anti-cancer drug irinotecan, which is often used in the treatment of colon cancer. Among patients exhibiting delayed diarrhea upon irinotecan treatment, 4 out of 11 patients responded to 100 mg racecadotril thrice daily in one cohort, whereas 9 of 10 patients responded to a combination of racecadotril and loperamide ($p < 0.02$ vs. racecadotril alone) (Saliba et al., 1998). In a randomized open-label study 136 patients receiving a total of 714 irinotecan chemotherapy cycles received 300 mg/d racecadotril for 15 days as a prophylactic treatment or no prophylactic treatment but the two groups did not differ significantly in the incidence or severity of delayed diarrhea (Ychou et al., 2000).

In an open, randomized cross-over study the efficacy of 100–300 mg racecadotril thrice daily and 50–150 μg octreotide thrice daily was compared in 13 treatment-resistant diarrhea in AIDS patients (Beaugerie et al., 1996). From a baseline of 7.0 ± 1.2 stools/day racecadotril caused a significant reduction to 4.6 ± 1.1 stools/day, whereas octreotide caused a non-significant reduction to only 5.6 ± 1.2 stools/day. Daily lipid output was increased non-significantly by racecadotril, but was nearly doubled with octreotide.

RACECADOTRIL CLINICAL STUDIES VS. PLACEBO AND OPEN STUDIES IN CHILDREN

Based upon the results of racecadotril in the treatment of acute diarrhea in adults, several studies have explored its use in the treatment of acute diarrhea in children. The data of all controlled studies with racecadotril in the treatment of acute diarrhea in children are summarized for efficacy in **Table 2** and for adverse events in **Figure 3**. In the first of such studies 135 boys aged 3–35 months including 73 with a verified rotavirus infection with 1.5 mg/kg racecadotril every 8 h or placebo in a randomized, double-blind study with both treatments being administered on top of oral rehydration solution (Salazar-Lindo et al., 2000). Total stool output in the first 48 h of treatment, the primary study endpoint, was 157 ± 27 g/kg with racecadotril as compared to 331 ± 39 g/kg with placebo ($p < 0.001$). The duration of diarrhea was significantly shorter with racecadotril (28 h regardless of rotavirus status) than with placebo treatment (72 and 52 h in rotavirus-positive and –negative boys, respectively). Moreover, the amount of required oral

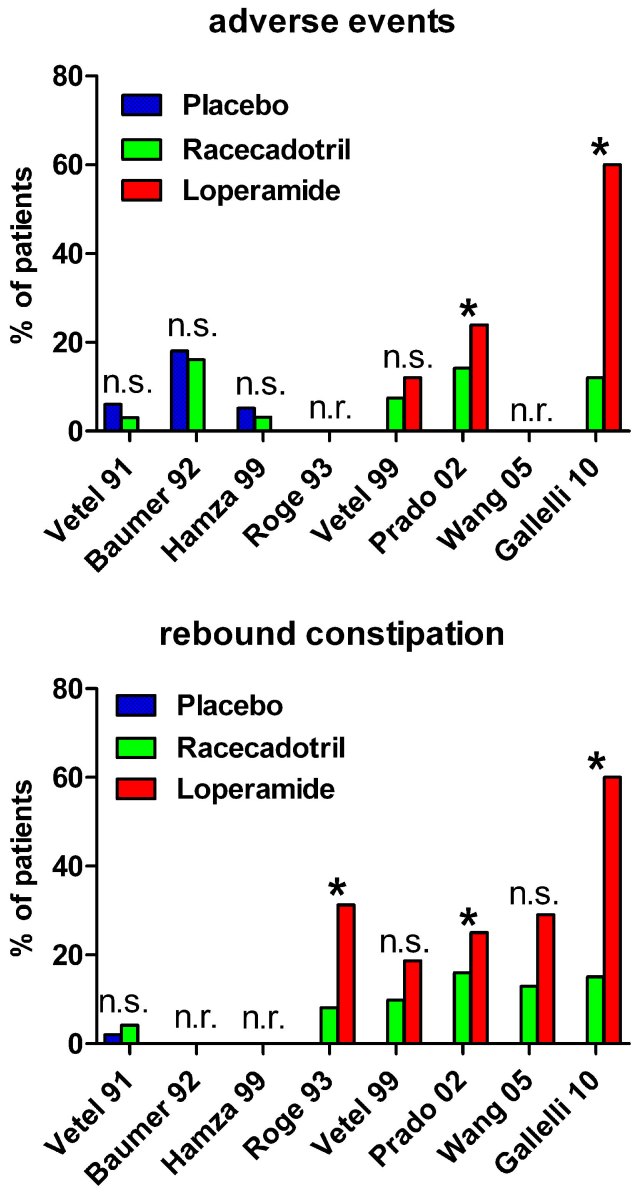

FIGURE 2 | Incidence of adverse events (upper panel) and rebound constipation (lower panel) in controlled studies with racecadotril in the treatment of acute diarrhea in adults. *$p < 0.05$ vs. comparator; n.s., not significant; n.r., not reported. For study details see main text, for corresponding efficacy results see **Table 1**.

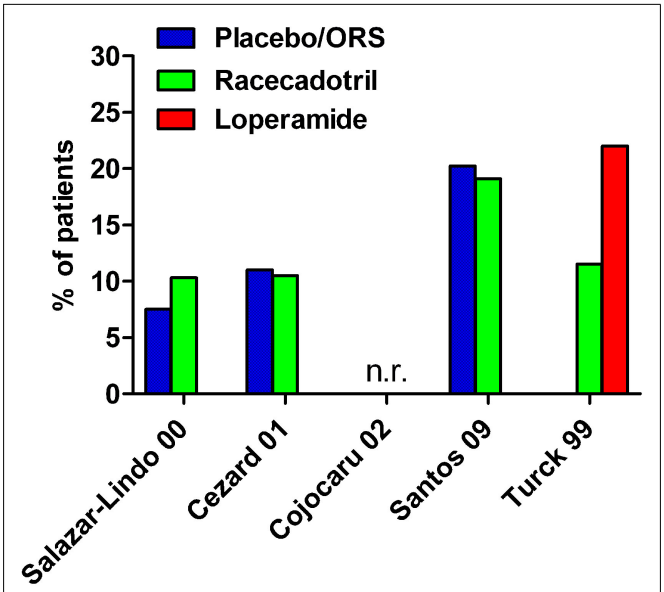

FIGURE 3 | Incidence of adverse events in controlled studies with racecadotril in the treatment of acute diarrhea in children. n.r., not reported; ORS, oral rehydration solution. Note that group differences did not reach statistical significance with the given number of patients. For study details see main text, for corresponding efficacy results see **Table 2**.

rehydration solution was also significantly less with racecadotril treatment. Adverse events were reported in 7 of 68 and 5 of 67 children receiving racecadotril and placebo, respectively; 51 and 52% reported vomiting at at least some point during treatment, and study withdrawal occurred in 9 and 14 patients, respectively.

A second double-blind, placebo-controlled randomized study of very similar design with racecadotril on top of oral rehydration solution was performed in 173 infants aged 3 months to 4 years, except that this study included children of both genders (Cezard et al., 2001). Total stool output was significantly lower by 60% (95% confidence interval 43–88%) with racecadotril as compared to placebo, and this was again independent of rotavirus status. Stool output in the first 24 h of treatment, a secondary endpoint, was also significantly less with racecadotril. The time to recovery was also significantly shorter with racecadotril in a Kaplan–Meier analysis. Moreover, the need for oral rehydration solution on the second day of treatment was also significantly lower with racecadotril treatment. Adverse events were reported by nine patients of each group, but abdominal distension was not noted in either treatment group.

In a third study the effect of racecadotril given as adjunct to oral rehydration solution was compared to rehydration alone in 166 children aged 3–34 months in a randomized open-label study (Cojocaru et al., 2002). The primary endpoint was the number of medical exams within a week after start of treatment which was significantly lower with racecadotril than without (14 vs. 27). Secondary endpoints the number of stools within the first 48 h (6.8 ± 3.8 vs. 9.5 ± 4.5) and the duration of diarrhea (97.2 ± 35.6 vs. 137.7 ± 42.4 h) were also significantly less in the racecadotril group.

In an open-label parallel group study 189 children aged 3–36 months were treated with oral rehydration solution or racecadotril (10 or 30 mg thrice daily for children with 9–13 or >13 kg body weight, respectively) on top of such solution until two normal stools were observed or no bowel movement occurred within 12 h for up to 7 days (Santos et al., 2009). The primary endpoint, number of bowel movements in the first 48 h after initiation of treatment, did not differ significantly between the two treatments (4.1 ± 2.7 vs. 3.8 ± 2.4 for control and racecadotril, respectively), and the duration of gastroenteritis, a secondary

Table 2 | Efficacy of racecadotril in the treatment of acute diarrhea in children.

Outcome parameter	Number of patients	Racecadotril	Comparator	Reference
DOUBLE-BLIND, PLACEBO-CONTROLLED STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Stool output, g/kg	68 vs. 65	157 ± 27*	331 ± 39	Salazar-Lindo et al. (2000)
Stool output, g/h	84 vs. 82	9*	15	Cezard et al. (2001)
OPEN-LABEL CONTROLLED STUDIES (VS. REHYDRATION ALONE) IN CHILDREN WITH ACUTE DIARRHEA				
Medical exams within 1 week of treatment	81 vs. 83	14*	27	Cojocaru et al. (2002)
Number of stools in first 48 h	88 vs. 91	3.8 ± 2.4	4.1 ± 2.7	Santos et al. (2009)
OPEN-LABEL OBERVATIONAL STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Time to relief, h	3873	18.5 ± 12.5	n.a.	Chacon (2010)
DOUBLE-BLIND, LOPERAMIDE-CONTROLLED STUDIES IN CHILDREN WITH ACUTE DIARRHEA				
Number of diarrhoic stools until recovery	52 vs. 50	2.7 ± 0.4	2.1 ± 0.4	Turck et al. (1999)

*n.a., Not applicable; *$p < 0.05$ vs. comparator.*

endpoint, also differed numerically but not significantly (4.7 ± 2.2 vs. 4.0 ± 2.1 days, respectively). The incidence of adverse events was similar in both treatment groups (20.2 vs. 19.1%, respectively).

In an open-label study 3873 children aged 3 months to 12 years were treated with 1.5 mg/kg thrice daily (Chacon, 2010). The primary endpoint of that study was time to relief, time from start of treatment to last watery bowel movement, which was reached after 18.5 ± 12.5 h (95% confidence interval 17.9–19.0 h). Using the very large number of children in this study, the authors have performed a multiple regression analysis to explore factors affecting drug performance. Among a range of potential explanatory variables diarrhea severity was the only with a significant and independent weight on racecadotril effectiveness, explaining 23% of time to relief variance, but even in severe cases mean time to relief was less than 24 h. Specific adverse event incidence was not reported, but overall tolerability was rated as excellent or good in 95.9% of cases.

While some of the above mentioned randomized pediatric studies have been systematically reviewed in the past (Szajewska et al., 2007; Tormo et al., 2008), those analyses covered only part of the existing trial databases. More importantly, an individual patient meta-analysis of nine pediatric studies with raw data available for analysis from 1384 children has been reported more recently which also included some studies that had not been reported before as full papers (Lehert et al., 2011). The proportion of children with recovery was higher with racecadotril treatment with a hazard ratio of 2.04 (95% confidence interval 1.85–2.32). For in-patient studies, the ratio of mean stool output racecadotril/placebo was 0.59 (0.51–0.74, $p < 0.001$), for out-patient studies, the ratio of the mean number of diarrhoic stools racecadotril/placebo was 0.63 (0.51–0.74, $p < 0.001$).

Accordingly, a survey of treatment patterns among all office-based pediatricians in France found racecadotril to be prescribed by 62% of pediatricians as compared to only 28% prescribing loperamide (Uhlen et al., 2004). In line with the above studies, as an addition to oral rehydration treatment, racecadotril is being recommended for the treatment of acute diarrhea in children by recent guidelines, e.g., from the World Gastroenterology Organisation (World Gastroenterology Association, 2008), the European Society of Pediatric Gastroenterology, Hepatology and Nutrition/European Society for Pediatric Infectious Diseases (Guarino et al., 2008), a guideline panel from Spain and Latin America (Gutierrez Castrelion et al., 2010), and the German Society for Pediatric Gastroenterology and Nutrition (Koletzko and Lentze, 2008). Similarly, a very recent international panel of experts from France, Ireland, Italy, Malaysia, Peru, Spain, USA, and Vietnam emphasized that oral rehydration solution is the basis of the treatment of acute diarrhea in children; the use of loperamide was discouraged, whereas racecadotril was recognized as an option for additional active treatment on top of rehydration solution (Guarino et al., 2012).

RACECADOTRIL CLINICAL STUDIES VS. LOPERAMIDE (ADULTS AND CHILDREN)

As loperamide has been the primary medical treatment of acute diarrhea for a long time, particularly in adults, six studies have reported direct comparisons of the efficacy and tolerability of racecadotril and loperamide including one study in children.

Roge et al. (1993) reported a double-blind controlled study in which 100 mg racecadotril was compared to 1.33 mg loperamide (two doses at start of treatment, followed by one dose every 8 h) in 37 vs. 32 patients, respectively. The study did not report a primary endpoint but rather several outcome parameters in parallel including physician evaluation of efficacy, time to diarrhea resolution, abdominal pain for more than 1 day, abdominal distension for more than 1 day, duration of abdominal distension and secondary constipation. Racecadotril was numerically superior to loperamide for all of these endpoints, and the difference reached statistical significance for the latter three. The authors did not specifically report incidence of adverse events but secondary constipation can be considered as such and was found significantly less frequent in racecadotril as compared to loperamide-treated patients (8.1 vs. 31.3%).

A second randomized, double-blind, double-placebo-controlled study compared the efficacy and safety of 100 mg racecadotril thrice daily with that of 2 mg loperamide after each diarrhoic stool in 82 and 75 patients, respectively, administered until recovery for a maximum of 7 days (Vetel et al., 1999). Both groups passed a similar number of stools (3.5 ± 0.5 vs. 2.9 ± 0.4) and had a similar duration of diarrhea (14.9 ± 2.0 vs. 13.7 ± 2.2 h). Adverse events

were reported in 7.4% of racecadotril and 12% of loperamide patients, and rebound constipation was experienced by 9.8% of racecadotril vs. 18.7% of loperamide patients.

The third and largest direct head-to-head study compared 473 patients receiving 100 mg racecadotril thrice daily with 472 patient receiving 2 mg loperamide thrice daily in a single-blind design (Prado, 2002). The primary efficacy criterion was duration of diarrhea, defined as time between start of treatment and appearance of first formed stool; this was 55.0 h in both groups (95% confidence interval 50.0–65.0 and 48.0–66.0 h in the racecadotril and loperamide group, respectively). The median duration of abdominal pain was similar in both groups, but difference in pain intensity between start and end of study was significantly in favor of racecadotril; moreover, a significantly greater percentage of patients reported residual pain at study end with loperamide as compared to racecadotril treatment (7 vs. 3%). Rebound constipation (objectively defined as 36 h without passing stool) was significantly more frequent with loperamide than with racecadotril treatment (25 vs. 16%). The incidence of reported adverse events was also significantly greater with loperamide than with racecadotril (23.9 vs. 14.2%).

A fourth randomized study compared 31 patients receiving 100 mg racecadotril thrice daily to 31 patients receiving 2 mg loperamide twice daily in a single-blind manner (Wang et al., 2005). The primary endpoint was duration of diarrhea, which did not differ significantly between treatments (median 19.5 vs. 13.0 h for racecadotril and loperamide, respectively). Duration of abdominal pain, abdominal distension, anal burning, and nausea also did not differ significantly between treatments. Adverse events were reported in 25.0% of racecadotril and 22.0% of loperamide patients. Among these rebound constipation was reported in four and nine racecadotril and loperamide patients, respectively, whereas itching was found in two racecadotril but no loperamide patients.

A fifth double-blind, randomized study compared 100 mg racecadotril thrice daily to loperamide (4 mg starting dose, followed by 2 mg after each unformed stool for a maximum of 8 mg/d) in 30 and 31 patients, respectively, being administered until recovery which was defined by two consecutive normal stools or no stools in a 12-h period (Gallelli et al., 2010). In contrast to the other studies, this one recruited only elderly nursing home residents with acute diarrhea (mean age 82 years). The primary endpoint was time to recovery which was met significantly earlier with racecadotril than with loperamide treatment (36 ± 4 vs. 63 ± 6 h). Secondary endpoints included duration of abdominal pain, number of diarrhea episodes, and total stool output in the intention-to-treat and in the per-protocol populations; racecadotril was numerically superior to loperamide all of these, and this reached statistical significance in several cases. In 50% of patients loperamide was ineffective within 4 days; these were switched to racecadotril resulting in rapid normalization of all symptoms. Adverse events were reported in 12% of racecadotril and 60% of loperamide patients; the latter percentage is much higher than in other loperamide studies, probably due to the elderly population being studied. Specifically, nausea and rebound constipation were noted more frequently with loperamide than with racecadotril, whereas abdominal pain, headache, and anorexia were seen similarly with both treatments. Based on genotyping for cytochrome P450 3A4 and 2C8 it was excluded that the group differences were not attributable to the presence of ultra-rapid or poor metabolizers. In a pharmacoeconomic analysis the average cost in the loperamide group was twice as high as in the racecadotril group (€ 91.99 vs. € 44.85).

A sixth randomized study directly comparing racecadotril and loperamide in the treatment of acute diarrhea was performed in a pediatric population with a mean age of 4.7 years (range 2–10 years) in a double-blind, double-placebo design (Turck et al., 1999). Fifty-two children received 1.5 mg/kg racecadotril thrice daily and 50 received 0.03 mg/kg loperamide thrice daily. The primary endpoint was number of passed stools until recovery which did not differ significantly between the two groups (2.7 ± 0.4 with racecadotril and 2.1 ± 0.4 with loperamide). The mean duration of diarrhea also did not differ significantly between groups (10.7 ± 1.7 h with racecadotril and 8.8 ± 2.3 h with loperamide). Adverse events were noted in 11.5% of racecadotril and 22% of loperamide patients. There were only statistically significant differences between the two treatments: rebound constipation (36.5% of racecadotril and 58% of loperamide patients) and need for concomitant medication (anti-emetics 5 vs. 8, analgesics 0 vs. 3 patients, oral rehydration 0 vs. 2, and laxatives 0 vs. 1 patient on racecadotril and loperamide, respectively.

Taken together these six studies demonstrate that the efficacy of racecadotril and loperamide in the treatment of acute diarrhea did not differ significantly for some endpoints in some studies, but that racecadotril was significantly more effective for at least some endpoints in some studies. The incidence of adverse events also was similar in some studies but significantly less frequent with racecadotril in some other studies. A much lower frequency of rebound constipation was seen with racecadotril in almost all studies. Thus, in comparison to loperamide racecadotril appeared to be superior in efficacy and tolerability when all studies are taken into consideration.

While the mechanisms underlying differential effects of racecadotril and loperamide in acute diarrhea have not been fully established, two candidates have emerged: Firstly, loperamide preferentially acts on μ-opioid receptors (Dehaven-Hudkins et al., 1999), whereas endogenous enkephalines active both μ- and δ-receptors (Huighebaert et al., 2003). Secondly, NEP inhibition will not only increase exposure to endogenous enkephalines but also to endogenous neuropeptide Y and, possibly, peptide YY, both of which have strong anti-secretory effects in the gut (Playford and Cox, 1996).

SAFETY AND TOLERABILITY

As summarized in **Figure 2** for adult and **Figure 3** for pediatric patients with acute diarrhea, the incidence of adverse events reported during treatment with racecadotril was consistently similar to that with placebo and similar to or significantly less frequent than that with loperamide. Of note secondary constipation was consistently less frequent with racecadotril than with loperamide; for details see specific study descriptions in section 6. Therefore, the following will summarize published findings on non-clinical toxicity studies and will highlight clinical findings with potential relevance for safety and tolerability.

With regard to general toxicity, no relevant findings were reported for single doses up to 2000 mg/kg and for chronic doses up to 100 mg/kg in dogs (Maertins et al., 2000). In mice no overt toxicity was observed upon i.p. treatment with 50 mg/kg racecadotril for 10 days (Lecomte et al., 1986). In a study with newborn gnotobiotic piglets an oral dose of 130 mg/kg racecadotril produced no signs of neurotoxicity and no deaths, whereas an equivalent high dose of 5 mg/kg loperamide resulted in death in three out of four piglets (Duval-Ilfah et al., 1999). In monkeys 12 months of treatment with up to 100 times the therapeutic human dose did not produce any toxic effects (Lecomte, 2000). Single doses of up to 2000 mg have been administered in healthy volunteers without ill effects (Lecomte, 2000).

Several studies have specifically explored potential adverse events related to airway function, breathing and allergy. As substance P also is a NEP substrate, the effect of racecadotril on responses to exogenous substance P has been explored. In guinea pigs racecadotril enhanced pulmonary substance P response (Lötvall et al., 1990). In humans 200 mg racecadotril enhanced the flare response to substance P in asthmatic subjects (Nichol et al., 1992), and a dose of 300 mg racecadotril enhanced the substance P-induced decrease in nasal conductance in healthy subjects and those with allergic rhinitis (Lurie et al., 1994). There is one case report of a 3-year-old boy weighing 20 kg and reporting generalized edema with itching and aphonia after 2 days of treatment with 30 mg racecadotril; upon additional testing this was classified as a non-allergic hypersensitivity response (Nucera et al., 2006). While respiratory depression is a typical effects of direct opioid receptor agonists with penetration to the central nervous system such as morphine, no respiratory depression was noted with acute i.v. or i.p. racecadotril doses of up to 100 mg/kg in mice (Lecomte et al., 1986).

Finally, there were a few isolated findings from animal studies. In rats racecadotril and thiorphan were reported to enhance the duration but not the frequency of uterine contractions in periparturient animals (Adjroud, 1995). Also in rats the angiotensin converting enzyme inhibitor captopril produced plasma extravasation; while racecadotril alone did not mimick this, it enhanced the captopril response (Sulpizio et al., 2004). However, both of these studies are difficult to place into context as no corresponding adverse events have been reported in patients. Thus, the overall non-clinical and clinical studies demonstrate that racecadotril is a safe drug with an overall tolerability profile similar to placebo.

CONCLUSION

Racecadotril is a low potency inhibitor of NEP, but upon oral administration it is rapidly and effectively metabolized to the potent NEP inhibitor thiorphan, with the latter not exhibiting penetration into the central nervous system. NEP inhibition affects the abundance of several endogenous peptides with enkephalins and ANP apparently being most important. Elevated exposure to ANP appears to underly most cardiovascular effects of racecadotril; while these tend to be beneficial they appear quantitatively insufficient to warrant therapeutic use in comparison to other available drug classes. Elevation of enkephalin exposure appears to underly most central nervous effects, most notably analgesia, but the pain relieving effects are inconsistent across animal models. Increased exposure to peripheral endogenous enkephalins appears to underly the gastro-intestinal racecadotril effects. Most prominent among them is an antisecretory effect in the gut which, in contrast to direct μ-opioid receptor agonists, occurs in the absence of effects on gastro-intestinal transit time. The clinical correlate of these findings is therapeutic efficacy against acute diarrhea in adults and children with a tolerability profile similar to that of placebo. In multiple direct comparative studies in different patients populations (children, adults, elderly), countries (Western Europea, Latin Amercia, Asia), and settings (out-patients, in-patients, nursing home residents) racecadotril was at least as effective as loperamide, and in several of those studies exhibited significantly better tolerability than loperamide. Most notably, rebound constipation was consistently less frequent with racecadotril than with loperamide; while this is primarily a tolerability benefit, it may also be relevant with regard to the efficacy of clearance of infectious organisms as demonstrated in one study. Of note, study designs and particularly treatment endpoints differed considerably between studies. This can be seen as a weakness because it makes inter-study comparisons more difficult; however, it can also be seen as a benefit because consistent therapeutic effects across so many different settings witness rather robust efficacy and tolerability. While additional studies appear warranted several guidelines, specifically in pediatric indications, now recommend including racecadotril in the management of acute diarrhea. Whether other forms of diarrhea, e.g., in the context of cancer chemotherapy, also benefit from racecadotril treatment is not fully clear.

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A distinct profile of tryptophan metabolism along the kynurenine pathway downstream of toll-like receptor activation in irritable bowel syndrome

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Irritable bowel syndrome (IBS), a disorder of the brain-gut axis, is characterised by the absence of reliable biological markers. Tryptophan is an essential amino acid that serves as a precursor to serotonin but which can alternatively be metabolised along the kynurenine pathway leading to the production of other neuroactive agents. We previously reported an increased degradation of tryptophan along this immunoresponsive pathway in IBS. Recently, altered cytokine production following activation of specific members of the toll-like receptor (TLR) family (TLR1-9) has also been demonstrated in IBS. However, the relationship between TLR activation and kynurenine pathway activity in IBS is unknown. In this study, we investigated whether activation of specific TLRs elicits exaggerated kynurenine production in IBS patients compared to controls. Whole blood from IBS patients and healthy controls was cultured with a panel of nine different TLR agonists for 24 h. Cell culture supernatants were then analyzed for both tryptophan and kynurenine concentrations, as were plasma samples from both cohorts. IBS subjects had an elevated plasma kynurenine:tryptophan ratio compared to healthy controls. Furthermore, we demonstrated a differential downstream profile of kynurenine production subsequent to TLR activation in IBS patients compared to healthy controls. This profile included alterations at TLR1/2, TLR2, TLR3, TLR5, TLR7, and TLR8. Our data expands on our previous understanding of altered tryptophan metabolism in IBS and suggests that measurement of tryptophan metabolites downstream of TLR activation may ultimately find utility as components of a biomarker panel to aid gastroenterologists in the diagnosis of IBS. Furthermore, these studies implicate the modulation of TLRs as means through which aberrant tryptophan metabolism along the kynurenine pathway can be controlled, a novel potential therapeutic strategy in this and other disorders.

Keywords: irritable bowel syndrome, kynurenine pathway, toll-like receptors, tryptophan, IDO, cytokine

INTRODUCTION

The diagnosis of irritable bowel syndrome (IBS), a highly prevalent functional gastrointestinal disorder (FGID), is currently made based on the presence of a characteristic symptom profile (abdominal pain/discomfort, bloating/distension, alterations in defecatory function) in the absence of a demonstrable organic disease of the gastrointestinal tract (GIT; Drossman and Dumitrascu, 2006). This diagnostic scheme reflects the lack of reproducible biological markers of this heterogeneous disorder, a serious impediment to advancing our understanding of its pathophysiology (Clarke et al., 2009b). The concept of IBS as a disorder of the brain-gut axis is now generally accepted and this has facilitated some progress in the area (Ohman and Simren, 2007, 2010). Recently, indices of a low grade immune activation have been reported in IBS, including elevations in circulating cytokines (Dinan et al., 2006; Liebregts et al., 2007; Dinan et al., 2008; Scully et al., 2010) and pro-inflammatory polyunsaturated fatty acids (Clarke et al., 2010) as well as evidence of enhanced immune cell activation, both systemically (Ohman et al., 2009a,b) and locally within the GIT (Cremon et al., 2009).

A growing appreciation of the potential impact of this increased inflammatory state on the brain-gut axis (Quigley, 2006; Dantzer et al., 2008; O'Malley et al., 2011) as well as an improved understanding of the influence of the GIT on mood and cognition (Forsythe et al., 2010; Grenham et al., 2011; Mayer, 2011; Bercik et al., 2012; Kennedy et al., 2012), has led to an exploration of a potential role for tryptophan and its associated metabolic

pathways in IBS. Tryptophan is an essential amino acid that serves as a precursor to serotonin (5-HT), a key neurotransmitter within both the enteric nervous system (ENS) and central nervous system (CNS; Ruddick et al., 2006; Forsythe et al., 2010). An alternative and physiologically dominant fate for tryptophan is degradation along the kynurenine pathway leading to the production of neuroprotective compounds like kynurenic acid and neurotoxic compounds like quinolinic acid (Schwarcz and Pellicciari, 2002). Dysregulation of tryptophan metabolism is thus poised to impact on mood and cognition within the CNS as well as secretion, motility, and perception in the ENS (Crowell, 2004; Forsythe et al., 2010). Crucially, indoleamine-2.3-dioxygenase (IDO), one of two key primary kynurenine pathway enzymes, is immunoresponsive and studies, to date, have indicated an increase in IDO activity in both male and female subjects with IBS (Fitzgerald et al., 2008; Clarke et al., 2009a).

Mechanistic insights into the inflammation observed in IBS and the associated downstream consequences are currently lacking. Toll-like receptors (TLRs) are pattern recognition receptors integral to the functioning of the innate immune system and respond to a variety of bacterial and viral cell components, resulting in increased production of inflammatory cytokines (Takeuchi and Akira, 2010). Recent evidence from both relevant animal models and biopsies from IBS sufferers have demonstrated altered expression of certain TLRs in the colonic mucosa (McKernan et al., 2009; Brint et al., 2011). Increased TLR2 expression on blood monocytes in IBS patients has also been reported (Ohman et al., 2012). Moreover it has recently been demonstrated that IBS patients have a distinct pattern of peripheral TLR activity as indicated by measurements of cytokine production following whole blood stimulation (McKernan et al., 2011). Although it is known that TLR activation can lead to alterations in IDO expression (Mahanonda et al., 2007), the potential consequences for tryptophan metabolism in IBS remain unknown.

In this study we investigated the potential functional consequences of TLR activation on brain-gut axis signaling in terms of perturbations in tryptophan metabolism. This was based on the hypothesis that IBS patients would exhibit a downstream pattern of tryptophan degradation along the kynurenine pathway subsequent to activation of TLRs with their specific ligands that was distinct from healthy control subjects.

MATERIALS AND METHODS

STUDY POPULATION

The study protocol was approved by the University College Cork (UCC) Clinical Research Ethics Committee. IBS patients were recruited from a university IBS database comprised of individuals who had either attended gastroenterology clinics at Cork University Hospital (CUH) or had responded to direct advertisement on the university campus or a local newspaper regarding participation in IBS research. Thirty seven healthy controls were recruited from staff at both UCC and CUH. Twenty five individuals aged between 18 and 65 years who satisfied Rome II criteria for IBS and in whom organic gastrointestinal diseases and clinically significant systemic diseases had been excluded, were considered for inclusion in the study. Subjects who had undergone any abdominal surgery, with the exception of hernia repair and appendectomy, were also excluded. No postinfectious IBS (PI-IBS) subjects were included in the recruitment and IBS patients were not selected on the basis of predominant bowel habit, although this was recorded according to the Rome II classifications. All subjects completed a questionnaire to assess both IBS severity (Francis et al., 1997) and current mood (Spitzer et al., 1999).

BIOLOGICAL ASSAYS

Fifteen milliliters of whole blood was collected between 11:00 and 13:00 hours from each healthy control and IBS patient. Collected whole blood (15 mL) was added to an equal volume of Histopaque 1077 (Sigma, St Louis, MO, USA) in a sterile 50 mL tube and centrifuged at 400 × *g* for 30 min at room temperature. Plasma on the upper layer was transferred to a separate tube and stored at −80°C for future analysis. Collected whole blood (2 mL) was diluted 1:10 in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Dublin, Ireland). Blood was aliquoted into 24 well plates and cultured in a 37°C incubator with 5% CO_2. Each blood sample was cultured in duplicate in DMEM cell culture medium supplemented with 10% Fetal Calf Serum (Sigma, Dublin, Ireland) with or without the following TLR ligands from a Human TLR agonist kit (Invivogen, San Diego, CA, USA) for 24 h:

TLR1/2-Palmitoyl-3-cysteine-serine-lysine 4 (Pam3Cys); TLR2-heat-killed *Listeria monocytogenes* (HKLM); TLR3-Polyriboinosinic polyribocytidylic acid (Poly I:C); TLR4-Lipopolysaccharide (LPS); TLR5-*Salmonella typhimurium* Flagellin; TLR6/2-FSL-1; TLR7-Imiquimod; TLR8-ssRNA40; TLR9-ODN2006. Agonists were reconstituted in endotoxin free water (supplied in kit) to a final concentration of 1 $\mu g\,mL^{-1}$ except for HKLM (10^8 cells) and Poly I:C (10 $\mu g\,mL^{-1}$). Subsequently, supernatants from both untreated and stimulated cells were aspirated and stored at −80°C for future analysis.

HPLC ASSAY FOR TRYPTOPHAN AND KYNURENINE

Tryptophan and kynurenine were determined by high performance liquid chromatography (HPLC): this involved using a system comprising a Waters 510 pump (Waters Ireland, Dublin, Ireland), 717plus cooled autosampler, a 996 PDA detector, a Hewlett Packard 1046A Fluorescent Detector (Waters Ireland, Dublin, Ireland), a waters bus SAT/IN module and a croco-cil column oven. System components were used in conjunction with Waters Empower software (Waters Ireland, Dublin, Ireland). All samples were injected onto a reversed phase Luna 3μ C18(2) 150 × 2 mm column (Phenomenex, Macclesfield, UK), which was protected by Krudkatcher disposable precolumn filters and security guard cartridges (Phenomenex). HPLC grade acetonitrile, acetic acid, and perchloric acid were obtained from Fisher Scientific Ireland (Dublin, Ireland). The analysis method was based on that by Herve et al. (1996). The mobile phase consisted of 50 $mmol\,L^{-1}$ acetic acid, 100 $mmol\,L^{-1}$ Zinc Acetate with 3% (v/v) acetonitrile and was filtered through a 0.45 μm Millipore filter (AGB, Dublin, Ireland) and vacuum degassed prior to use. Separations were achieved by isocratic elution at 0.3 $mL\,min^{-1}$. The fluorescent detector was set to an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The PDA detector start wavelength was 210 nm and the end wavelength was 400 nm with chromatogram extraction at 330 nm. Working standard dilutions

were prepared from millimolar stock solutions of each standard and stored at −80°C until required for analysis. Samples were deproteinized by the addition of 20 μL of 4 mol L^{-1} perchloric acid to 200 μL of plasma spiked with 3-nitro-L-tyrosine as internal standard. Twenty microliters of either sample or standard was injected onto the HPLC system and chromatograms generated were processed using Waters Empower software. Analytes were identified based on their characteristic retention time and their concentrations determined using Analyte:Internal standard peak height ratios; these were measured and compared with standard injections which were run at regular intervals during the sample analysis. Results were expressed at ng analyte per mL of supernatant/plasma.

STATISTICS

The sample size was determined by a power calculation based on our previous data and aimed at detecting differences between IBS patients and controls at the 0.05 level. Data was expressed as mean ± SEM. Statistical analysis was carried out using SPSS 18 for Windows (SPSS, Inc., Chicago, IL, US). Plasma tryptophan, kynurenine, and the kynurenine:tryptophan ratio were compared using an unpaired two-tailed Student's *t*-test and differences considered significant at the $p < 0.05$ level. Bonferroni corrections for multiple comparisons were applied as required. TLR agonist induced alterations in, kynurenine and the kynurenine:tryptophan ratio were determined using a two-way ANOVA and by Bonferroni *post hoc* tests.

RESULTS

BASELINE CHARACTERISTICS

There were no significant differences between IBS patients (5M, 20F) and controls (13M, 25F) in terms of age (41.32 ± 2.234 vs. 36.63 ± 1.821, $p = 0.11$) or body mass index (BMI; 24.88 ± 0.7089 vs. 23.96 ± 0.8668, $p = 0.45$). According to Rome II sub classification of predominant bowel habit, nine had constipation-predominant IBS (IBS-C), eight had diarrhea-predominant IBS (IBS-D), and eight had alternating IBS (IBS-A). There were three current smokers in each group. Eleven IBS subjects (44%) met criteria for a current psychiatric co-morbidity. According to IBS symptom severity scores, 5 patients rated their symptoms as mild, 12 as moderate and 8 as severe.

PLASMA TRYPTOPHAN AND KYNURENINE CONCENTRATIONS

There was no significant difference in plasma tryptophan levels between controls and IBS groups (10.0 ± 0.4 vs. 10.5 ± 0.7 μg mL^{-1}, $p = 0.51$). Plasma kynurenine levels were significantly elevated in comparison to controls levels in IBS patients (0.39 ± 0.02 vs. 0.50 ± 0.05 μg mL^{-1}, $p < 0.05$) as was the kynurenine:tryptophan ratio (0.03991 ± 0.00222 vs. 0.04795 ± 0.00315, $p < 0.05$), an index of IDO activity (**Figures 1A–C**).

TLR AGONIST INDUCED ALTERNATIONS IN TRYPTOPHAN DEGRADATION.

Concentrations of tryptophan and kynurenine following TLR stimulation are given in **Tables 1** and **2** respectively.

Two-way ANOVA analysis revealed a significant interaction between disease state and treatment ($F_{3,126} = 5.867$, $p < 0.05$) for the kynurenine:tryptophan ratio following stimulation of whole blood with the TLR1/2 agonist Pam3Csk. *Post hoc* analysis indicated a significant reduction in this ratio, in the IBS group, in stimulated preparations compared to the unstimulated samples (0.01774 ± 0.00089 vs. 0.01493 ± 0.00098, $p < 0.05$) with no alteration in this ratio in the samples taken from the healthy controls (**Figure 2A**). Two-way ANOVA analysis revealed a significant interaction between disease state and treatment ($F_{3,126} = 8.475$, $p < 0.01$) following stimulation of whole blood with the TLR2 agonist HKLM. *Post hoc* analysis revealed a significant increase in this ratio in the healthy control group in stimulated vs. unstimulated samples (0.01681 ± 0.0006 vs. 0.02159 ± 0.00098, $p < 0.001$) with no alteration in this ratio in the samples taken from IBS patients (**Figure 2B**). There was a trend toward a significant interaction between disease state and treatment ($F_{3,126} = 2.887$, $p = 0.092$) following stimulation with the TLR3 agonist Poly I:C. *Post hoc* analysis indicated a significant increase in the ratio in the healthy control group in stimulated vs. unstimulated samples (0.01681 ± 0.0006 vs. 0.01907 ± 0.00092, $p < 0.05$) with no alteration in the ratio being demonstrated in the samples taken from IBS patients (**Figure 2C**).

There was no significant interaction between disease state and treatment following stimulation with the TLR4 agonist LPS ($F_{3,125} = 0.157$, $p = 0.962$). *Post hoc* analysis indicated a significant increase in the ratio in the stimulated compared to unstimulated samples from both controls (0.01681 ± 0.0006 vs. 0.02354 ± 0.00112, $p < 0.001$) and IBS patients (0.01774 ± 0.00089 vs. 0.02367 ± 0.00137, $p < 0.001$; **Figure 3A**). There was a trend toward a significant interaction between disease state and treatment following stimulation with the TLR5 agonist flagellin ($F_{3,124} = 3.604$, $p = 0.06$). The kynurenine:tryptophan ratio was significantly elevated from unstimulated levels only in the samples taken from IBS patients (0.01774 ± 0.00089 vs. 0.02259 ± 0.0015, $p < 0.01$; **Figure 3B**).

There was no significant interaction between disease state and treatment following stimulation with the TL6/2 agonist FSL1 ($F_{1,124} = 1.026$, $p = 0.313$) not was there any alteration in the kynurenine:tryptophan ratio in either healthy controls or IBS subjects (**Figure 3C**).

There was no significant interaction between disease state and treatment following stimulation with the TLR7 agonist imiquimod ($F_{3,125} = 0.146$, $p = 0.703$) although this agonist did induce an increase in the kynurenine:tryptophan ratio in stimulated samples from control subjects (0.01681 ± 0.0006 vs. 0.01954 ± 0.00087, $p < 0.05$) that was not evident in their IBS counterparts (**Figure 4A**). There was a trend toward a significant interaction between disease state and treatment following stimulation of samples with the TLR8 agonist ssRNA40 ($F_{3,124} = 3.491$, $p = 0.064$). This TLR ligand induced an increase in the kynurenine:tryptophan ratio only in the stimulated samples from IBS patients (0.01774 ± 0.00089 vs. 0.02166 ± 0.00161, $p < 0.05$; **Figure 4B**). There was no significant interaction between disease state and treatment following stimulation with the TLR9 agonist ODN2006 ($F_{3,125} = 2.324$, $p = 0.131$) nor was there any alteration in the kynurenine:tryptophan ratio in the stimulated samples from either healthy control or IBS subjects (**Figure 4C**).

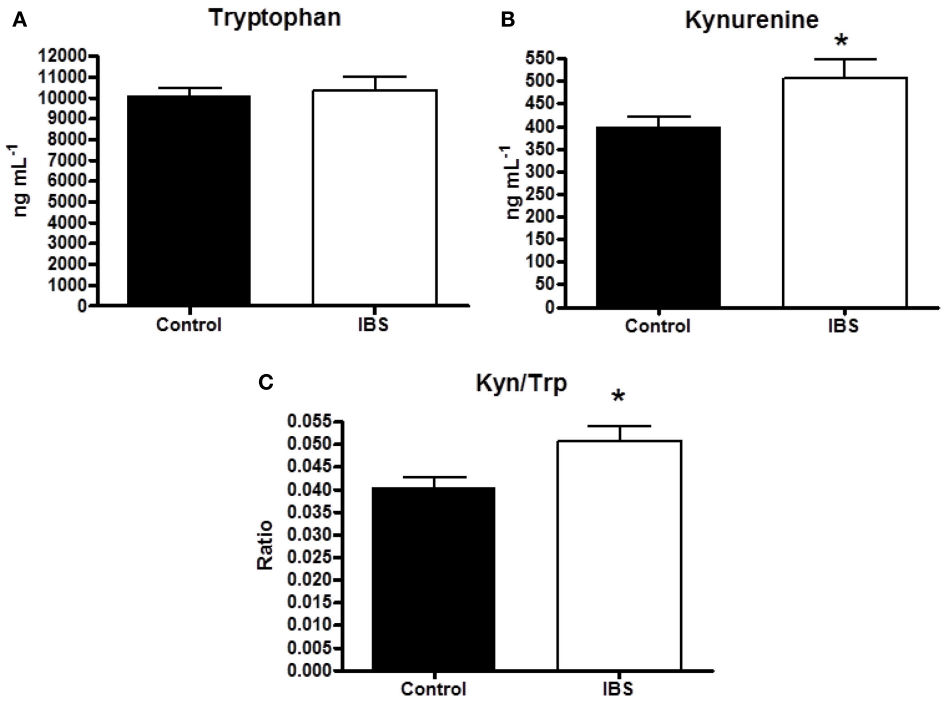

FIGURE 1 | (A) Plasma tryptophan concentrations ($\mu g\,mL^{-1}$) in healthy controls and IBS patients. **(B)** Plasma kynurenine concentrations ($\mu g\,mL^{-1}$) in healthy controls and IBS patients. **(C)** Plasma kynurenine:tryptophan (Kyn:Tryp) ratio in healthy controls and IBS patients. Data are expressed as means ± SEM. Statistical differences between healthy controls and IBS patients were determined using Student's *t*-test. $*p < 0.05$.

Table 1 | Tryptophan concentrations ($\mu g\,mL^{-1}$) in unstimulated and TLR agonist stimulated whole blood supernatants from healthy controls and IBS patients.

	Control		IBS	
TLR stimulation	**Tryptophan**	***p***	**Tryptophan**	***p***
Unstimulated	13.5 ± 0.2	N/A	13.7 ± 0.3	N/A
1/2 (Pam3Cys)	11.7 ± 0.2	***	12.0 ± 0.3	$$
2 (HKLM)	13.1 ± 0.3	—	12.1 ± 0.3	$$
3 (Poly I:C)	11.8 ± 0.3	***	12.3 ± 0.2	$$
4 (LPS)	13.7 ± 0.4	—	13.7 ± 0.4	—
5 (Flagellin)	13.3 ± 0.3	—	13.3 ± 0.3	—
6/2 (FSL1)	12.6 ± 0.2	*	13.2 ± 0.3	—
7 (Imiquimod)	13.0 ± 0.2	—	13.0 ± 0.2	—
8 (ssRNA40)	12.6 ± 0.3	*	12.9 ± 0.2	—
9 (ODN2006)	13.4 ± 0.5	—	13.0 ± 0.5	—

*Data are expressed as means ± SEM. Statistical differences between stimulated and unstimulated groups were determined using Bonferroni post hoc test. *$p < 0.05$, Healthy control unstimulated vs. TLR agonist stimulated; ***$p < 0.001$, healthy control unstimulated vs. TLR agonist Stimulated; $$ $p < 0.01$, IBS unstimulated vs. stimulated; — $p > 0.05$.*

Table 2 | Kynurenine concentrations ($\mu g\,mL^{-1}$) in unstimulated and TLR agonist stimulated whole blood supernatants from healthy controls and IBS patients.

	Control		IBS	
TLR stimulation	**Kynurenine**	***p***	**Kynurenine**	***p***
Unstimulated	0.22 ± 0.01	N/A	0.24 ± 0.01	
1/2 (Pam3Cys)	0.21 ± 0.01	—	0.18 ± 0.01	$$$
2 (HKLM)	0.27 ± 0.01	***	0.20 ± 0.01	$
3 (Poly I:C)	0.22 ± 0.01	—	0.19 ± 0.01	$$
4 (LPS)	0.33 ± 0.01	***	0.32 ± 0.02	$$$
5 (Flagellin)	0.23 ± 0.01	—	0.30 ± 0.02	$$
6/2 (FSL1)	0.21 ± 0.01	—	0.21 ± 0.01	—
7 (Imiquimod)	0.25 ± 0.01	—	0.26 ± 0.02	—
8 (ssRNA40)	0.23 ± 0.01	—	0.28 ± 0.02	—
9 (ODN2006)	0.25 ± 0.01		0.21 ± 0.01	—

*Data are expressed as means ± SEM. Statistical differences between stimulated and unstimulated groups were determined using Bonferroni post hoc test. ***$p < 0.001$, Healthy control unstimulated vs. TLR agonist stimulated; $ $p < 0.05$, IBS unstimulated vs. TLR agonist stimulated; $$ $p < 0.01$, IBS unstimulated vs. stimulated; $$$ $p < 0.001$ IBS unstimulated vs. TLR agonist stimulated; — $p > 0.05$.*

DISCUSSION

Previous reports from our laboratory have indicated increased degradation of tryptophan along the kynurenine pathway and highlighted the potential utility of these indices as biological markers of IBS (Fitzgerald et al., 2008; Clarke et al., 2009a). Here we have confirmed these findings by demonstrating increased plasma kynurenine concentrations as well as an elevation in the

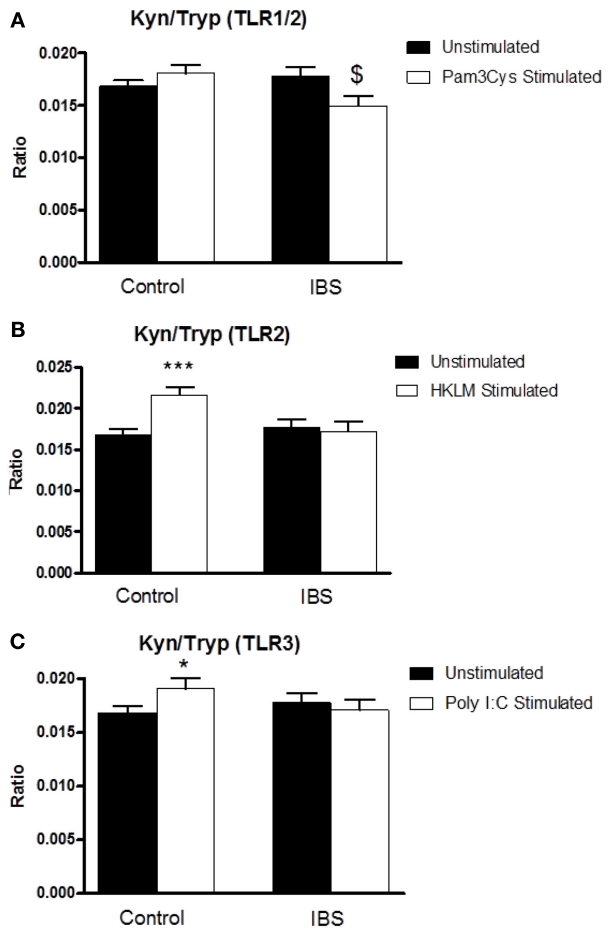

FIGURE 2 | Kynurenine:tryptophan (Kyn:Tryp) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR1/2 agonist Pam3Csk, (B) TLR2 agonist HKLM, and (C) TLR3 agonist Poly I:C. Data are expressed as mean ± SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni *post hoc* test. *$p < 0.05$, Healthy control stimulated vs. unstimulated; ***$p < 0.001$, healthy control stimulated vs. unstimulated; $ $p < 0.05$, IBS stimulated vs. unstimulated.

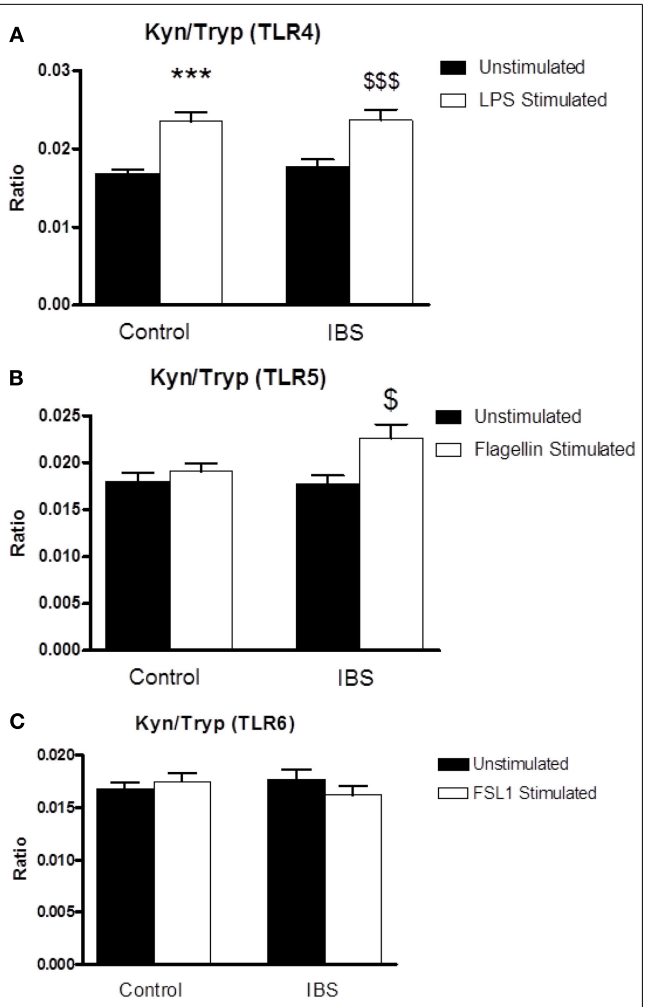

FIGURE 3 | Kynurenine:tryptophan (Kyn:Tryp) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR4 agonist LPS, (B) TLR5 agonist flagellin, and (C) TLR6/1 agonist FSL1. Data are expressed as mean ± SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni *post hoc* test. ***$p < 0.001$, Healthy control stimulated vs. unstimulated; $ $p < 0.05$, IBS stimulated vs. unstimulated; $$$ $p < 0.001$, IBS stimulated vs. unstimulated. There was no significant interaction between disease state and treatment following stimulation with the TL6/2 agonist FSL1 ($F_{1,124} = 1.026$, $p = 0.313$) not was there any alteration in the kynurenine:tryptophan ratio in either healthy controls or IBS subjects (**Figure 3C**).

kynurenine:tryptophan ratio in our IBS cohort compared to controls. Moreover, we have demonstrated, for the first time, that IBS patients exhibit a distinct tryptophan degradation profile downstream of TLR activation that is different from that of healthy controls, as indicated by the kynurenine:tryptophan ratio in the supernatants of whole blood preparations.

The pathophysiological relevance of increased activity along the kynurenine pathway in the plasma of IBS patients remains to be defined as does the source of such alterations. Of the TLRs that have previously been demonstrated to be upregulated in the colon in IBS (Brint et al., 2011) and to have an elevated cytokine release profile (McKernan et al., 2011), both TLR5 and TLR8 induced, on activation, an increase in the kynurenine:tryptophan ratio in our experimental system. It has recently been shown that alterations in gastrointestinal IDO activity in Crohns's disease correlate with systemic indices of kynurenine pathway activation (Gupta et al., 2011). Moreover, it is becoming increasingly apparent that peripheral blood alterations in kynurenine pathway metabolites can manifest at the CNS level (Raison et al., 2009) and that modulation of systemic pathway activity might be a useful therapeutic strategy (Reinhart and Kelly, 2011; Zwilling et al., 2011). Nevertheless, it will be important to determine whether the findings presented here are indeed reflected at the level of the intestinal compartment, an important site of tryptophan metabolism and serotonergic signaling in the periphery and further studies using supernatants from mucosal biopsies will be of value in this regard.

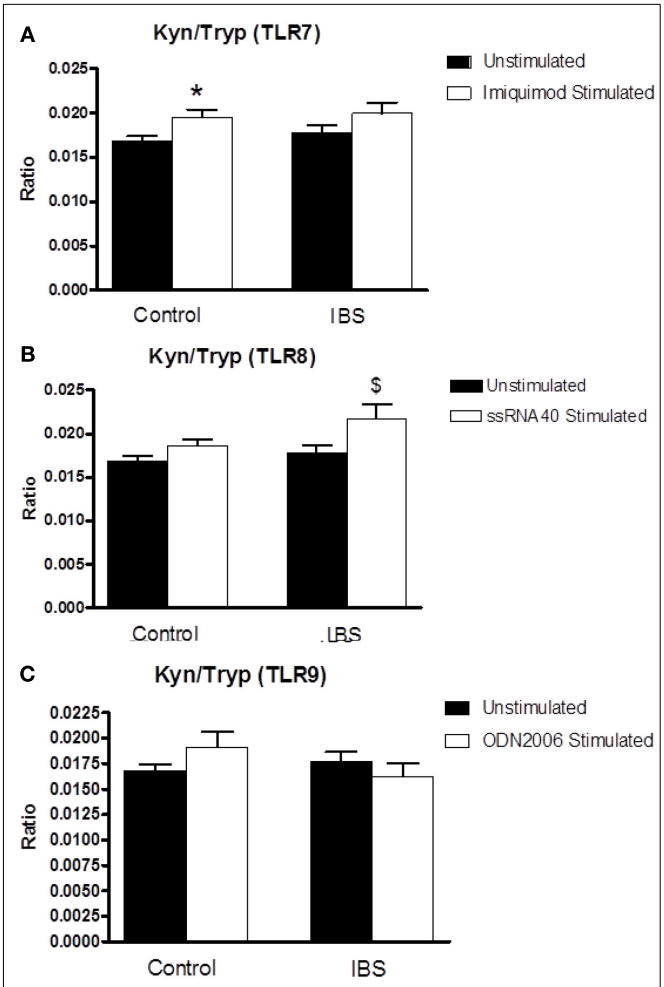

FIGURE 4 | Kynurenine:tryptophan (Kyn:Tryp) ratio in unstimulated whole blood supernatants from healthy controls and IBS patients and following stimulation with: (A) TLR 7 agonist Imiquimod, (B) TLR8 agonist ssRNA40, and (C) TLR9 agoinst ODN2006. Data are expressed as mean ± SEM. Statistical differences between treatments and disease states were determined using two-way ANOVA with Bonferroni *post hoc* test. *$p < 0.05$, Healthy control stimulated vs. unstimulated; $ $p < 0.05$, IBS stimulated vs. unstimulated.

The differential tryptophan degradation profile at TLR1/2, TLR2, and TLR3 is an important finding. These receptors are expressed at both intracellular and extracellular domains (Akira and Takeda, 2004; Sioud, 2006) and our data suggests the presence, in IBS, of TLR dysfunction at the level of both the cell membrane and the endosome. It is also noteworthy that TLRs can interact at a functional level to limit or inhibit the normal response to a particular ligand (Hajjar et al., 2001), a feature which may be of relevance to the reduced kynurenine:tryptophan ratio observed in IBS patients following stimulation with the TLR1/2 ligand. An alternative explanation, given that our ratio alterations following stimulation of TLR1/2 and TLR3 are derived from reductions in tryptophan concentrations in conjunction with *decreased* kynurenine, may involve the sequestering of tryptophan for the diverse cellular processes in which it is involved. Certain cytokines, for example, can upregulate tryptophan hydroxylase expression, the rate-limiting enzyme in the conversion of tryptophan to serotonin (Lisak et al., 2011). Future studies will also need to address whether a differential leukocyte distribution might account for our findings although recent studies suggest that cellular activation might be a more important factor (Ohman et al., 2009a,b).

In any case, the blunted kynurenine production at TLR2 and TLR3 in our IBS cohort is perplexing given the enhanced production of TNF-α and IL-8 demonstrated at those receptor subtypes, respectively, in our earlier study (McKernan et al., 2011) and the recently reported increased TLR2 expression on blood monocytes in IBS patients (Ohman et al., 2012). It is, however, worth noting that combinations of elevated cytokines may favour kynurenine production over the singular increases described at these receptor subtypes (Taylor and Feng, 1991) and that the availability of certain co-factors is also required (Muller and Schwarz, 2007). Additionally, the spontaneous release of IL-8 from unstimulated samples, a previously reported phenomenon (Molina et al., 2006; Horton and Remick, 2010), from both groups and the baseline differences in the concentration of this cytokine may have obscured the impact that post-stimulation alterations in IL-8 production may have produced. Also of relevance is that repeated stimulation of specific TLRs with their ligands can induce unresponsiveness or immunotolerance, at least in cell line studies (Ehlers and Ravetch, 2007; Gomez-Llorente et al., 2010). This raises the possibility that the defective response at TLR2 and TLR3 in IBS subjects might alternatively be due to prolonged stimulation by endogenous ligands. This is in line with suggestions that activation of TLRs by their endogenous ligands might have a role in the promotion of systemic inflammation (Marshak-Rothstein, 2006). Interestingly host mRNA can activate TLR3 (Kariko et al., 2004) and high mobility group box 1 (HMGB1) is a ligand for TLR2 (Yu et al., 2006). Total plasma mRNA levels remain to be profiled in IBS and although fecal HMGB1 has been proposed as a novel marker of intestinal inflammation, it is also uncharacterised in the disorder.

Interestingly, we found that the downstream consequences of TLR4 activation for tryptophan metabolism are equivalent in both healthy controls and IBS patients. Previously we reported an increased release of the same two cytokines (IL-1B, TNF-α) in IBS patients following stimulation of this receptor with LPS as occurred following TLR5 activation with flagellin (McKernan et al., 2011). At first glance, it appears unusual that an exaggerated release of the same two cytokines would elicit differential responses in terms of kynurenine pathway indices at different TLRs if the immunoresponsive enzyme IDO mediates the effects we have observed. However, it should be noted that the magnitude of the cytokine response following TLR4 stimulation is greater than that induced following TLR5 activation suggesting that the more modest cytokine release pattern at the latter receptor may be required to tease apart the differential downstream effects of TLR stimulation on kynurenine production. The cellular distribution of the TLRs may also be of importance: it is worth noting that TLR4 is much more highly expressed than TLR5 in monocytes (Hornung et al., 2002) and LPS stimulation of whole blood primarily results in cytokine release from this cell type (Pace and Heim, 2011). In contrast, TLR5 is more highly expressed in NK cells and T cells

than TLR4, although the magnitude of dominance TLR5 enjoys in these cell populations does not match the superiority of TLR4 in terms of monocyte expression patterns. Although we did not analyze the cellular composition of our whole blood preparations, this theory is consistent with the observation that IBS subjects display an increased level of T-cell activation (Ohman et al., 2009a). This explanation also reflects the view in the literature that IBS is a disorder characterised by a low grade inflammation as opposed to an immune disorder *per se* (Clarke et al., 2009b). However it should also be noted that TLR4 stimulation increased kynurenine concentrations without altering tryptophan concentrations which may suggest the involvement of enzymatic components of the kynurenine pathway other than IDO. Indeed differential activation of kynurenine pathway enzymes can be a feature of a systemic immune challenge with LPS (Connor et al., 2008).

A limitation of the current study is that whole blood stimulations of TLRs may only partially model the complexity of the *in vivo* response. This is especially pertinent when one considers that the entire family of receptors may be simultaneously susceptible to activation *in vivo* depending on the combination of ligands present at physiologically relevant concentrations. Indeed, it is known that combinations of TLR ligands can either synergistically induce cytokine gene expression (Makela et al., 2011) or result in a blunted response (Marshall et al., 2007), depending on the particular ligand combination employed. Consequently, our results do not clarify whether the cumulative, sometimes opposing and sometimes synergistic, downstream effects of multiple simultaneous TLR activations are responsible for the enhanced resting state degradation of tryptophan along the kynurenine pathway in IBS. Moreover, it is unclear at present which receptor has the greatest biological influence on kynurenine metabolism *in vivo*. Nevertheless whole blood stimulation of single receptor subtypes is considered a valid strategy to interrogate their responsiveness in general and to determine whether particular patient populations are primed for aberrant responses (Pace and Heim, 2011). Moreover, an assessment of the kynurenine:tryptophan ratio is a previously validated method for the assessment of IDO activity in cell culture supernatants (Schroecksnadel et al., 2005; Mahanonda et al., 2007; Schroecksnadel et al., 2011). The fact that IBS populations show both basal alterations in this pathway and a distinct profile subsequent to stimulation of individual TLRs with their various ligands only adds to their potential utility as biomarkers of the disorder, albeit as components of a biomarker panel of other promising indices rather than as unique identifiers themselves. Further studies are urgently required, both to define such a biomarker panel and to determine the sensitivity and specificity of such an approach.

In conclusion, we have provided novel evidence demonstrating that TLR activation induces a pattern of downstream tryptophan degradation along the kynurenine pathway that differentiates IBS patients from healthy controls. This bolsters current and previous findings that highlighted baseline disturbances in this pathway and illuminates a mechanism through which TLR responses can functionally impact on brain-gut axis signaling in this disorder. Moreover, this implicates the modulation of TLRs as a novel therapeutic strategy in this debilitating condition.

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The pharmacology of TD-8954, a potent and selective 5-HT$_4$ receptor agonist with gastrointestinal prokinetic properties

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This study evaluated the *in vitro* and *in vivo* pharmacological properties of TD-8954, a potent and selective 5-HT$_4$ receptor agonist. TD-8954 had high affinity (pK$_i$ = 9.4) for human recombinant 5-HT$_{4(c)}$ (h5-HT$_{4(c)}$) receptors, and selectivity (>2,000-fold) over all other 5-hydroxytryptamine (5-HT) receptors and non-5-HT receptors, ion channels, enzymes and transporters tested (n = 78). TD-8954 produced an elevation of cAMP in HEK-293 cells expressing the h5-HT$_{4(c)}$ receptor (pEC$_{50}$ = 9.3), and contracted the guinea pig colonic longitudinal muscle/myenteric plexus preparation (pEC$_{50}$ = 8.6). TD-8954 had moderate intrinsic activity in the *in vitro* assays. In conscious guinea pigs, subcutaneous administration of TD-8954 (0.03–3 mg/kg) increased the colonic transit of carmine red dye, reducing the time taken for its excretion. Following intraduodenal dosing to anesthetized rats, TD-8954 (0.03–10 mg/kg) evoked a dose-dependent relaxation of the esophagus. Following oral administration to conscious dogs, TD-8954 (10 and 30 μg/kg) produced an increase in contractility of the antrum, duodenum, and jejunum. In a single ascending oral dose study in healthy human subjects, TD-8954 (0.1–20 mg) increased bowel movement frequency and reduced the time to first stool. It is concluded that TD-8954 is a potent and selective 5-HT$_4$ receptor agonist *in vitro*, with robust *in vivo* stimulatory activity in the gastrointestinal (GI) tract of guinea pigs, rats, dogs, and humans. TD-8954 may have clinical utility in patients with disorders of reduced GI motility.

Keywords: constipation, serotonin, 5-HT$_4$, prokinetic, TD-8954

INTRODUCTION

5-Hydroxytryptamine (5-HT) plays a critical role in coordinating gastrointestinal (GI) transit (Hansen and Skadhauge, 1997; Grider et al., 1998; Jin et al., 1999; Baker, 2005). In response to intestinal stretching and mucosal stimulation, 5-HT is released from enterochromaffin cells of the mucosal epithelium, and promotes peristalsis via activation of intrinsic primary afferent neurons located in the submucous plexus (Kirchgessner et al., 1992; Foxx-Orenstein et al., 1995; Gershon and Tack, 2007). Of the 5-HT receptors believed to influence GI motility (e.g., 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_3$, and 5-HT$_4$; Beattie and Smith, 2008), the 5-HT$_4$ receptor subtype is considered particularly important, both physiologically and pathophysiologically (Kadowaki et al., 1996; Kim and Camilleri, 2000; Baker, 2005). The peristaltic reflex, for example, is dependent on activation of 5-HT$_4$ receptors on intrinsic primary afferent neurons, interneurons, and motor neurons within the gut wall, which results in the coordinated release of acetylcholine, substance P, and calcitonin gene-related peptide. The release of these agents is associated with synchronized contraction and relaxation of GI smooth muscle, and propulsion of luminal contents (Jin et al., 1999; Gershon and Tack, 2007). Additionally, activation of 5-HT$_4$ receptors in the smooth muscle of the human distal colon, and on enteric neurons or enterocytes promotes a direct relaxant effect and fluid secretion, respectively, further supporting GI transit (Hillier et al., 1994; Hansen and Skadhauge, 1997).

Agents interacting with 5-HT$_3$ and 5-HT$_4$ receptors have provided meaningful benefit to patients with GI functional disorders (Sanger, 2008). The GI prokinetic activity of 5-HT$_4$ receptor agonists, such as tegaserod (Zelnorm®), cisapride (Propulsid®), velusetrag (TD-5108), prucalopride (Resolor®), and mosapride has been demonstrated in a variety of species (Jin et al., 1999; Briejer et al., 2001b; Inui et al., 2002; Ji et al., 2003; Manini et al., 2009), and clinical efficacy has been established in patients with irritable bowel syndrome with constipation (IBS-C), chronic idiopathic constipation, functional dyspepsia, or gastroparesis (Deruyttere et al., 1987; Muller-Lissner, 1987; Abell et al., 1991; Camilleri, 2001; Johanson, 2004; Patel et al., 2004; Camilleri et al., 2008; Goldberg et al., 2010). Cisapride and tegaserod were used widely to treat upper and lower GI disorders of reduced motility, respectively, although their clinical efficacy in many patients was modest (Kellow et al., 1995; Evans et al., 2004), possibly reflecting their interactions with receptors other than the 5-HT$_4$ subtype (Briejer et al., 1995; Beattie et al., 2004; Beattie and Smith, 2008; De Maeyer et al., 2008). The clinical use of cisapride and tegaserod is now restricted on the basis of cardiovascular safety concerns

(Barbey et al., 2002; Pasricha, 2007). Cisapride was associated with serious cardiac arrhythmias including ventricular tachycardia, ventricular fibrillation, and torsades de pointes as a result of its potent cardiac human ether-à-go-go-related gene (hERG) potassium channel inhibitory activity (Mohammad et al., 1997). The marketing of tegaserod was suspended in the United States due to concerns of an increased risk of serious ischemic cardiovascular events, although two recent epidemiological studies failed to identify such an association (Anderson et al., 2009; Loughlin et al., 2010). The newer generation, highly selective 5-HT_4 receptor agonists, velusetrag and prucalopride, produce robust clinical efficacy in chronic idiopathic constipation patients, have been well tolerated to date, and offer renewed hope for patients experiencing disorders of reduced GI motility (Camilleri et al., 2008; Sanger, 2009; Goldberg et al., 2010; Manabe et al., 2010). Prucalopride is approved in Europe for the treatment of women with chronic idiopathic constipation who have not responded adequately to laxatives.

In this study, the *in vitro* and *in vivo* pharmacodynamic properties of a structurally novel 5-HT_4 receptor agonist, TD-8954 (4-{(4-[(2-isopropyl-1*H*-benzoimidazole-4-carbonyl)amino]methyl}- piperidin-1-ylmethyl)piperidine-1-carboxylic acid methyl ester; **Figure 1**), have been investigated. The preclinical activity of several standard 5-HT_4 agonists was evaluated in parallel for comparison to that of TD-8954.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the principles of good laboratory animal care provided by the Institutional Animal Care and Use Committees of Theravance, Inc. (rodent studies) or Drug Research Laboratories (dog study). The study protocol and consent form for the human single ascending dose study were reviewed by the clinical site's Institutional Review Board, and each volunteer provided written informed consent prior to initiation of study procedures. The study was conducted at a single site in accordance with the United States Code of Federal Regulations and the principles of the Declaration of Helsinki.

HUMAN RECOMBINANT 5-HT_4 RECEPTORS

Radioligand binding

Radioligand binding studies were conducted as described previously (Smith et al., 2008). Inhibition of [^{3}H]-GR113808 binding was measured using membranes prepared from HEK293 cells stably expressing the human 5-$HT_{4(c)}$ (h5-$HT_{4(c)}$) receptor splice variant (HEK293-h5-$HT_{4(c)}$) (Kaumann and Levy, 2006).

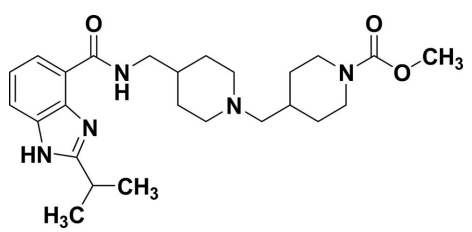

FIGURE 1 | TD-8954 (4-(4-{[(2-isopropyl-1*H*-benzoimidazole-4-carbonyl)amino]methyl}-piperidin-1-ylmethyl)piperidine-1-carboxylic acid methyl ester).

5-$HT_{4(c)}$ receptor mRNA was been detected in human GI tissue by RT-PCR (Blondel et al., 1998; Bender et al., 2000; Medhurst et al., 2001; Ito et al., 2003), and the rank order of affinities or potencies for several agonist and antagonists is maintained across 5-HT_4 receptor splice variants in published reports (Blondel et al., 1998; Smith et al., 2008), supporting the use of the 5-$HT_{4(c)}$ variant for the current studies. Unlabeled compound (10 pM to 100 μM) was incubated for 1 h at room temperature with [^{3}H]-GR113808 (0.15 nM) and h5-$HT_{4(c)}$ membranes (2 μg protein) in a total assay volume of 400 μL. Binding reactions were terminated by rapid filtration over GF/B filter plates and bound radioactivity quantitated by liquid scintillation spectroscopy in Microscint-20 using a TopCount Scintillation Counter (Packard BioScience, Meriden, CT, USA).

Binding data were analyzed by non-linear regression analysis using GraphPad Prism™ software (GraphPad Software, Inc., San Diego, CA, USA) and a three-parameter model for one-site competition. The pK_i (negative decadic logarithm of K_i) values for test compounds were calculated from the best-fit IC_{50} values, and the K_d value of the radioligand, using the Cheng–Prusoff equation [Cheng and Prusoff, 1973; $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] = radioligand concentration], and are reported as the mean ± SEM. Standard compounds were evaluated in parallel with TD-8954; the pK_i values were consistent with those reported previously (Vickery et al., 2007; Smith et al., 2008).

Whole cell cAMP accumulation

Whole cell cAMP accumulation assays were performed as described previously (Smith et al., 2008) using a homogeneous radioimmunoassay (Flashplate Adenylyl Cyclase Activation Assay System; Perkin Elmer Life Sciences, Boston, MA, USA). HEK293-h5-$HT_{4(c)}$ cells were lifted in Versene and collected by centrifugation (1,200 × *g*, 5 min) in phosphate-buffered saline (PBS). The cell pellet was resuspended gently in warm "stimulation buffer" (provided in the assay kit) and diluted to 5×10^5 cells/mL. Cells (25,000 per well) were incubated with test compound (10 pM to 100 μM) for 15 min at 37°C in a 96-well Flashplate, in a total volume of 0.1 mL. In antagonist inhibition studies, cells were preincubated in the absence or presence of GR113808 (0.6, 1.7, or 5 nM) for 20 min at 37°C prior to the addition of the test agonist. After the incubation period, [^{125}I]-cAMP was added in 100°μL of ice-cold "detection buffer" to each well, according to the manufacturer's instructions. Bound radioactivity was quantified by scintillation counting and the amount of cAMP produced was extrapolated from a cAMP standard curve.

Data were analyzed by non-linear regression analysis with GraphPad Prism™ using the three-parameter sigmoidal concentration–effect model (slope constrained to unity). The potency of test agents was reported as a mean (±SEM) pEC_{50} value (negative decadic logarithm of the effective concentration producing 50% of the maximum response), and the intrinsic activity (IA) as a mean (±SEM) percentage of the maximum 5-HT-evoked response. The pEC_{50} and IA values for standard compounds (tested in parallel with TD-8954) were consistent with those reported previously (Vickery et al., 2007; Smith et al., 2008). Schild regression analysis was used to determine pK_b values for GR113808 in the antagonist inhibition studies for 5-HT and TD-8954 (Kenakin, 1997). Concentration ratios (CR) were calculated

as the ratio of the EC_{50} values in the presence and absence of antagonist. The log[CR-1] was plotted against the log[GR113808], resulting in a linear relationship with the slope not significantly different from unity. The slope was therefore constrained to unity, and the X-intercept was extrapolated to provide a measure of the pK_b value for GR113808.

5-HT$_4$ receptor selectivity

Off-target selectivity screening was conducted at Theravance, Inc. or at a contract research organization (CEREP, Paris, France). Conventional radioligand binding studies were conducted using, in the majority of assays, membranes prepared from cell lines transfected with the respective human recombinant receptor, ion channel, or transporter. The percent inhibition of specific binding by TD-8954, at a single concentration (1 μM, in duplicate), was determined. Conventional whole cell voltage-clamp techniques were used to examine the interaction of TD-8954 (3 μM) with neuronal (rat $Na_v1.2$) and cardiac (human $Na_v1.5$) voltage-gated sodium channels and hERG potassium channels (see Smith et al., 2006, 2008).

Functional 5-HT$_4$ receptor activity in the guinea pig colonic longitudinal muscle/myenteric plexus

Adult, male Dunkin Hartley guinea pigs (200–350 g, Harlan, Chicago, IL, USA) were euthanized by CO_2 asphyxiation. The distal colon was removed and placed in Krebs–Henseleit physiological buffer containing (in mM): KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ anhydrous (1.2), NaCl (118.1), D-glucose (11.1), $NaHCO_3$ (25.0), $CaCl_2$ (2.6), ondansetron (0.003; to block 5-HT_3 receptors), methysergide (0.001; to antagonize 5-HT_1 and 5-HT_2 receptors), and indomethacin (0.001; to inhibit prostaglandin synthesis). The colon was cut into 5 cm lengths, and the longitudinal muscle/myenteric plexus (LMMP) carefully peeled off. The LMMP was then mounted, under a tension of 10 mN, in a 10-mL tissue bath filled with Krebs–Henseleit buffer (37°C, aerated continuously with 95% O_2/5% CO_2).

Tissues were washed three times (at 0, 15, and 30 min after mounting) and then challenged (at 45 min) with 5-HT at a concentration (0.3 μM) previously established to evoke a maximal contractile response. Once the contraction had reached its maximum, tissues were washed four times (2 min between each wash), and once more 10 min later. An additional priming challenge of 5-HT (0.3 μM) was made 15 min later. Following further washing prior to, and after a third 5-HT (0.3 μM) challenge, a cumulative concentration–response curve to 5-HT, tegaserod, mosapride, cisapride, prucalopride, or TD-8954 was constructed. TD-8954 concentration–response curves were also constructed in tissues previously exposed for 15 min to the selective 5-HT_4 receptor antagonist, piboserod (0.3 μM; Gaster et al., 1995) or vehicle. Changes in colonic isometric force were recorded by means of a force transducer (World Precision Instruments, model Fort 100), amplifier (Astro Med., model S48), and a data acquisition system (Biopac MP100, Acknowledge™ Waveform Acquisition and Analysis software). Contractile responses to test compounds were normalized to those of the last, primed 5-HT (0.3 μM) response in each tissue. Data were analyzed by non-linear regression analysis with GraphPad Prism using the four-parameter sigmoidal concentration–effect model. The pEC_{50} and IA (i.e., the maximum compound-evoked response expressed as a percentage of the fully primed 5-HT maximum) were derived and reported for each test agent.

GUINEA PIG COLONIC TRANSIT

Adult, male Dunkin Hartley guinea pigs (220–300 g, Harlan, Chicago, IL, USA) were acclimated to their holding room (temperature controlled at 21 ± 1°C and 12:12 h light–dark cycle commencing at 7 a.m.) for approximately 1 week prior to surgery. A standard guinea pig diet (7006, Harlan Teklad) and drinking water were provided *ad libitum*. Guinea pigs were anesthetized with isoflurane (2–3%, in oxygen) in an induction chamber. Maintenance of anesthesia was achieved with isoflurane (2–3%) administered via a nose cone. The mid-scapular area and abdomen were shaved and cleansed with betadine and 70% isopropanol. A small incision was made in the lower abdomen to expose the proximal colon. After isolation, a small incision was made in the proximal colon (approximately 2 cm from the cecum) and a cannula consisting of micro-renathane (MRE-040) tubing with a 2-cm silicone rubber tip (RenaSil™; 0.047″ OD × 0.025″ ID) was introduced and advanced approximately 2 cm toward the aboral end. A purse-string suture (Ethicon, 6-0 silk) was used to anchor the cannula in the colon and Baytril antibiotic (2.27%) was then applied topically to the colonic surgical site. The muscle layer was closed with a 4-0 Vicryl suture. The cannula was then secured to the nearby musculature with a 6-0 silk suture, tunneled under the skin and exteriorized at the mid-scapular region. The cannula was flushed and locked with sterile saline (Baxter), sealed with a sterile stainless steel pin, and secured to the back of the neck with a wound clip. The incisions in the peritoneum and abdomen were cleansed of blood and closed with a 3-0 Ethilon suture (Ethicon). Subcutaneous (s.c.) warmed lactated-Ringer's solution (3 mL) was administered immediately after surgery in addition to an intramuscular injection of the opioid analgesic, Buprenex® (buprenorphine, 0.03 mg/kg).

At least 5 days after surgery, guinea pigs were assigned randomly to a study group. Animals were dosed with test agent or vehicle (2 mL/kg s.c.), and 5 min later, each guinea pig was gently restrained and a non-absorbable marker (0.2 mL) was infused into the proximal colon via the implanted cannula. The marker consisted of 6 g of carmine red dye per 15 mL of carboxymethyl cellulose (0.5%). The study personnel were blinded to the treatment that each animal received. Animal cages were visually inspected for the presence of excreted red fecal pellets at 30-min intervals until each guinea pig had excreted pellets containing the red marker, or until 10 h had lapsed from the time of the marker injection. In the case that an animal failed to produce red fecal pellets within 10 h, the animal was left overnight in a clean cage and inspected the following morning. If excretion of dye occurred overnight, a value of 10 h was assigned. The whole colonic transit was defined as the time that lapsed between marker injection and the appearance of dye in the feces. Data for each treatment group were expressed as a mean percent increase (±SEM) in colonic transit time relative to vehicle-treated animals. Differences between treatment groups were determined using one-way analysis of variance

(ANOVA) with a Dunnett's *post hoc* test ($p < 0.05$ considered to be statistically significant).

RAT ESOPHAGEAL RELAXATION

The technique of digital sonomicrometry was used (Adelson and Million, 2004; Armstrong et al., 2006). Adult, male Sprague-Dawley rats (250–350 g, Harlan, Chicago, IL, USA) were acclimated to the colony room (temperature controlled at $21 \pm 1^{\circ}$C and 12:12 h light–dark cycle commencing at 7 a.m.) for at least 5 days prior to investigation. Standard rat diet (Harlan Teklad) and drinking water were available *ad libitum*. Rats were anesthetized with isoflurane (2–3%) in an induction chamber and anesthesia was then maintained with isoflurane (2–3%) *via* a nose cone for the duration of each experiment. Animals were placed, in a supine position, on a heated pad to maintain body temperature at 37–38°C [monitored rectally with a sensor (Physitemp BAT-12)]. A midline incision was made in the skin and muscle layers of the abdomen, and the stomach and esophagus were exposed. A small incision was made in the upper duodenum approximately 1 cm from the pyloric sphincter, to permit intraduodenal (i.d.) administration of test agents. A micro-renathane catheter (MRE-40 with a 1 cm RenaSil rubber tip) was inserted approximately 1.5 cm into the duodenum via the incision and closed with 6-0 silk purse-string suture. Two piezoelectric crystals (1 mm diameter; Sonometrics Corp.) were gently glued, in a longitudinal orientation, to the distal esophagus (1 cm from the lower esophageal sphincter) using Vetbond tissue adhesive. The inter-crystal distance was approximately 2 mm. The wires connecting the crystals to the measurement device (Sonometrics Corp. TRX series 8) were exteriorized through the abdominal incision site, which was then closed with 4-0 Ethilon suture.

Following surgery, baseline esophageal tone was allowed to stabilize over approximately 30 min, prior to drug administration. The settings for the Sonometrics system were fixed within the Sonoview software (Sonometrics Corp., version 3.2.1) as follows: sampling rate = 99.4 Hz, transmit pulse = 375 ns, inhibit delay = 1.2–1.5 mm, velocity of sound through biological tissue = 1.59 mm/μs. Drug vehicle, followed by increasing doses of test compounds (0.5 mL/kg) were administered cumulatively via the duodenal cannula. Each dose was administered only when the esophageal response to the preceding dose had reached a maximum (typically 15–20 min). Changes in inter-crystal distance (in mm) from resting levels were averaged for each treatment group. The data were fitted to a sigmoidal dose–response relationship with variable slope, and an ED_{50} value (i.e., the dose resulting in 50% of the maximum response) was calculated using Prism graphics software 3.0 (GraphPad, Inc.).

DOG GASTROINTESTINAL CONTRACTILITY

Five adult, female beagle dogs (6.5–8.5 kg, Marshall BioResources, North Rose, NY, USA), were maintained in a temperature controlled holding room at 21–24°C and 12:12 h light–dark cycle commencing at 7 a.m. for at least 7 days prior to surgery. Dogs were fed at 8 a.m. and 6 p.m. with commercial dog food, while water was available *ad libitum*. Under pentobarbital anesthesia (30 mg/kg i.v. for induction, 1–2 mg/kg i.v. for maintenance), a strain gauge transducer (R. B. Products, Stillwater, MN, USA, calibrated with weights of 10–200 g) was sutured on to the antrum of the stomach (1 cm above the pylorus), duodenum, and jejunum (10–12 cm distal to the ligament of Treitz). Strain gauges were positioned to record circular muscle activity (Bass and Wiley, 1972). The strain gauge wiring was anchored securely to abdominal muscle with four stainless steel sutures. The abdominal incision was then closed around the wiring assembly. All dogs were treated with a fentanyl (25 μg) patch, which was applied 1 day prior to surgery and removed 3–4 days post-surgery. At least 2 weeks were allowed to lapse after surgery, before testing.

Dogs were fasted for 18 h prior to each study. The animals were positioned in slings, and the strain gauges were connected to a Grass (model 7P) polygraph to allow recording of the GI motility patterns. In fasted beagles, three phases of activity are evident in the antrum, duodenum and jejunum: quiescence followed by a pre-burst and then a burst period. After recording data for a full motility cycle, and confirming commencement of a quiescent period, each dog was dosed orally with TD-8954 (0.01 and 0.03 mg/kg), tegaserod (0.1 and 0.3 mg/kg), or vehicle (1 mL/kg over 5 s), followed by a 10 mL flush of water. After dosing, GI motility was monitored for 3 h. Dogs were allowed at least 4 days between treatments, the sequence of which was based on a Latin square design.

The area under the contractile force-time curve (AUC) of GI muscle contractions was measured directly from the polygraph tracings by means of a Numonics™ 2210 digitizing tablet in conjunction with an IBM 80486 computer and Sigma Scan™ analysis software (Jandel Scientific). Data were expressed as mean values (±SEM) for each treatment group.

HUMAN SINGLE ASCENDING DOSE STUDY

A Phase 1, double-blind, randomized, placebo-controlled, single ascending dose study was performed, consisting of seven sequential cohorts. In each cohort, eight healthy male and female subjects (18–50 years old) were randomized such that six subjects received a single oral dose of TD-8954 (0.1, 0.5, 1, 2, 5, 10, or 20 mg) and two subjects received placebo. The lowest dose of TD-8954 was formulated as an aqueous solution, while the other doses were administered as a powder in capsule. All subjects were confined to the clinical research unit from day 1 (admission) until after the 48-h post-dose safety assessments, and then returned on day 7 for a follow-up visit. A bowel diary was used to record the date and time of each bowel movement after dosing. The bowel diary was reviewed daily and before discharge. Blood samples were collected to assess the pharmacokinetics of TD-8954, and safety and tolerability were closely monitored.

MATERIALS

Standard biochemical and tissue culture reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA) respectively. [^{3}H]GR113808 was purchased from Amersham Biosciences (Newark, NJ, USA). The Flashplate Adenylyl Cyclase Activation Assay System was purchased from Perkin-Elmer (Boston, MA, USA). TD-8954, prucalopride, mosapride, and piboserod were synthesized at Theravance, Inc. GR113808, tegaserod and cisapride were purchased from Tocris Cookson (Ellisville, MO, USA), Apin Chemicals (Abingdon, Oxon, UK) and

Sequoia Research Products (Pangbourne, UK), respectively. Carbachol, 5-HT, and ketanserin were purchased from Sigma-Aldrich. For *in vitro* radioligand binding and cAMP accumulation studies, stock agonist solutions (10 mM) were prepared in DMSO, diluted to 400 μM with 50 mM HEPES (pH 7.4) at 25°C, containing 0.1% BSA, and serial dilutions prepared in the same buffer. For isolated tissue studies, 10 mM solutions, prepared in DMSO, were diluted serially in sterile water. For *in vivo* studies, TD-8954 and prucalopride were dissolved in 5% dextrose in distilled water (D5W) or 0.9% saline, while tegaserod and mosapride were prepared in 10% sulfobutyl ether-beta cyclodextrin (10% SBE/CD), and cisapride was dissolved in 10% SBE/CD containing citrate (20 mM). Doses were expressed with respect to the free base weights of each compound.

RESULTS

5-HT$_4$ RECEPTOR BINDING AND cAMP ACCUMULATION

TD-8954, tegaserod, prucalopride, cisapride, and mosapride produced a concentration-dependent inhibition of [^{3}H]-GR113808 binding to HEK293-h5-HT$_{4(c)}$ cell membranes. Comparison of the mean pK$_i$ values for the compounds (**Table 1**) indicated a rank order of affinity of TD-8954 (pK$_i$ = 9.4) > tegaserod (pK$_i$ = 8.6) > prucalopride (pK$_i$ = 7.6) > cisapride (pK$_i$ = 7.1) > mosapride (pK$_i$ = 6.8). In cAMP accumulation assays, using HEK-293 cells stably transfected with the h5-HT$_{4(c)}$ receptor, TD-8954, tegaserod, cisapride, prucalopride, and mosapride produced a concentration-dependent increase in cAMP. The rank order of potency was TD-8954 (pEC$_{50}$ = 9.3) > tegaserod (pEC$_{50}$ = 8.7) > prucalopride (pEC$_{50}$ = 7.9) > cisapride (pEC$_{50}$ = 7.4) > mosapride (pEC$_{50}$ = 6.3). The mean IAs of TD-8954, tegaserod, cisapride, prucalopride, and mosapride, relative to 5-HT (100%) were 83, 120, 101, 109, and 22%, respectively; **Table 1**). In antagonist inhibition studies, increasing concentrations of GR113808 produced rightward shifts in the 5-HT and TD-8954 concentration–response curves, resulting in pK$_b$ values (with 95% confidence intervals) of 10.3 (95% CI: 10.2–10.4) and 10.3 (95% CI: 10.2–10.5), respectively.

5-HT$_4$ RECEPTOR SELECTIVITY

TD-8954 was >2,000-fold selective for h5-HT$_{4(c)}$ receptors over other 5-HT receptors (**Table 2**), and all non-5-HT receptors, transporters, ion channels, and enzymes tested (**Table 3**). TD-8954 (3 μM) had no effect on hERG potassium currents (n = 6 cells) while cisapride (20 nM) was associated with a mean inhibition of 65% in the same cells. Exposure to TD-8954 (3 μM) for 3 min had no effect on the magnitude of the inward rat Na$_v$1.2a or human Na$_v$1.5 sodium currents (n = 3 cells for each).

GUINEA PIG COLONIC LONGITUDINAL MUSCLE/MYENTERIC PLEXUS

TD-8954, tegaserod, cisapride, prucalopride, and mosapride produced concentration-dependent contraction of the guinea pig colonic LMMP (**Figure 2**). Comparison of the mean pEC$_{50}$ (±SEM) values for the compounds indicated a rank order of potency of TD-8954 (pEC$_{50}$ = 8.6) > tegaserod (pEC$_{50}$ = 7.9) = prucalopride (pEC$_{50}$ = 7.7) > cisapride (pEC$_{50}$ = 7.0) > mosapride (pEC$_{50}$ = 5.4). TD-8954 had a mean IA (55% of the 5-HT maximum) lower than that of cisapride and prucalopride (75 and 81%, respectively), but higher than that of tegaserod (45%) and mosapride (37%; **Table 1**). Incubation of tissues with the selective 5-HT$_4$ receptor antagonist, piboserod (0.3 μM), resulted in a 614-fold shift (apparent pK$_b$ value = 9.3) of the TD-8954 concentration–response curve (data not shown).

Table 2 | Binding affinity (pK$_i$) of TD-8954 at 5-HT receptor subtypes.

Receptor	Radioligand	[Radioligand] (nM)	pK$_i$
Human 5-HT$_{1A}$	[^{3}H]-8-OH-DPAT	0.5	<6
Rat 5-HT$_{1B}$	[^{125}I]-CYP	0.1	<6
Bovine 5-HT$_{1D}$	[^{3}H]-serotonin	2	<6
Human 5-HT$_{2A}$	[^{3}H]-ketanserin	0.5	<6
Human 5-HT$_{2B}$	[^{3}H]-LSD	1.2	<6
Human 5-HT$_{2C}$	[^{3}H]-mesulergine	1	<6
Human 5-HT$_{3a}$	[^{3}H]-GR65630	0.5	<4
Human 5-HT$_{4(c)}$	[^{3}H]-GR113808	0.15	9.4
Human 5-HT$_{5a}$	[^{3}H]-LSD	1	<6
Human 5-HT$_6$	[^{3}H]-LSD	2	<6
Human 5-HT$_7$	[^{3}H]-LSD	4	<6

Inhibition of radioligand binding to non-5-HT$_4$ receptor subtypes was determined in duplicate at a single test concentration (1 μM, with the exception of the 5-HT$_{3a}$ receptor subtype, at which concentrations up to 100 μM were evaluated).

Table 1 | Human 5-HT$_{4(c)}$ binding affinity (HEK293-h5-HT$_{4(c)}$; pK$_i$), and human (HEK293-h5-HT$_{4(c)}$) and guinea pig colonic longitudinal muscle/myenteric plexus (LMMP) agonist potency (pEC$_{50}$) and intrinsic activity (IA; % 5-HT maximum) values for TD-8954, cisapride, mosapride, prucalopride, and tegaserod.

	Human 5-HT$_{4(c)}$ receptor affinity		Human 5-HT$_{4(c)}$ agonist activity			Guinea pig colonic LMMP contractile activity		
	pK$_i$ (mean ± SEM)	n	pEC$_{50}$ (mean ± SEM)	IA (mean % 5-HT max ± SEM)	n	pEC$_{50}$ (mean ± SEM)	IA (mean % 5-HT max ± SEM)	n
TD-8954	9.4 ± 0.04	6	9.3 ± 0.11	83 ± 6	6	8.6 ± 0.1	55 ± 2	24
Cisapride	7.1 ± 0.05	11	7.4 ± 0.11	101 ± 4	11	7.0 ± 0.1	75 ± 3	10
Mosapride	6.8 ± 0.09	6	6.3 ± 0.11	22 ± 3	6	5.4 ± 0.1	37 ± 2	3
Prucalopride	7.6 ± 0.03	11	7.9 ± 0.11	109 ± 5	11	7.7 ± 0.1	81 ± 2	24
Tegaserod	8.6 ± 0.03	11	8.7 ± 0.07	120 ± 5	11	7.9 ± 0.3	45 ± 3	13

Table 3 | Binding data (% inhibition of specific binding and pK_i values) of TD-8954 at non-5-HT receptors, transporters, ion channels, and enzymes.

Receptor	Radioligand	[Radioligand] (nM)	% Inhibition of specific binding (1 μM)	pK_i
BIOGENIC AMINE RECEPTORS				
Human A_1	[^{3}H]-DPCPX	1	−4	<6
Human A_{2A}	[^{3}H]-CGS 21680	6	16	<6
Rat α_1 (non-selective)	[^{3}H]-prazosin	0.25	−1	<6
Rat α_2 (non-selective)	[^{3}H]-RX 821002	0.5	7	<6
Human D_1	[^{3}H]-SCH 23390	0.3	−2	<6
Human D_{2S}	[^{3}H]-spiperone	0.3	6	<6
Human $D_{4.4}$	[^{3}H]-spiperone	0.3	6	<6
Human D_5	[^{3}H]-SCH 23390	0.3	−12	<6
Guinea pig H_1	[^{3}H]-pyrilamine	1	−7	<6
Guinea pig H_2	[^{125}I]-APT	0.1	4	<6
Human M_1	[^{3}H]-NMS	1	–	<6
Human M_2	[^{3}H]-NMS	1	–	6.1
Human M_3	[^{3}H]-NMS	1	–	<5
Human M_4	[^{3}H]-NMS	1	–	5.5
Human M_5	[^{3}H]-NMS	1	–	<5
Human β_1	[^{3}H]-DHA	0.8	−8	<6
Human β_2	[^{3}H]-DHA	0.8	−17	<6
PEPTIDE RECEPTORS				
Human AT_1	[^{125}I]-[Sar^1, Ile^8]-ATII	0.05	2	<6
Rat BB (non-selective)	[^{125}I]-[Tyr^4]-bombesin	0.01	4	<6
Human B_2	[^{3}H]-bradykinin	0.2	3	<6
Human CGRP	[^{125}I]-hCGRPα	0.03	−18	<6
Human CCK_1 (CCK_A)	[^{125}I]-CCK-8	0.08	−8	<6
Human CCK_2 (CCK_B)	[^{125}I]-CCK-8	0.06	−1	<6
Rat CRF_1	[^{125}I]-Tyr^0-CRF	0.1	−6	<6
Human ET_A	[^{125}I]-endothelin-1	0.03	0	<6
Human ET_B	[^{125}I]-endothelin-1	0.03	5	<6
Rat galanin (non-selective)	[^{125}I]-galanin	0.05	1	<6
Human motilin	[^{125}I]-motilin	0.05	14	<6
Human NK_1	[^{125}I]-[Sar^9, Met$(O_2)^{11}$]-SP	0.15	−2	<6
Human NK_2	[^{125}I]-NKA	0.1	−8	<6
Human NK_3	[^{3}H]-SR 142801	0.4	−3	<6
Human Y_1	[^{125}I]-peptide YY	0.025	−11	<6
Human Y_2	[^{125}I]-peptide YY	0.015	−5	<6
Rat NT (non-selective)	[^{125}I]-Tyr^3-neurotensin	0.05	4	<6
Rat PACAP (PAC_1)	[^{125}I]-$PACAP_{1–27}$	0.02	−3	<6
Mouse sst (non-selective)	[^{125}I]-Tyr^{11}-somatostatin	0.05	−6	<6
Human $VPAC_1$ (VIP_1)	[^{125}I]-VIP	0.04	2	<6
Human $VPAC_2$ (VIP_2)	[^{125}I]-VIP	0.05	−19	<6
Human V_{1a}	[^{3}H]-AVP	0.3	1	<6
Human V_2	[^{3}H]-AVP	0.3	−11	<6
OPIATE RECEPTORS				
Human δ_2	[^{3}H]-DADLE	0.5	0	<6
Guinea pig κ kappa	[^{3}H]-U69593	0.7	22	<6
Human μ	[^{3}H]-DAMGO	0.5	−30	<6
TRANSPORTERS				
Human norepinephrine	[^{3}H]-nisoxetine	1	6	<6
Rat dopamine	[^{3}H]-GBR12935	0.8	3	<6
Human 5-HT	[^{3}H]-imipramine	2	0	<6

(*Continued*)

Table 3 | Continued

Receptor	Radioligand	[Radioligand] (nM)	% Inhibition of specific binding (1 μM)	pK_i
ION CHANNELS				
Rat AMPA	[^{3}H]-AMPA	8	−4	<6
Rat kainate	[^{3}H]-kainic acid	5	−6	<6
Rat NMDA	[^{3}H]-CGP 39653	5	−6	<6
Rat $\alpha_4\beta_2$ nAChR (α-BGTX-insensitive)	[^{3}H]-cytisine	1.5	−2	<6
Rat α_7 nAChR (α-BGTX-sensitive)	[^{125}I]-α-bungarotoxin	1	3	<6
Rat Ca^{2+} channel (L, DHP site)	[^{3}H]-(+)PN 200-110	0.04	7	<6
Rat Ca^{2+} channel (L, diltiazem site)	[^{3}H]-diltiazem	5	13	<6
Rat Ca^{2+} channel (L, verapamil site)	[^{3}H]-(−)D 888	0.5	21	<6
Rat Ca^{2+} channel (N)	[^{125}I]-ω-conotoxin	0.001	−11	<6
K^+_{ATP} channel	[^{3}H]-glibenclamide	0.1	5	<6
K^+_V channel	[^{125}I]-α-dendrotoxin	0.01	−5	<6
SK^+_{Ca} channel	[^{125}I]-apamin	0.004	−4	<6
OTHER				
Human CB_1	[^{3}H]-WIN 55212-2	2	8	<6
Rat GABA (non-selective)	[^{3}H]-GABA	10	−6	<6
Rat P2Y	[^{35}S]-dATP αS	10	8	<6
Rat σ (non-selective)	[^{3}H]-DTG	8	30	<6
Enzyme	**Substrate/stimulus/ tracer**	**[Substrate/stimulus/ tracer] (μM)**	**% Inhibition of control (1 μM)**	**pIC_{50}**
Human COX_1	Arachidonic acid	0.3	2	<6
Human COX_2	Arachidonic acid	50	−36	<6
Human PDE4	[^{3}H]-cAMP + cAMP	1	−19	<6
Rat adenylyl cyclase	ATP	500	5	<6
Human acetylcholinesterase	AMTCh	50	2	<6

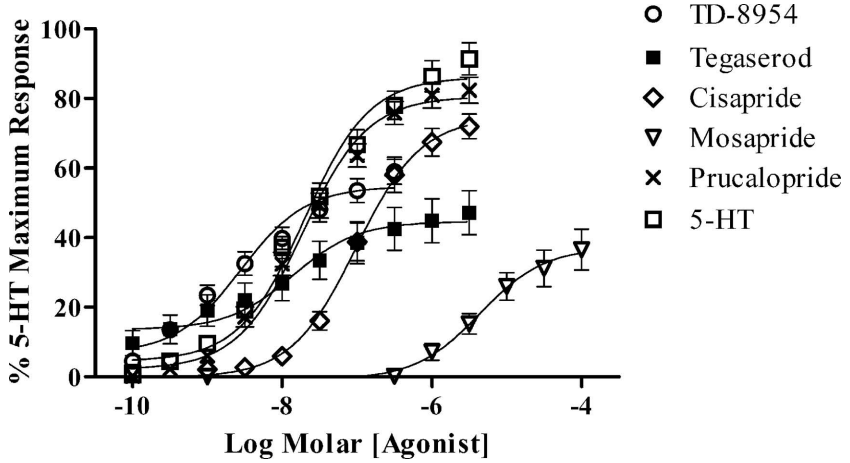

FIGURE 2 | Concentration–response curves to TD-8954 ($n = 24$), tegaserod ($n = 13$), cisapride ($n = 10$), mosapride ($n = 3$), prucalopride ($n = 24$), and 5-HT ($n = 26$) in the guinea pig isolated colonic LMMP preparation. Values are expressed as the mean (±SEM) change in tension, as a percentage of the primed 5-HT (0.3 μM) response in the same tissue.

GUINEA PIG COLONIC TRANSIT

In vehicle (2 mL/kg s.c.)-treated guinea pigs, the mean time taken for excretion of the first fecal pellet containing red dye was typically between 220 and 310 min. Following s.c. dosing, TD-8954, tegaserod, cisapride, mosapride (each at 0.03–3 mg/kg) and prucalopride (0.03–10 mg/kg) increased colonic transit, reducing the time taken for excretion of the dye, compared to vehicle-treated animals (**Figure 3**), although statistical significance ($p < 0.05$, one-way ANOVA with a Dunnett's *post hoc* test) was achieved only for all of the TD-8954 doses and for the 0.3 and 3 mg/kg prucalopride

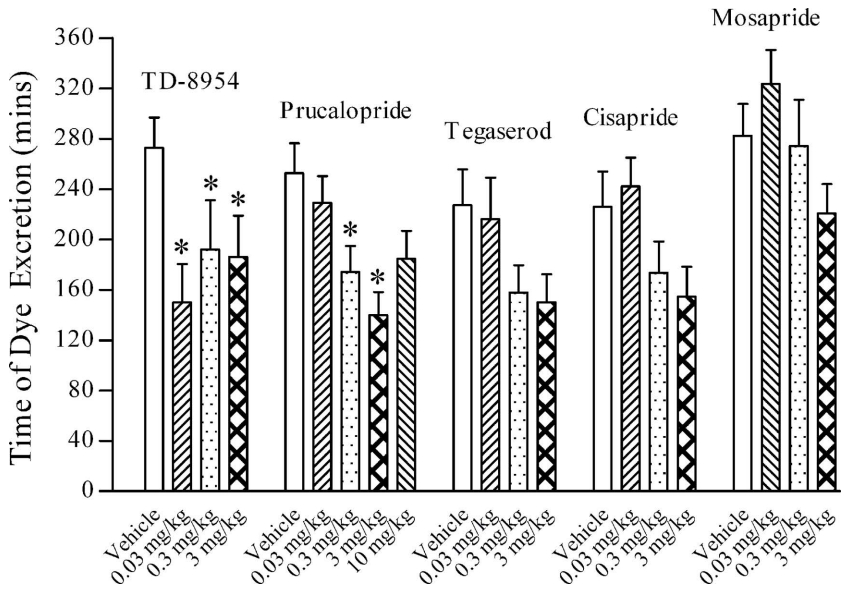

FIGURE 3 | The effects of TD-8954, tegaserod, cisapride, mosapride (each at 0.03–3 mg/kg), prucalopride (0.03–10 mg/kg), and vehicle (2 mL/kg), administered s.c., on the colonic transit of dye in conscious guinea pigs ($n = 9$–22 for each group; *$p < 0.05$; ANOVA followed by Dunnett's *post hoc* test vs. vehicle).

doses. TD-8954 was more potent than tegaserod, prucalopride, cisapride, and mosapride, being significantly active at the lowest dose tested (0.03 mg/kg). At 0.03 mg/kg, TD-8954 had already achieved its maximum effect.

RAT ESOPHAGEAL RELAXATION

Following crystal placement on the rat esophagus, 30 min proved sufficient to establish a stable sonomicrometry recording. No spontaneous changes in esophageal muscle length were observed after this stabilization period. Following cumulative i.d. dosing, TD-8954, prucalopride, tegaserod (each 0.03–10 mg/kg), cisapride (0.3–10 mg/kg), and mosapride (0.3–10 mg/kg), but not their vehicles (1–10 mL/kg) evoked a dose-dependent increase in inter-crystal distance, consistent with esophageal relaxation (**Figure 4**). The ED_{50} values (with 95% confidence limits) for TD-8954 and prucalopride were 0.15 (0.08–0.26) and 0.18 (0.13–0.25) mg/kg, respectively. Accurate ED_{50} values could not be calculated for tegaserod, cisapride, and mosapride as solubility limitations precluded verification that their maximum relaxations had been achieved. To compare the potencies of each compound, the doses of TD-8954, prucalopride, tegaserod, cisapride, and mosapride associated with a relaxation response of 0.1 mm were calculated (i.e., 0.23, 0.30, 2.43, 2.66, and 4.37 mg/kg, respectively; **Figure 4**). TD-8954 was therefore equieffective, on a dose basis, with prucalopride following i.d. dosing, and 11-, 12-, and 19-fold more potent than tegaserod, cisapride, and mosapride, respectively.

DOG GASTROINTESTINAL CONTRACTILITY

The quiescent phase of the antrum, duodenum, and jejunum motility cycle generally lasted for 50–60 min before transitioning into the pre-burst period (muscle contractions of gradually increasing magnitude occurring at random, with a duration of 30–60 min), followed by the burst period (vigorous and frequent contractions, with a duration of 5–15 min). Following oral administration of vehicle (1 mL/kg), there was little or no change in the activity of the antrum, duodenum, or jejunum; the expected motility patterns characteristic of fasted beagles were maintained throughout the observation period (**Figures 5** and **6**). TD-8954 (0.01 and 0.03 mg/kg) and tegaserod (0.1 and 0.3 mg/kg) produced increases in contractility in the antrum, duodenum, and jejunum (**Figure 5**). The onset of contractile activity with TD-8954 occurred typically within 10 min of dosing (**Figure 6**). Comparison of the activities of TD-8954 and tegaserod indicated that TD-8954 was statistically significantly more potent than tegaserod in the antrum, duodenum, and jejunum following oral administration ($p < 0.05$, ANOVA, followed by Dunnett's *post hoc* test).

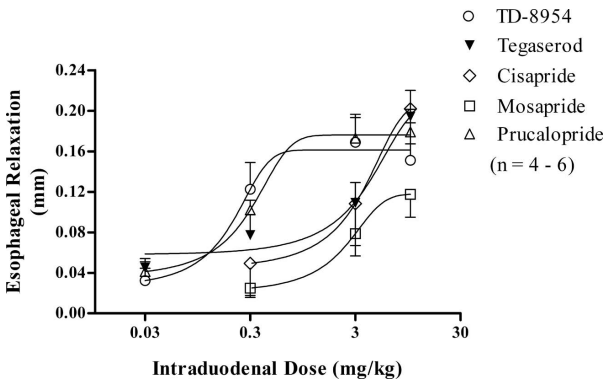

FIGURE 4 | Dose-dependent relaxation [mean (±SEM) change in inter-crystal distance (in mm)] of the rat esophagus following i.d. administration of TD-8954 (0.03–10 mg/kg; $n = 4$), prucalopride (0.03–10 mg/kg; $n = 4$), tegaserod (0.03–10 mg/kg; $n = 6$), cisapride (0.3–10 mg/kg; $n = 4$), and mosapride (0.3–10 mg/kg; $n = 4$).

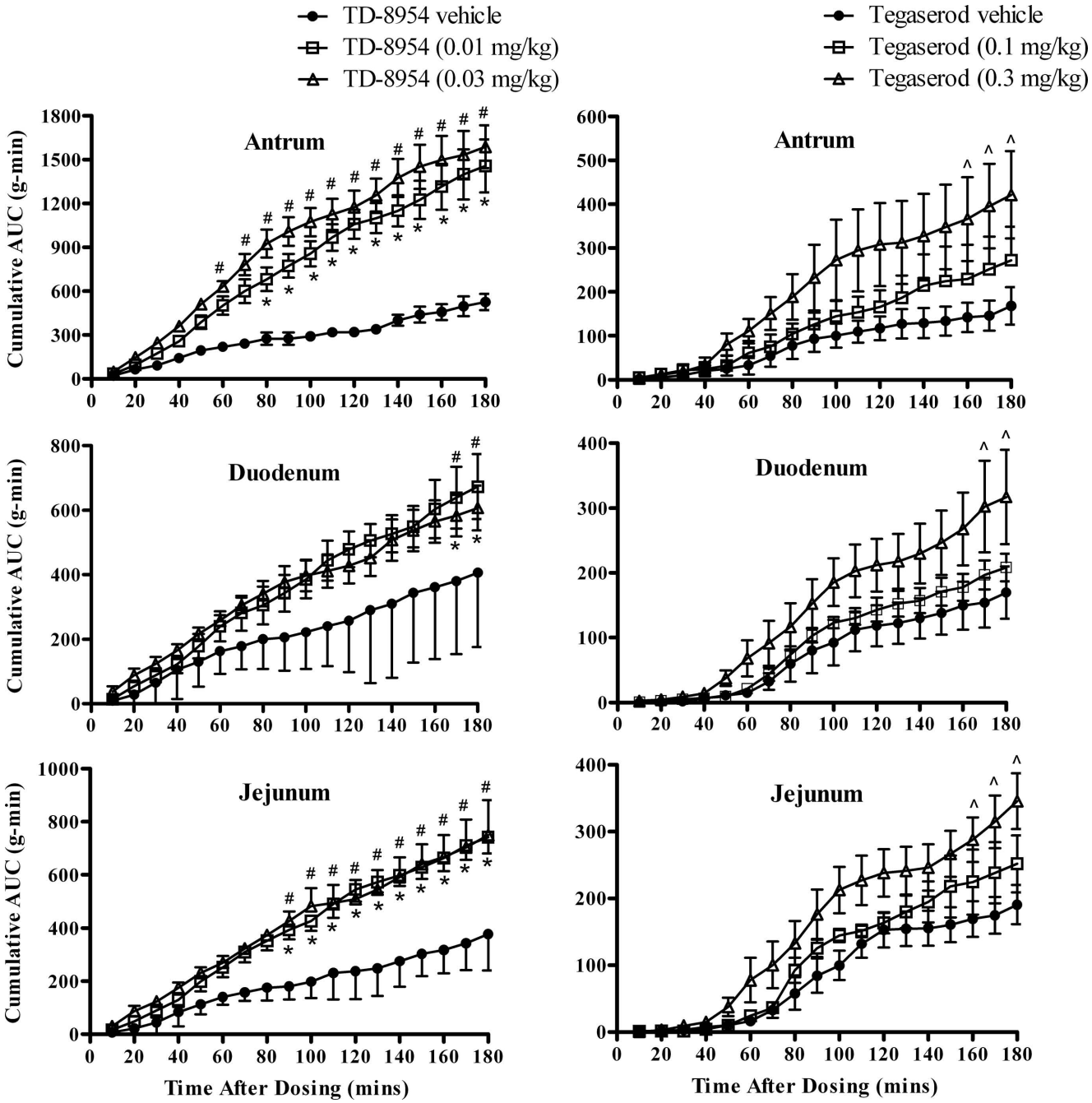

FIGURE 5 | Contractile activity following oral dosing of TD-8954 (0.01 and 0.03 mg/kg; $n = 5$; left-hand columns), tegaserod (0.1 and 0.3 mg/kg; $n = 6$; right-hand columns), and vehicles ($n = 5$ or 6) in the canine antrum, duodenum, and jejunum. Data are expressed as the mean (±SEM) cumulative contractile AUC in 10-min periods for 3 h post-dosing (at 0 min). Data are analyzed using a two-way analysis of variance with a Bonferroni *post hoc* test ($^{*,\#}p < 0.05$ for the 0.01, 0.03, and 0.3 mg/kg treatment groups, respectively, vs. vehicle.

HUMAN SINGLE ASCENDING DOSE STUDY

In healthy human subjects, GI prokinetic effects of TD-8954 (0.1–20 mg) were observed (**Figure 7**). The number of bowel movements from 0 to 24 h after each TD-8954 dose was increased significantly relative to placebo ($p < 0.03$ upon comparison of each TD-8954 treated group and placebo, based on Wilcoxon rank sum test). Compared to placebo, each TD-8954 dose was associated with a statistically significant reduction in the time to first bowel movement ($p < 0.05$ for all treatment groups comparing difference in survival function between each TD-8954 treated group and placebo obtained by log-rank test).

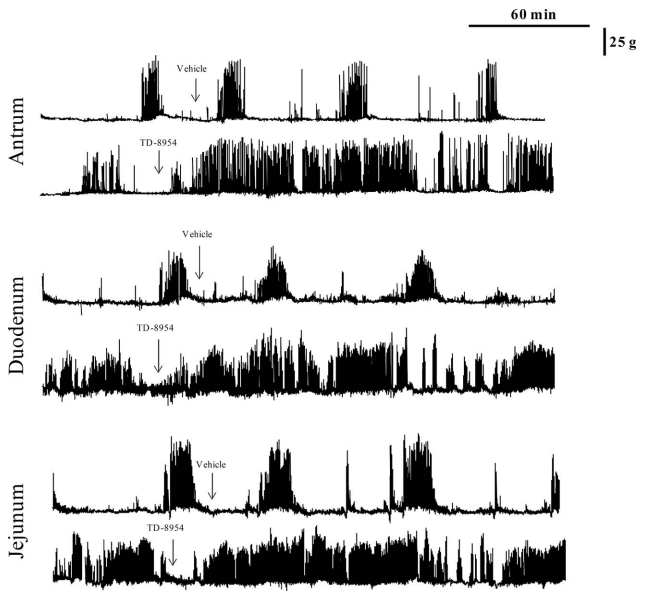

FIGURE 6 | Representative traces demonstrating the effects of oral dosing of TD-8954 (0.01 mg/kg) and vehicle on the contractility of the antrum, duodenum, and jejunum of conscious, fasted dogs.

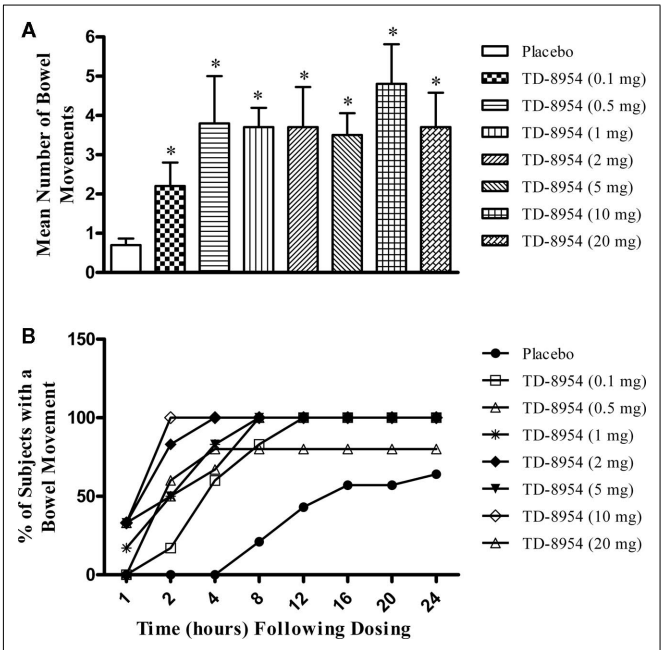

FIGURE 7 | (A) The mean number of bowel movements, and **(B)** the cumulative percentage of subjects responding with a bowel movement within 24 h following the oral dosing of TD-8954 (0.1–20 mg) and placebo to healthy human subjects ($n = 5$–14 per group). Each dose of TD-8954 produced a statistically significant effect compared to placebo ($^*p < 0.03$, Wilcoxon rank sum test vs. placebo).

DISCUSSION

Disorders of reduced GI motility, such as IBS-C, chronic idiopathic constipation, gastroparesis, post-operative ileus, and functional dyspepsia have a significant impact on the quality of life of affected individuals. Prior to their removal from the market, cisapride and tegaserod provided some relief to patients afflicted with these disorders (Prather et al., 2000; Evans et al., 2004; Ford et al., 2009). Limitations in their efficacy have been attributed to a lack of selectivity for the 5-HT_4 receptor subtype (Beattie and Smith, 2008; De Maeyer et al., 2008). Cisapride is a potent 5-HT_{2A} and 5-HT_{2B} receptor antagonist, while tegaserod has affinity for, and/or antagonist potency at, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors (Buchheit et al., 1995; Beattie et al., 2004; De Maeyer et al., 2008). Interactions of cisapride or tegaserod with non-5-HT receptors have also been proposed to underlie cardiac arrhythmic or ischemic adverse events. It is now well established that cisapride can induce ventricular tachycardia, ventricular fibrillation, and torsades de pointes as a result of potent cardiac hERG potassium channel inhibitory activity, particularly when cytochrome P_{450} 3A4 substrates are co-administered (Mohammad et al., 1997). The perceived risk of cardiovascular ischemic events with tegaserod, initially identified upon reviewing clinical trial data, has been questioned recently (Anderson et al., 2009; Loughlin et al., 2010). It is clear, however, that a significant unmet medical need remains for new therapeutic agents that will be efficacious and well tolerated in patients with functional GI disorders. One approach has been the development of selective 5-HT_4 receptor agonists, such as velusetrag (Beattie et al., 2008a; Smith et al., 2008; Goldberg et al., 2010), prucalopride (Briejer et al., 2001a; Camilleri et al., 2008), and TD-8954 (Beattie et al., 2008b), which, it is believed, will provide robust efficacy with acceptable tolerability and safety for patients. TD-8954, the subject of this study, is one of the most potent and selective 5-HT_4 receptor agonists described to date.

The *in vitro* data demonstrated that TD-8954 had high affinity and potency at the human 5-HT_4 receptor (pK_i and pEC_{50} values of 9.4 and 9.3, respectively). In each assay, TD-8954 was significantly more potent than each of the comparator 5-HT_4 agonists tested in parallel, an observation consistent with that made previously in experiments with human isolated colonic circular muscle preparations (Beattie et al., 2008b). Activation of the 5-HT_4 receptor by TD-8954 was confirmed *in vitro* by antagonist inhibition, where the GR113808 pK_b value was consistent with the [^{3}H]-GR113808 binding pK_d and antagonist pK_b values reported previously against 5-HT and tegaserod (Smith et al., 2008), providing further evidence of a 5-HT_4 receptor-mediated elevation of cAMP. TD-8954 had moderate IA at human recombinant 5-HT_4 receptors and endogenous 5-HT_4 receptors in the guinea pig colonic LMMP. Optimal target activation to induce a physiological response is dependent upon, amongst other things, the IA of an agonist and the receptor reserve in the target tissue (Grimwood and Hartig, 2009). While TD-8954 had moderate 5-HT_4 receptor agonist IA *in vitro*, robust *in vivo* GI prokinetic or 5-HT_4 agonist activity, to at least the same degree as standards with higher IA *in vitro*, was noted in guinea pigs, rats, and dogs. The data suggest that a particularly low level of agonist activity in the *in vitro* assays (e.g., such as that evident with mosapride) is required before reduced efficacy is observed *in vivo*, consistent with a large 5-HT_4 receptor reserve. TD-8954 had >2,000-fold selectivity for the human 5-$HT_{4(c)}$ receptor over all other targets tested. Notably, TD-8954, unlike tegaserod or cisapride, had no

affinity for 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, or 5-HT_3 receptors. Also, in contrast to cisapride, TD-8954 had no inhibitory effect at the hERG potassium channel.

The high 5-HT_4 receptor agonist potency of TD-8954 was also evident *in vivo*. TD-8954 (0.03–3 mg/kg s.c.) produced a statistically significant increase in colonic transit in conscious guinea pigs. Following TD-8954 administration and injection of carmine red dye into the proximal colon, the time for excretion of the first fecal pellet containing the marker was markedly reduced. The potent prokinetic activity of TD-8954 in this guinea pig model is in keeping with the proposed role of 5-HT and 5-HT_4 receptor activation in promoting GI motility (Muller-Lissner, 1987; Jin et al., 1999; Briejer et al., 2001b; Ji et al., 2003). The TD-8954-induced relaxation of the rat esophagus in this study is also consistent with agonist activity at the 5-HT_4 receptor (Triggle et al., 1988; Reeves et al., 1991). The technique of digital sonomicrometry provides a sensitive method to demonstrate the 5-HT_4 receptor agonist-mediated changes in rat esophageal tone (Armstrong et al., 2006). In conscious, fasted dogs, TD-8954 (10 and 30 μg/kg) produced a dose-dependent increase in contractility of the antrum, duodenum, and jejunum following oral administration. TD-8954 was clearly more potent than tegaserod throughout the canine GI tract, consistent with its superior 5-HT_4 agonist potency *in vitro* and oral pharmacokinetic properties (Theravance, Inc., data on file) in this species. Considering the published data from similar models (Gullikson et al., 1993; Briejer et al., 2001b; Tazawa et al., 2002), the findings of this study are entirely consistent with activation of 5-HT_4 receptors by TD-8954 in the canine GI tract.

Based on the positive preclinical pharmacodynamic effects of TD-8954, a single ascending dose study was performed in healthy human subjects. The data demonstrated that TD-8954 was associated with an increase in bowel movement frequency and a reduction in the time to first stool compared to placebo. The high potency noted in the preclinical assays was also noted clinically; a dose as low as 0.1 mg was associated with a prokinetic effect, and 0.5 mg produced a maximal response. The free plasma C_{max} and $AUC_{0–24}$ values for TD-8954 following a single dose of 0.5 mg to humans are 3.4 and 36.2 nM/h, respectively. Assuming that the free concentration of TD-8954 in plasma is equivalent to that at its site of action in the GI tract, mean and maximal 5-HT_4 receptor occupancies in the 24-h period following a 0.5 mg dose should be approximately 80 and 90%, respectively. This apparent requirement for a high level of receptor occupancy to achieve a maximal agonist response (Grimwood and Hartig, 2009) is consistent with the moderate IA of TD-8954 demonstrated preclinically in the *in vitro* assays.

CONCLUSION

TD-8954 is a potent and selective 5-HT_4 receptor agonist *in vitro* with robust *in vivo* GI activity in guinea pigs, rats, dogs, and notably humans. As a result of its demonstrated prokinetic activity in healthy human subjects, TD-8954 may have value in the treatment of patients with disorders of reduced GI motility.

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Interleukin-6 modulates colonic transepithelial ion transport in the stress-sensitive Wistar Kyoto rat

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Immunological challenge stimulates secretion of the pro-inflammatory cytokine interleukin (IL)-6, resulting in variety of biological responses. In the gastrointestinal tract, IL-6 modulates the excitability of submucosal neurons and stimulates secretion into the colonic lumen. When considered in the context of the functional bowel disorder, irritable bowel syndrome (IBS), where plasma levels of IL-6 are elevated, this may reflect an important molecular mechanism contributing to symptom flares, particularly in the diarrhea-predominant phenotype. In these studies, colonic ion transport, an indicator of absorption and secretion, was assessed in the stress-sensitive Wistar Kyoto (WKY) rat model of IBS. Mucosa-submucosal colonic preparations from WKY and control Sprague Dawley (SD) rats were mounted in Ussing chambers and the basal short circuit current (I_{SC}) was electrophysiologically recorded and compared between the strains. Exposure to IL-6 (1 nM) stimulated a secretory current of greater amplitude in WKY as compared to SD samples. Furthermore, the observed IL-6-mediated potentiation of secretory currents evoked by veratridine and capsaicin in SD rats was blunted in WKY rats. Exposure to IL-6 also stimulated an increase in transepithelial resistance in both SD and WKY colonic tissue. These studies demonstrate that the neuroexcitatory effects of IL-6 on submucosal plexi have functional consequences with alterations in both colonic secretory activity and permeability. The IL-6-induced increase in colonic secretory activity appears to neurally mediated. Thus, local increases in IL-6 levels and subsequent activation of enteric neurons may underlie alterations in absorpto-secretory function in the WKY model of IBS.

Keywords: capsaicin, veratridine, interleukin-6, irritable bowel syndrome, Wistar Kyoto, ussing chambers, electrophysiology

INTRODUCTION

The functional gastrointestinal (GI) disorder, irritable bowel syndrome (IBS) is characterized by episodic bouts of abdominal pain, bloating, and altered bowel habit including diarrhea, constipation, or both. Although the pathophysiological changes underlying IBS are still being investigated, stress has been attributed a role in the initiation, exacerbation, and persistence of IBS symptom flares (Lydiard et al., 1993; Spiller, 2004; Fitzgerald et al., 2008). Additionally, a growing body of data implicates local activation of gut immune factors in the development and persistence of IBS symptoms (Quigley, 2006; O'Malley et al., 2011c). Mucosal biopsies from IBS patients express higher levels of T-cells, lymphocytes, and mast cells (Chadwick et al., 2002) and plasma samples from IBS patients exhibit altered pro-inflammatory cytokine profiles (Macsharry et al., 2008). Indeed, interleukin (IL)-6 has reproducibly been found to be elevated in plasma samples from IBS patients (Dinan et al., 2006, 2008; Liebregts et al., 2007; Clarke et al., 2009; Scully et al., 2010; McKernan et al., 2011).

As yet, the mechanisms that link altered cytokine profiles with the development of functional GI disorders such as IBS are poorly understood. However, there is growing evidence that IBS patients have altered GI permeability (Camilleri et al., 2012) and most pro-inflammatory cytokines have the capacity to influence intestinal epithelial permeability (Al-Sadi et al., 2009). Indeed, the importance of cytokines in neuromuscular dysfunction in the inflamed intestine has been demonstrated (Hurst et al., 1993; Ruhl et al., 1994), thus, with particular relevance to post-infective IBS, immunomodulation of enteric neurons by cytokines released from within the GI milieu may be important in the persistence of IBS symptomatology (Ruhl et al., 2001).

Increased IL-6 synthesis following administration of a cholinesterase inhibitor has been correlated with increased abdominal pain and bloating (Dinan et al., 2008) and IL-6 can modulate mucosal ion transport and epithelial permeability, in addition to enhancing cholinergically mediated neurotransmission in rodents (Natale et al., 2003). Moreover, both IL-1β and IL-6 act as excitatory neuromodulators in a subset of myenteric neurons via presynaptic inhibition of acetylcholine release (Kelles et al., 2000). IL-6 has also been shown to suppress nicotinic and noradrenergic neurotransmission in guinea-pig submucosal neurons (Xia et al., 1999). Previous studies from our group have shown expression of IL-6 receptors on a subset of rat colonic submucosal

neurons. Exposure of these neurons to recombinant IL-6 results in increases in intracellular calcium [$(Ca^{2+})_i$] levels, which in turn results in increased colonic secretion (O'Malley et al., 2011b).

The Wistar Kyoto (WKY) rat has been validated (Greenwood-Van Meerveld et al., 2005; Gibney et al., 2010; O'Malley et al., 2010a) as an appropriate pre-clinical model of IBS, displaying increased visceral sensitivity to colorectal distension and enhanced colonic motility and fecal output following exposure to a psychological stressor (Gibney et al., 2010; O'Malley et al., 2010a). Colonic morphology and goblet cell expression is also altered in this rat (O'Malley et al., 2010a) and it exhibits evidence of altered cytokine expression. Although plasma levels of IL-6 are not different between WKY and SD rats (unpublished observation), mucosal scrapings from WKY colons contain higher levels of IL-6 and excised WKY colons secrete more IL-6 than control Sprague Dawley (SD) colons. Moreover, these secretions stimulate calcium responses of greater amplitude in naïve submucosal neurons than the SD secretions (O'Malley et al., 2011a). These observations are comparable to studies carried out in the maternal separation (MS) model of IBS where MS secretions stimulated a larger response in submucosal neurons than control non-separated colonic secretions. Moreover, recombinant IL-6 was shown to stimulate an increase in secretory activity (O'Malley et al., 2011b).

Evidence is mounting that IL-6 has neuromodulatory effects that contribute to altered GI function, however it is currently unclear whether these effects translate into functional changes. The current studies use Ussing chamber electrophysiology to investigate absorpto-secretory function in WKY rats following exposure to IL-6 and compare these effects to the SD control strain, which has normal GI function.

MATERIALS AND METHODS

ANIMALS

Sprague Dawley and WKY rats (200–250 g) purchased from Harlan, UK were group-housed 4–6/cage and maintained on a 12/12 h dark-light cycle (08.00–20.00). All experiments were in full accordance with the European Community Council Directive (86/609/EEC) and the local University College Cork animal ethical committee.

TISSUE PREPARATION

Distal segments of colon were excised from naïve SD or WKY rats and maintained in ice-cold bubbled (95% O_2/ 5% CO_2) Krebs saline consisting of (in mmol/L) NaCl, 117; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and D-glucose, 11. Longitudinal and circular muscle layers were removed to prepare a mucosa-submucosal preparation for Ussing chamber electrophysiology as previously described (O'Malley et al., 2011b).

USSING CHAMBER ELECTROPHYSIOLOGY

Mucosa-submucosal preparations of distal colon were mounted in Ussing chambers (exposed area of 0.12 cm^2) with 5 ml of Krebs solution (95% O_2/5% CO_2, 37°C) in the basolateral and luminal reservoirs. Tissues were voltage-clamped at 0 mV using an automatic voltage clamp (EVC 4000, World Precision Instruments, Sarasota, FL, USA) and the short circuit current (I_{SC}) required to maintain the 0 mV potential was monitored as a recording of the net active ion transport across the epithelium. Experiments were carried out simultaneously in all chambers and connected to a PC equipped with DataTrax II software (WPI). This software was used to measure the peak response and resistance was calculated using Ohms law.

Based on previous evaluations of the pro-secretory effects of IL-6 in SD tissues (O'Malley et al., 2011b), it was determined that the peak response to IL-6 occurred within 10 min of application. Thus, this time point was used to compare the effects of IL-6 on secretion in WKY versus SD rats. Following a period of stabilization (30–60 min) and prior to addition of any reagents, transepithelial resistance (TER) was measured. Another measurement of TER was taken at the end of the experiment (60–90 min later) and the difference (Δ resistance) between the two measurements was calculated.

STATISTICS

The data are represented as mean values ± the standard error of the mean (SEM). Students' *t*-test and one-way ANOVA with Neumann Keuls *post hoc* test were used to compare groups. Two-way ANOVA was used to analyze strain and treatment effects as independent variables. $p \leq 0.05$ was considered significant. All experiments were conducted in tissue taken from at least six different animals.

RESULTS

IL-6 EVOKES INCREASED COLONIC SECRETION AND TRANSEPITHELIAL RESISTANCE IN SD AND *WKY* RATS

Electrophysiological Ussing chamber studies were used to compare colonic transepithelial ion transfer and tissue resistance in the stress-sensitive WKY rats with the widely used SD comparator strain. Short circuit current (I_{SC}) was measured and used as an indicator of net ionic movement across the tissue. In control colonic sections, not treated with IL-6, basal I_{SC} was found to be lower in WKY ($n = 9$) colonic sections as compared to SDs ($n = 18$, $p < 0.05$, **Figure 1A**). However, TER, an indicator of colonic permeability, was not different between SD ($n = 17$) and WKY ($n = 8$, $p > 0.05$, **Figure 1B**) tissues.

Previous studies in SD tissues determined that a peak increase in colonic I_{SC} was observed at ∼10 min during a 30 min application of IL-6 (1 nM) to the serosal reservoir (O'Malley et al., 2011b). Therefore, all measurements were taken at the 10 min timepoint in both SD and WKY colons. Replicating our previous findings (O'Malley et al., 2011b), IL-6 evoked a small increase in I_{SC} in SD controls ($n = 23$). Application of IL-6 to WKY tissue samples ($n = 9$) also induced a secretory current. However, the amplitude of the secretory response was larger in WKY tissues than SDs ($p = 0.07$, **Figure 1C**).

The change in TER was calculated by comparing a measurement of TER at the beginning and end of each experiment (60–90 min). In control tissue, not exposed to IL-6, no change was observed in TER in either SD or WKY rats (**Figure 1D**). However, the continued presence of IL-6 stimulated a significant increase in TER in both SD ($12.6 \pm 5.3\ \Omega\ cm^2$, $n = 18$) and WKY ($27.8 \pm 11.7\ \Omega\ cm^2$, $n = 6$, $p > 0.05$, **Figure 1D**) tissues.

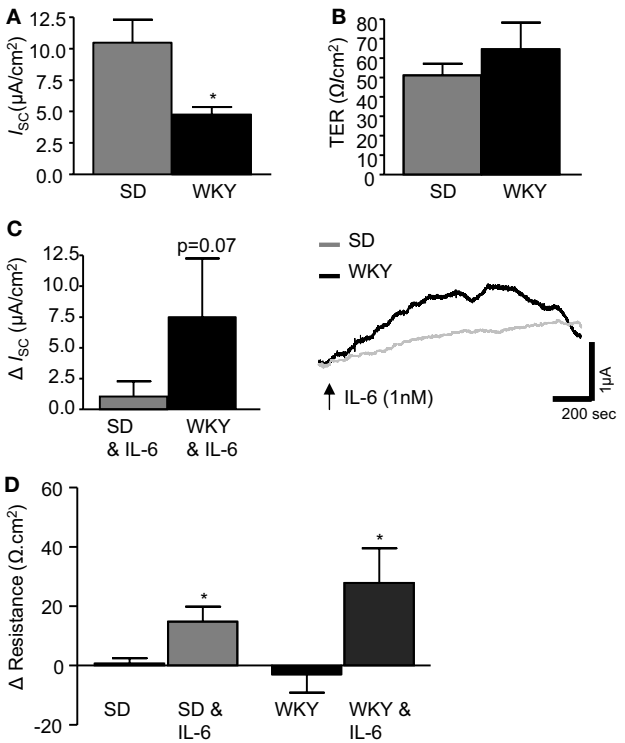

FIGURE 1 | IL-6 modulates transepithelial current and resistance. (A) The histograms illustrates basal short circuit current (I_{SC}, μA cm², SD: $n = 18$, WKY: $n = 9$) and **(B)** transepithelial resistance (TER, Ω cm², SD: $n = 17$, WKY: $n = 8$) in distal colon segments from naïve Sprague Dawley (SD) and Wistar Kyoto (WKY) rats. **(C)** The histograms and representative trace shows the effect of basolaterally applied IL-6 (1 nM, measured 10 min after application) on basal I_{SC} (SD: $n = 23$, WKY: $n = 9$). **(D)** This histogram illustrates the change (Δ) in tissue resistance over the course of the experiment (60–90 min, SD: $n = 18$, WKY: $n = 6$). Values = mean ± SEM. * Indicates $p < 0.05$.

Two-way ANOVA analysis demonstrated a clear effect of the IL-6 treatment on tissue resistance [$F_{(1, 44)} = 14.2$, $p < 0.001$] but there were not any strain differences or interaction between the factors despite a trend toward a larger effect in the WKY tissue.

IL-6 POTENTIATES VERATRIDINE-STIMULATED SECRETORY CURRENTS IN SD BUT NOT *WKY* RATS

To assess differences in the sensitivity of neuronally mediated colonic secretion between SD and WKY rats, the sodium channel activator, veratridine (10 μM) was applied to the basolateral reservoir. In non-stimulated colon samples no differences were noted in the peak response to veratridine between SD ($n = 19$) and WKY ($n = 10$, $p > 0.05$, **Figures 2A,B**) rats. In paired experiments we found that exposure to IL-6 (1 nM, 30 min) potentiates the secretory effects of veratridine in SD tissues when compared to control non-stimulated samples ($n = 15$, $p < 0.05$), which is consistent with previous findings (O'Malley et al., 2011b). However, in WKY tissues, IL-6 exposure had no effect on veratridine-induced currents ($n = 10$, $p > 0.05$, **Figures 2A,B**).

IL-6 POTENTIATES BETHANECHOL-STIMULATED SECRETORY CURRENTS

To investigate strain differences in cholinergically mediated currents, the muscarinic receptor agonist, bethanechol (10 μM) was added to the basolateral chamber. The agonist evoked a rapid biphasic current in control SD tissues ($n = 12$). The peak response in WKY rats was comparable ($n = 9$, $p > 0.05$, **Figures 3A,B**). The modulatory effects of IL-6 on the bethanechol response were subsequently examined in both tissues. As we have previously demonstrated (O'Malley et al., 2011b), IL-6 potentiated the evoked bethanechol response in SD tissues ($n = 12$, $p < 0.05$). IL-6 also enhanced bethanechol-evoked secretion in WKY tissues ($n = 9$, $p = 0.05$, **Figures 3A,B**). Two-way ANOVA analysis demonstrated a significant effect of IL-6 treatment [$F_{(1, 38)} = 5.4$, $p < 0.05$], but no differences in strain or any interaction between the factors was identified.

Muscarinic acetylcholine receptors can be present on both epithelial cells and neurons in the gut. To determine which cell type excited by bethanechol were sensitive to the effects of IL-6, control experiments in SD tissues were carried out in the presence of the sodium channel blocker, tetrodotoxin. In paired experiments ($n = 5$ each), I_{SC} in IL-6-treated (98.8 ± 22.4 μA/cm²) and control (104 ± 29.7 μA/cm²) tissues following administration bethanechol in the presence of tetrodotoxin (100 nM, 15 min) were similar ($p > 0.05$). These data indicate that the potentiating effect of IL-6 on the bethanechol response appears to be mediated through neuronal activation.

IL-6 POTENTIATES THE ANTI-SECRETORY PHASE OF CAPSAICIN-STIMULATED SECRETORY CURRENTS IN SD BUT NOT *WKY* RATS

Finally, the sensory nerve stimulant capsaicin was examined in SD and WKY tissues. In control tissues, addition of capsaicin (1 μM) caused a rapid biphasic response as previously described (Yarrow et al., 1991). The early secretory phase (phase I) was comparable in both SD ($n = 10$) and WKY samples ($n = 10$, $p > 0.05$, **Figures 4A,B**). In phase II, where capsaicin evokes an anti-secretory current, I_{SC} values in non-stimulated SD and WKY tissues were also comparable ($p > 0.05$, **Figures 4A,B**). Pretreatment with IL-6 (1 nM, 30 min) did not affect I_{SC} in phase I in either SD ($n = 11$) or WKY ($n = 7$) colons such that they remained comparable ($p > 0.05$, **Figures 4C,D**). However, IL-6 potentiated the capsaicin-evoked anti-secretory current in SD rats but not WKY rats such that a significant difference was apparent between the strains ($p < 0.05$, **Figures 4C,D**). Using two-way ANOVA, a difference in strain is approaching significance in the secretory phase [$F_{(1, 36)} = 3.7$, $p = 0.06$] with no effect of IL-6 and no interaction. In the anti-secretory phase, a strain difference is also apparent [$F_{(1, 31)} = 7.7$, $p < 0.01$] but there is no effect of the treatment itself and no interaction between the factors.

DISCUSSION

This series of electrophysiological studies builds on previous work from our group in which we demonstrated the capacity of IL-6 to directly stimulate a secretory current and decrease membrane permeability in colons from SD rats (O'Malley et al., 2011b). These

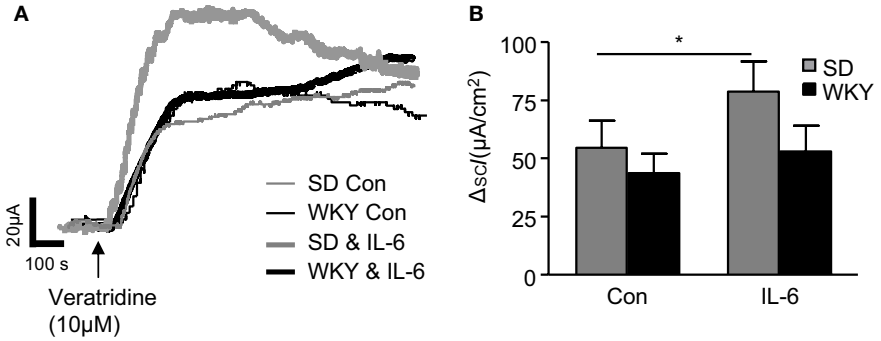

FIGURE 2 | Veratridine-induced currents in Sprague Dawley (SD) and Wistar Kyoto (WKY) colons. (A) Representative traces illustrate the secretory current (I_{SC}) induced by exposure to veratridine (10 μM) in non-stimulated (con) SD (thin gray line, $n = 7$) and WKY (thin black line, $n = 9$) colonic sections and IL-6-stimulated SD (thick gray line, $n = 7$) and WKY (thick black line, $n = 9$) colonic sections. **(B)** The histogram shows the pooled data. Values = mean ± SEM. * Indicates $p < 0.05$.

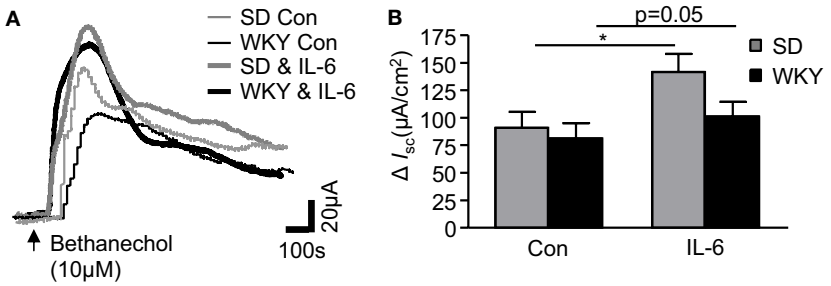

FIGURE 3 | Cholinergically driven currents in Sprague Dawley (SD) and Wistar Kyoto (WKY) rats are altered by IL-6 exposure. (A) The representative traces show bethanechol-induced increases in short circuit current (I_{SC}) in control (Con, thin gray line, $n = 12$) and IL-6-(1 nM) stimulated (IL-6, thick gray line, $n = 12$) SD colonic tissue. WKY traces are also included for control (thin black line, $n = 9$) and Il-6-stimulated (thick black line, $n = 9$) colonic tissues. **(B)** Histograms show the pooled data. Values = mean ± SEM. * Indicates $p < 0.05$.

studies have investigated the effects of IL-6 on colonic secretory and permeability parameters in WKY rats which exhibit several markers of GI dysfunction and have been used as an animal model of IBS. By comparing changes in colonic I_{SC} and TER between the strains, we have demonstrated that IL-6-induced changes in secretory activity and colonic permeability differ in WKY rats, thereby revealing a possible immune-mediated mechanism which could contribute to the dysfunctional bowel activity described in this rat (O'Malley et al., 2010a).

The WKY rat has been well characterized as a suitable preclinical model of IBS (Gunter et al., 2000; Gosselin et al., 2009; Gibney et al., 2010; O'Malley et al., 2010a). The GI dysfunction exhibited by the WKY rat includes an innate hypersensitivity to visceral pain stimuli such as that induced by colorectal distension (Gibney et al., 2010) and altered defecation patterns, particularly when exposed to stress (O'Malley et al., 2010a). Expression of the stress-related peptide, corticotropin-releasing factor (CRF) receptors are altered both in the colon (O'Malley et al., 2010b) and centrally (O'Malley et al., 2011d) in this strain. Given that amygdalar CRFR1 activation can contribute to visceral hypersensitivity in WKY rats (Johnson et al., 2012) these changes may have direct effects on the IBS-like symptom profile. Moreover, colonic toll-like receptor expression is also altered (McKernan et al., 2009) in this strain.

With regard to their colonic secretory parameters, WKY rats appear to display a pro-absorptive phenotype, which is thought to be reliant on decreased epithelial cholinergic sensitivity (Hyland et al., 2008). Under resting conditions we also observed this pro-absorptive phenotype as indicated by lower I_{SC} in the WKY rat as compared to SD. Interestingly, this relationship is reversed in the presence of IL-6, with a larger current being evoked from the WKY colonic tissues. The mechanisms underlying this secretory event are as yet unclear, however we have previously determined that submucosal neurons prepared from WKY colons display increased sensitivity to the neuroexcitatory effects of IL-6 (O'Malley et al., 2011a). As submucosal neurons have been attributed a primary role in regulating mucosal secretion and absorption, IL-6-mediated neural activation of colonic secretion may override the pro-absorptive phenotype regulated by epithelial cholinergic activity at rest (Hyland et al., 2008). This change in absorpto-secretory function would be consistent with the increase in mucus secretion and stress-induced fecal output evident in these animals (O'Malley et al., 2010a).

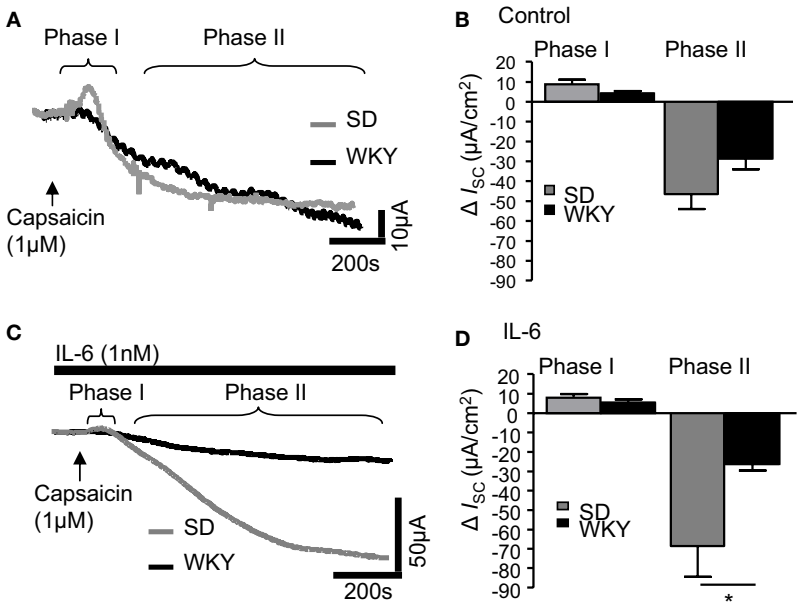

FIGURE 4 | IL-6 alters capsaicin-induced currents in Sprague Dawley (SD) and Wistar Kyoto (WKY) rat colons. (A) The representative traces illustrate the secretory (phase I) and anti-secretory (phase II) responses to capsaicin (1 μM) in control SD (gray line, $n = 10$) and WKY (black line, $n = 10$) distal colons. **(B)** The histogram illustrates the pooled data for changes in current (I_{SC}). **(C)** The traces are representative of capsaicin-evoked responses following exposure to IL-6 (1 nM) in SD ($n = 11$) and WKY ($n = 7$). **(D)** The pooled data are plotted in a histogram. Values = mean ± SEM. * indicates $p < 0.05$.

To further assess the importance of neurally evoked changes in secretion following application of IL-6 to the basolateral side of the tissue, pharmacological stimulators were applied as previously described (Julio-Pieper et al., 2010). Veratridine evokes neuronally mediated secretory currents by depolarizing intrinsic neurons via increased permeability through voltage-gated Na^+ channels. This secretory response is caused by a net increase in Cl^- secretion (Sheldon et al., 1990). Under control conditions the veratridine-evoked responses were of similar amplitude in both SD and WKY colonic tissues and unlike SD tissue (O'Malley et al., 2011b), IL-6 had no effect on currents evoked in WKY tissue. Veratridine has been shown to stimulate the release of enteric neurotransmitters such as substance P, VIP, (Belai and Burnstock, 1988) and acetylcholine (Yau et al., 1986). However, further investigation will be required to determine the neurotransmitters underlying the IL-6-induced modulation of veratridine-stimulated ion secretion in SD rats. Evidently, this potentiating mechanism is not active in WKY rats.

The contribution of the cholinergic system to IL-6 secretion has been demonstrated in IBS patients (Dinan et al., 2008). Indeed, activation of secretomotor neurons may underlie neurogenic secretory diarrhea (Liebregts et al., 2007). In SD rats we provided evidence that IL-6 exposure potentiated currents induced by the muscarinic receptor agonist, bethanechol that were sensitive to the sodium channel blocker, tetrodotoxin. This effect was intact in WKY tissues as IL-6 similarly enhanced the bethanechol current.

Finally, currents evoked by activating transient receptor potential cation channels (TRPV1) were examined by exposing the tissue to capsaicin. Capsaicin stimulates visceral afferent neurons in the GI tract causing the subsequent release of nerve terminal neuropeptides which in turn, stimulate mucosal electrolyte transport and fluid secretion (Holzer et al., 1990; Vanner and MacNaughton, 1995), motility (Takaki and Nakayama, 1989), mucus secretion (Moore et al., 1996), and mucosal blood flow (Akiba et al., 1999) in addition to playing a protective role in maintaining mucosal integrity (Evangelista and Meli, 1989; Esplugues et al., 1990; Holzer et al., 1990). Indeed, TRPV1 is increased in inflammatory diseases of the GI tract (Yiangou et al., 2001) and in patients with rectal hypersensitivity (Chan et al., 2003). Application of capsaicin-evoked a large biphasic response in SD tissues which was comprised of an initial secretory phase followed by a larger more sustained anti-secretory phase consistent with previous studies (Yarrow et al., 1991). Interestingly, responses evoked by capsaicin in WKY tissues did not have such distinct phases. Rather than a small secretory response, there appeared to be a delay prior to the longer-lasting anti-secretory event, possibly indicating a balance between the two opposing mechanisms. Indeed, the differences between the strains came into sharper focus following addition of IL-6, which potentiated the anti-secretory phase in SD tissues only. Thus, in SD GI tissue, IL-6 exposure enhances the anti-secretory activity of afferent nerves whereas WKY rats have lost this regulatory response to IL-6 which could underlie the overall increased secretory activity in this strain. Moreover, the extrinsic, afferent innervation of the GI tract conveys information to the CNS that gives rise to the sensations of pain and discomfort. Thus, the insensitivity of WKY rats to IL-6-evoked potentiation of

the capsaicin anti-secretory response may also be important in the increased sensitivity to visceral pain (Gibney et al., 2010).

Although the mechanisms of this effect require further elucidation, it is feasible that low-grade inflammation in the WKY rat may result in constant stimulation of capsaicin-sensitive nerve terminals causing neurotransmitter depletion or that IL-6 directly inhibits neurotransmitter release in these neurons. Alternatively, altered sensitivity to stress may contribute to these observations. CRF1 receptor antagonists alleviate visceral sensitivity in the WKY rat (Greenwood-Van Meerveld et al., 2005) but evidence exists for crosstalk between IL-6 and CRF (O'Malley et al., 2011c). Therefore, alterations in stress-induced expression of CRF receptors (O'Malley et al., 2010b; O'Malley et al., 2011d), may be linked to the increase in IL-6 sensitivity observed in the WKY rats.

Tissue resistance was also measured in these animals as a marker of membrane permeability and was found to be similar between strains. Interestingly, IL-6 stimulated an increase in TER in both strains. Changes in permeability can occur as a result of alterations in the expression of tight junctions (Chen et al., 2010; Martinez et al., 2012), dysbiosis of microbiota (Fukuda et al., 2011) or through the increased presence of pro-inflammatory cytokines (Arrieta et al., 2006). Furthermore, stress can contribute to changes in permeability as has previously been demonstrated in WKY rats (Saunders et al., 2002). Over the 90 min duration of these recordings, one possibility might be an increase in mucus secretion evoked by IL-6, which could influence membrane permeability. Indeed, we have previously observed increased expression of goblet cell number in the WKY strain (O'Malley et al., 2010a). We demonstrated that acute application of IL-6 increases TER, appearing to help maintain the integrity of the epithelial cell layer in both SD and WKY rats. This is consistent with one previous study (Wang et al., 2007). On the other hand, chronic exposure to elevated IL-6 levels may result in increased gut permeability (Hiribarren et al., 1993; Natale et al., 2003). As mucosal levels of the pro-inflammatory cytokine, IL-6 are elevated in WKY colons (O'Malley et al., 2011a), one might have expected that continuous exposure to IL-6 would have resulted in increased colonic permeability in this strain. However, TER at rest is comparable between SD and WKY rats. This may indicate that there are increased numbers of IL-6-containing cells in the mucosa of WKY rats but not necessarily increased levels of IL-6 secretion.

At a functional level, these studies have demonstrated that IL-6-evoked secretion is enhanced in WKY colons and this is likely to be due to increased sensitivity of submucosal neurons to the pro-inflammatory cytokine. We have provided evidence that inhibition of the potentiating effect of IL-6 on capsaicin-evoked anti-secretory currents is a likely contributor to the changes in colonic secretion. These data further demonstrate the neuromodulatory effects of IL-6 in colonic function and provide mechanistic evidence of how elevations in systemic IL-6 in IBS patients could be a contributory factor in the pathophysiology of the disorder.

CONTRIBUTION OF EACH AUTHOR

Dervla O'Malley: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. Timothy G. Dinan: critical revision of the manuscript for important intellectual content; study supervision. John F. Cryan: critical revision of the manuscript for important intellectual content; study supervision.

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Small intestinal cannabinoid receptor changes following a single colonic insult with oil of mustard in mice

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Cannabinoids are known to be clinically beneficial for control of appetite disorders and nausea/vomiting, with emerging data that they can impact other GI disorders, such as inflammation. Post-inflammatory irritable bowel syndrome (PI-IBS) is a condition of perturbed intestinal function that occurs subsequent to earlier periods of intestinal inflammation. Cannabinoid 1 receptor (CB1R) and CB2R alterations in GI inflammation have been demonstrated in both animal models and clinically, but their continuing role in the post-inflammatory period has only been implicated to date. Therefore, to provide direct evidence for CBR involvement in altered GI functions in the absence of overt inflammation, we used a model of enhanced upper GI transit that persists for up to 4 weeks after a single insult by intracolonic 0.5% oil of mustard (OM) in mice. In mice administered OM, CB1R immunostaining in the myenteric plexus was reduced at day 7, when colonic inflammation is subsiding, and then increased at 28 days, compared to tissue from age-matched vehicle-treated mice. In the lamina propria CB2R immunostaining density was also increased at day 28. In mice tested 28 day after OM, either a CB1R-selective agonist, ACEA (1 and 3 mg/kg, s.c.) or a CB2R-selective agonist, JWH-133 (3 and 10 mg/kg, s.c.) reduced the enhanced small intestinal transit in a dose-related manner. Doses of ACEA and JWH-133 (1 mg/kg), alone or combined, reduced small intestinal transit of OM-treated mice to a greater extent than control mice. Thus, in this post-colonic inflammation model, both CBR subtypes are up-regulated and there is increased efficacy of both CB1R and CB2R agonists. We conclude that CBR remodeling occurs not only during GI inflammation but continues during the recovery phase. Thus, either CB1R- or CB2-selective agonists could be efficacious for modulating GI motility in individuals experiencing diarrhea-predominant PI-IBS.

Keywords: colitis, pathophysiology, motility, receptor up-regulation, endocannabinoids, inflammation, cannabinoid receptors, enteric nervous system

INTRODUCTION

Debate about the medical benefits of cannabinoids in a wide array of diseases is based on anecdotal evidence from patients who have used medical marijuana, experimental animal models, and an increasing number of clinical studies. In gastrointestinal (GI) diseases there is overwhelming evidence that cannabinoids increase appetite and improve weight gain in patients during cancer chemotherapy or those suffering from AIDS wasting syndrome. However, as reviewed recently (Izzo and Sharkey, 2010), cannabinoids in the gut modulate gastric secretion and gastroprotection, GI motility, ion transport, visceral sensation, intestinal inflammation, and cell proliferation. In addition, evidence is emerging that exogenous and endogenous cannabinoids, have an important role in pathophysiology, such as in GI inflammation (D'Argenio et al., 2007; Izzo and Camilleri, 2009).

The primarily active constituent of marijuana, Δ^9-tetrahydrocannabinol, acts on cannabinoid 1 (CB1R) and 2 (CB2R) receptors, which are G-protein coupled receptors (Glass and Northup, 1999; Bosier et al., 2010). There is a differential localization and distribution of CBR, where CB1R is located primarily on neuronal tissue and CB2R is located on peripheral blood leukocytes. In the GI tract, CB1R highly expressed in the submucosal and myenteric plexus neurons, as well as visceral afferent nerves (Kulkarni-Narla and Brown, 2000; Coutts et al., 2002; Pertwee and Ross, 2002; Casu et al., 2003). Immune cells in the intestinal lamina propria express CB2R, including plasma cells and activated macrophages (Wright et al., 2005).

In normal GI motility states, there is substantial experimental evidence that CB1R inhibits contractility and GI motility at multiple levels, reviewed in (Izzo and Sharkey, 2010). An enhanced role of CB1R has been reported in a number of rodent GI inflammatory models. For example, in mice with colitis induced by intracolonic dinitrobenzene sulfonic acid (Sibaev et al., 2006) and with ileitis-induced small intestinal croton oil (Izzo et al., 2001). Compared to CB1R, there is little evidence that CBR2 is involved in the control of normal GI motility, although CBR2 tonic inhibitory activity via endogenous cannabinoids has been reported in rat stomach (Storr et al., 2002), but not mouse stomach (Mule et al., 2007). However, in inflamed mice and rats models, there is now clearly an inhibitory role of CB2R in GI motility. For example, CB2R up-regulation and inhibition of GI transit have been reported in models of inflammation such as, croton oil-induced diarrhea (Izzo et al., 2000), trinitrobenzene sulfonic acid (TNBS)-induced colitis (Storr et al., 2009) and lipopolysaccharide (LPS)-induced GI transit *in vivo* and in isolated segments of ileum (Mathison et al., 2004; Duncan et al., 2008). Interestingly, in LPS-treated rat tissue, CB2R is localized in

enteric neurons (Duncan et al., 2008) and in inflammatory bowel disease patients (Wright et al., 2005) CB2R is expressed on intestinal epithelium. Both of these are cell types that do not typically express CB2R in normal tissue and suggests extensive CBR remodeling in GI inflammation in diseased human tissue and experimental animal models.

There is also human genetic evidence of CBR in GI-related disorders. A frequent silent mutation is a common polymorphism (1359 G/A) of the CB1R gene (CNR1) in Caucasians (Gadzicki et al., 1999). This has been associated with metabolic syndrome in a Chinese population (Hu and Feng, 2010) and with irritable bowel syndrome (IBS) in Korean patients (Park et al., 2010). Although endocannabinoids and CBR have been linked to the underlying physiological processes of IBS (Storr et al., 2008), the association to the CNR1 gene is the first evidence linking CBR to IBS, to our knowledge. According to the Rome II and III criteria (Drossman, 2007), IBS is a spectrum of disorders characterized by abdominal discomfort and increased pain perception associated with altered bowel habits. Animal models of IBS have focused on the pain component and demonstrated that CBR agonists ameliorate visceral hyperalgesia during colorectal distention (Sanson et al., 2006; Brusberg et al., 2009) with increased efficacy after intracolonic TNBS (Kikuchi et al., 2008). However, there is little direct experimental evidence suggesting that cannabinoids may be beneficial for the dysmotility of IBS.

We have characterized a mouse model of accelerated transit (Kimball et al., 2005) that persists after the resolution of colonic inflammation (Kimball et al., 2006). In this mouse model, intracolonic oil of mustard (OM) produces an acute (3 day) colitis (Kimball et al., 2006) that is associated with an early increased mRNA expression of soluble inflammatory mediators and cytokine levels in the colon (Kimball et al., 2007). During the inflammatory stage, CB1R mRNA is rapidly up-regulated by 6 h but then down-regulated 2–3 days after OM (Kimball et al., 2007). After these inflammatory changes have resolved, mice exhibit increased small bowel transit 4 weeks after the initial insult (Kimball et al., 2005). Since this model may provide insight into CBR in the underlying pathophysiology of post-inflammatory IBS (PI-IBS), and we do not know the role of cannabinoids at this stage in the model, we used it to examine the time course of expression of CB1R and CB2R immunostaining in mouse small bowel following OM colitis induction, as well as compare the effects of cannabinoid agonists on intestinal transit after resolution of colonic inflammation.

MATERIALS AND METHODS

Male CD-1 mice (Charles River Laboratories, Kingston, NC, USA), 10- to 12-weeks old, were used throughout these studies. All treatments were carried out in accordance with the Federal Animal Welfare Act and with methods approved by the Institutional Animal Care and Use Committee of Johnson and Johnson Pharmaceutical Research and Development, LLC.

INDUCTION OF COLITIS

Freshly opened OM (95 or 98% pure allyl isothiocyanate, Sigma-Aldrich St. Louis, MO, USA), was used in each experiment. Mice ($N = 12–15$ per drug or vehicle treatment group) were briefly anesthetized with ketamine/xylasine (Sigma, St. Louis, MO, USA), and held vertically in a head down position so that 50 µl of a solution of 0.5% OM in 30% ethanol could be administered intracolonically. The OM administration occurred to a depth of 4 cm via a syringe equipped with a ball-tipped 22 G needle. The mice were allowed to recover from anesthesia under a warming light, and then were maintained with normal feed and water for 28 days at which time they were tested for small intestinal transit rate.

DRUG TREATMENT

Cannabinoid agonists arachadonyl chloroethyl amide (ACEA), and JWH-133 (Tocris-Cookson, St. Louis, MO, USA) were administered subcutaneously, 30 min prior to carmine dye (cochineal powder; Sigma-Aldrich St. Louis, MO, USA) administration. In all experiments, CBR agonists were dissolved in a vehicle consisting of 5% Tween 80, 5% ethanol, and 5% dextrose in water. Drug-treated groups were compared against an OM control, which was administered vehicle only. The doses of agonists (ACEA and JWH-133) used in the present study were similar to that shown to be selectively acting at CB1R and CB2R in mice (Arevalo-Martin et al., 2003; Mathison et al., 2004).

UPPER GI TRANSIT

Mice received 250 µl of a 6% solution of carmine dye in 0.5% methylcellulose (w/v) by oral gavage. After 20 min, the mice were rapidly euthanized by cervical dislocation according to accepted procedures. The large and small intestines were resected, starting with the distal colon first, and working toward the pylorus until the entire small intestine, cecum, and colon were removed intact. Excess connective tissue was trimmed, the resected bowel was arranged lengthwise without being stretched, and the length of the entire small intestine was recorded. Percent transit was determined to be the distance that the carmine dye front traveled and then converted into a percentage of the length of the entire small intestine. For some comparisons values were normalized to their own respective non-drug-treated (i.e., vehicle) control group whose transit rate was set as 100% of control. Transit was compared in OM-treated mice vehicle-treated and naïve age-matched controls. Mice were not fasted prior to gavage and all were feeding normally at the start of the experiment. In pilot experiments with this model we determined that food deprivation has no effect on transit measured by carmine red. Potential inter-animal variability in the severity of colitis and small intestinal transit induced by OM were inconsequential for $N = 12–15$ per experimental treatment group, as previously reported (Kimball et al., 2005). When results were combined from multiple experiments each experimental treatment group contained data from multiples of $N = 12–15$. Throughout the text small intestinal transit is used interchangeably with upper GI transit though it is recognized that both gastric emptying and small bowel transit contribute to the distance traveled down the small intestine.

IMMUNOHISTOCHEMISTRY

CB1R- and CB2R immunostaining was performed in 5 cm-long jejunoileal tissues, starting 5 cm proximal of the cecum. Tissues were collected from untreated mice, and from mice 7 and 28 days after OM-treatment. The tissues were formalin-fixed, and paraffin-embedded prior to immunohistochemical treatments. Briefly, 5 µm

tissue sections were mounted on microscope slides and then routinely dewaxed and rehydrated. After a 5 min exposure in a microwave in Target Buffer (Dako, Carpinteria, CA, USA), slides were subsequently treated in 3% H_2O_2 for 5 min, followed by treatment with avidin–biotin reagent (Serotec, Raleigh, NC, USA) to eliminate endogenous peroxidase and biotin activity, respectively. The slides were then routinely processed for immunohistochemistry. All incubations were performed at room temperature for 30 min. After a 10 min blocking step with normal goat serum, the tissues were incubated with the primary antibodies. Rabbit anti-CB1R (1:20 dilution) and rabbit anti-CB2R (1:2 dilution; Chemicon, Temecula, CA, USA) polyclonal antibodies were used to identify CBR-immunoreactivity in tissue sections. Biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA), avidin–horseradish peroxidase (AbD Serotec, Raleigh, NC, USA) and 3,3′diaminobenzidine (Biomeda Foster City, CA, USA) used to detect immunostaining. Staining controls lacked primary antibodies, but included all other reagents. Since both primary antibodies were rabbit polyclonals the same staining controls could serve for both CB1R and CB2R, as reported in previous studies using these antibodies (Kimball et al., 2006).

Semi-quantitative analysis of CB1R- and CB2R staining was performed by a histologist who was blinded as to the treatment. For CB1R immunostaining myenteric ganglia were counted in the entire section ($N = 8$ sections) using a 40X objective, and assigned as weak-negative, moderate or intense staining. CB2R + ve cells in lamina propria were quantified as number of villi from eight high-powered fields showing either fewer or greater than five positively stained cells in the LP.

STATISTICS

Experimental groups were analyzed for significance of differences between the means of treatment groups and control groups by ANOVA with Bonferroni's post-test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A P-value < 0.05 was considered statistically significant.

RESULTS

CB1R and CB2R immunostaining was analyzed in jejunoileal tissues from normal mice and OM-treated mice taken 7 and 28 days after OM colitis induction. These times were based on our previous work, where at day 7 mice showed no alterations in intestinal transit with diminished inflammation in the large intestine and at day 28 mice had no inflammation but increased transit (Kimball et al., 2005). Representative photomicrographic images illustrate the marked changes observed in CB1R and CB2R immunostaining noted in myenteric ganglia and lamina propria (**Figure 1**). CB1R immunostaining in myenteric ganglia was moderately intense in untreated mouse tissue (**Figure 1A**) compared with little to no staining observed in tissues 7 days after OM administration (**Figure 1C**). However, by day 28 post-OM treatment (**Figure 1E**), more intense CB1R immunostaining was visualized in myenteric ganglia neurons, which appeared to exceed that seen in normal tissues. The myenteric ganglia also appeared to be larger in size, though size of ganglia was not quantified. CB2R immunostaining was most notably changed in the lamina propria. Lamina propria CB2R + ve cells were low to absent in untreated (**Figure 1B**) and day 7 post-OM administration tissues (**Figure 1D**), but were strongly positive and abundant 28 days post-OM administration (**Figure 1F**). Positively stained cells in the lamina propria appeared to be mononuclear (**Figure 1F** inset), but further work would be required to identify specific cell type.

Semi-quantitative analysis of the changes in CB1R- and CB2R staining, by someone blinded as to treatment, illustrate these time dependent observations (**Figure 2**). Compared to untreated tissue, at day 7 post-OM tissues contained significantly fewer ganglia with moderate and intense CB1R + ve neuronal staining, whereas at day 28 post-OM these were significantly increased (**Figure 2A**). The number of villi with > 5 CB2R + ve cells in lamina propria cells was significantly increased at 28 days post-colitis (**Figure 2B**).

In a separate group of animals small intestinal transit studies were performed with CBR agonists administered to mice 28 days after intracolonic vehicle (30% ethanol) or OM. As expected from our earlier study (Kimball et al., 2005), normal untreated mice upper GI transit (56 ± 2 and 56 ± 2% of small bowel length) was less than in mice 28 days after treatment with intracolonic OM (71 ± 2 and 68 ± 2% of small bowel length). Administration of the CB1R-selective agonist ACEA (1 and 3 mg/kg) to OM-treated mice effectively reduced the enhanced small intestinal transit to 59 ± 2 and 58 ± 4%, respectively, resulting in upper GI transit similar to that in normal mice (**Figure 3A**). Administration of the CB2R-selective agonist JWH-133 (3 and 10 mg/kg, s.c.) to OM-treated mice also reduced small intestinal transit to 49 ± 3 and 53 ± 4%, respectively. In this case, upper GI transit after CB2R agonist (3 mg/kg) administration in OM-treated mice appeared lower than that of normal mice but this was not reproduced at the highest dose (10 mg/kg; **Figure 3B**).

In **Figures 3C,D**, all values for upper GI transit in CBR-treated mice were normalized to their respective CBR vehicle-treated control group, to enable comparison of CBR agonists in both intracolonic OM-and vehicle-treated mice. This illustrates a dose-related inhibition of accelerated transit in OM-treated mice by both CBR agonists. In intracolonic vehicle-treated control mice there was no effect of increasing doses of ACEA (**Figure 3C**). There was a trend for JWH-133 to increase upper GI transit in intracolonic vehicle-treated group (**Figure 3D**); however, the apparent increases did not attain the acceptable level of statistical significance.

Small intestinal transit is stable in normal (untreated) mice and those administered intracolonic vehicle (30% ethanol) after 28 days (**Figure 4**). Administration of the CB1R-selective agonist, ACEA, or the CB2R-selective agonist, JWH-133, individually at 1 mg/kg inhibited transit in OM-treated mice, but had no significant effect in normal mice (**Figure 4**). When the doses were combined they reduced the distance the marker traveled down the intestine to 65 ± 6 and 49 ± 7% of total length in normal and OM-treated mice, respectively ($P < 0.001$ compared to their respective controls). Although there was a trend to greater inhibition by the combination of CBR agonist in OM-treated mice this was not different than the same treatment in normal mice.

DISCUSSION

In the present study we have made the novel observations that CB1R in myenteric neurons and CB2R immunostaining in lamina propria are altered in the small intestine of mice up to 4 weeks after intracolonic OM. These changes are observed not only in

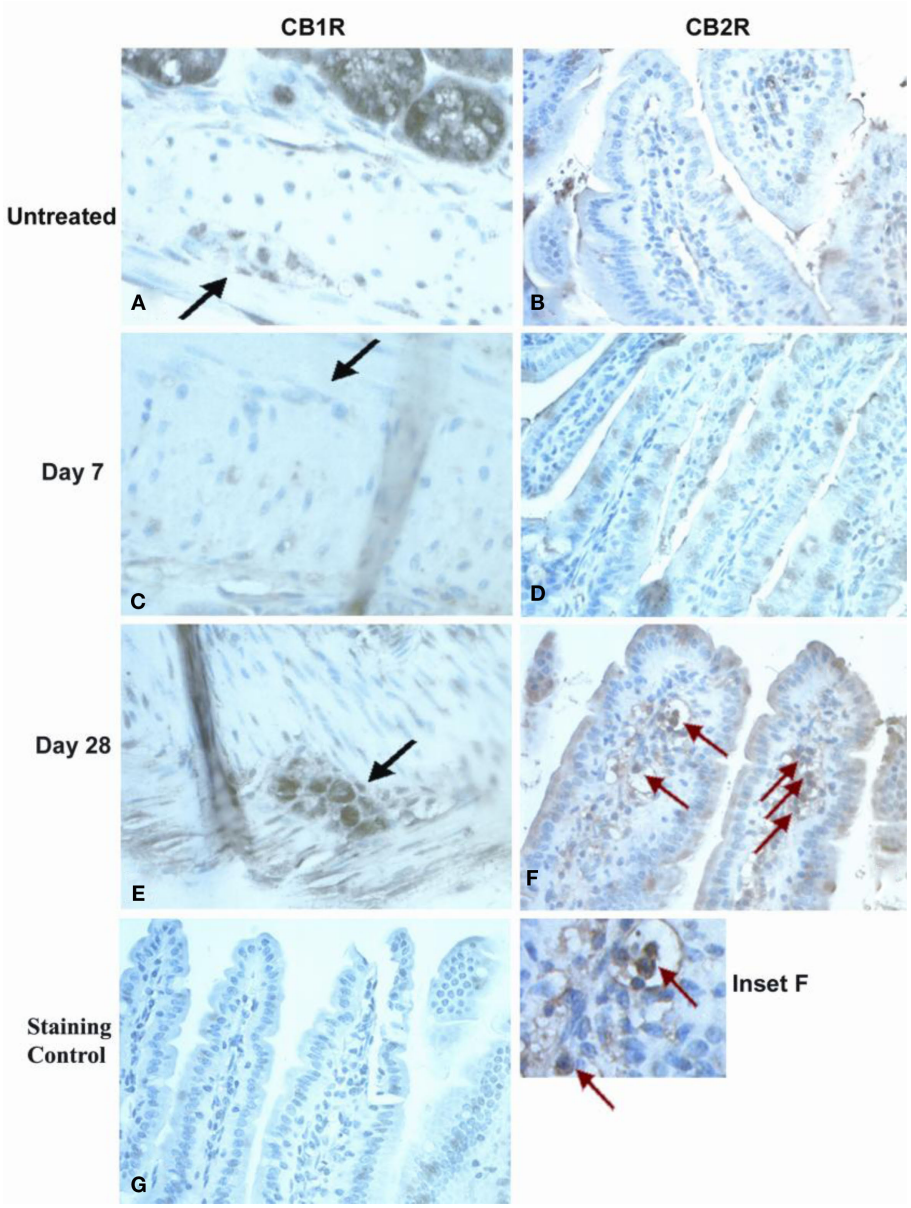

FIGURE 1 | Immunohistochemical staining of small intestine tissues for CBR expression. Neuronal staining in myenteric plexus in normal controls **(A)**, day 7 post-OM **(C)** and day 28 post-OM **(E)**. Black arrows indicate myenteric ganglia. Moderate staining was observed in normal controls, deeply reduced at day 7, and restored to higher levels of expression at day 28. Immunohistochemical staining of small intestine tissues for CB2R expression is shown for lamina propria (LP) cells. Red arrows indicate stained cells in LP. LP cell staining is shown for normal controls **(B)**, day 7 post-OM **(D)** and day 28 post-OM (F). Dense CB2R + staining was observed in numerous cells in day 28 tissues, but not in untreated or in day 7 tissues. Inset **(F)** was enlarged to highlight CB2R + LP cells with monocytic appearance. Non-specific staining in tissue where primary antiserum was omitted **(G)**.

the immediate post-colitis period (7 days) but also after all overt signs of inflammation have resolved (4 weeks) in mice that exhibit increased upper GI transit, which is a symptom consistent with clinical pathophysiology of PI-IBS (Kimball et al., 2005). Our results also demonstrate that both CB1R and CB2R agonists normalize upper GI transit in the OM-treated model and that the effect of these agonists is enhanced in the pathophysiological model compared to normal mice. The differences in efficacy by CBR agonists in the post-inflammatory compared to normal are consistent with cooperativity between CB1R and CB2R and amplification of signaling in a perturbed post-inflammatory system. Altogether these data suggest that altered CBR responsiveness is maintained long after an initial inflammatory period, and suggest a role in the underlying pathophysiology of PI-IBS.

We have previously characterized this model during acute inflammation (Kimball et al., 2006) and used it to determine the effects of CBR agonists in colitis (Kimball et al., 2006). We selected this model to study the CBR changes in post-inflammatory condi-

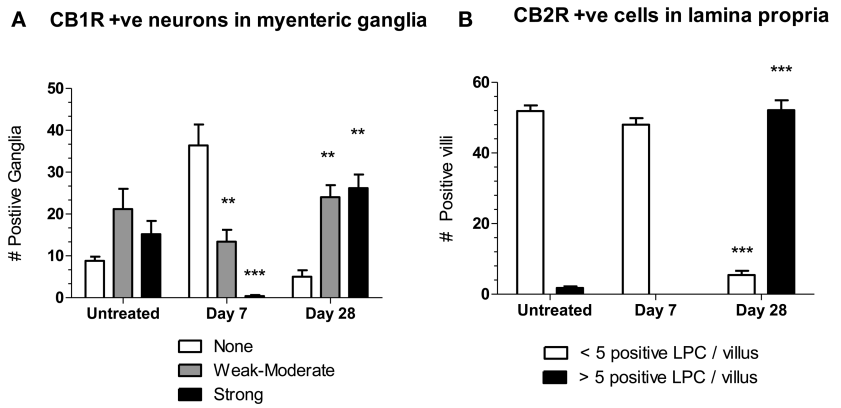

FIGURE 2 | (A) The number of myenteric ganglia in jejunoileal tissue with moderate (gray bars) and intense (black bars) CB1R-stained neurons are decreased at day 7 and increased at day 28 compared to untreated tissue. Changes in the number of ganglia in which CB1R staining was absent (clear bars) were not significantly different in post-OM compared to untreated tissue. **(B)** The number of villi with < 5 CB2R + ve cells (clear bars) compared to > 5 CB2R + ve cells (black bars) in lamina. Differences two-way ANOVA and by Bonferroni's post-test as a function of time were statistically significant and are shown as $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to corresponding column for untreated.

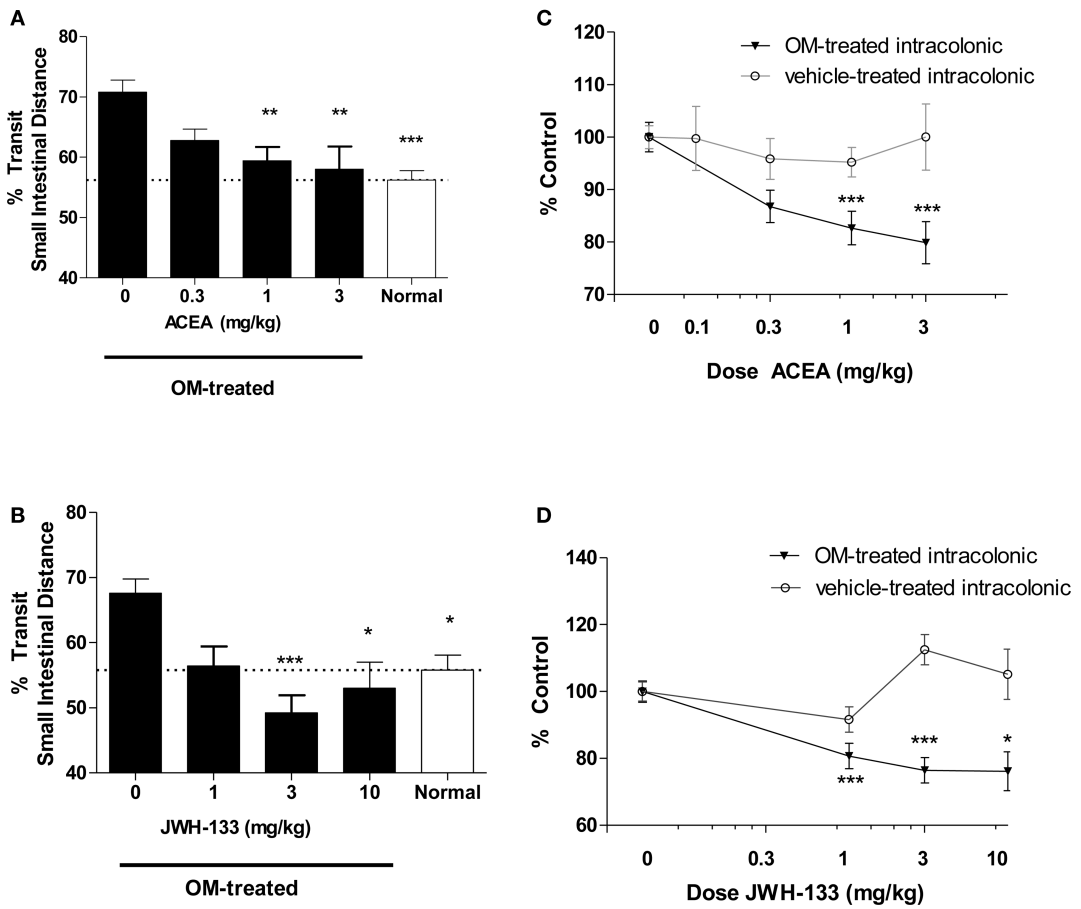

FIGURE 3 | Effects on small intestinal transit of a range of doses of CB1R (A,C) and CB2R agonists **(B,D)** in mice 28 days after intracolonic 0.5% OM, vehicle (30% ethanol) and normal (untreated) age-matched mice. In **(A)** and **(B)** the % small intestinal distance traveled by carmine red is illustrated, with statistical comparisons to intracolonic vehicle-treated mice (i.e., first column in **A**, **B**). Effective doses (1 and 3 mg/kg) ACEA in OM-treated mice resulted in less upper GI transit than control (OM-treated only) mice and transit distance was similar to normal mice. Both ACEA **(C)** and JWH-133 **(D)** decrease transit in a dose-related fashion, when the values for CBR-treated groups are normalized to % of vehicle-treated control mice within each treatment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

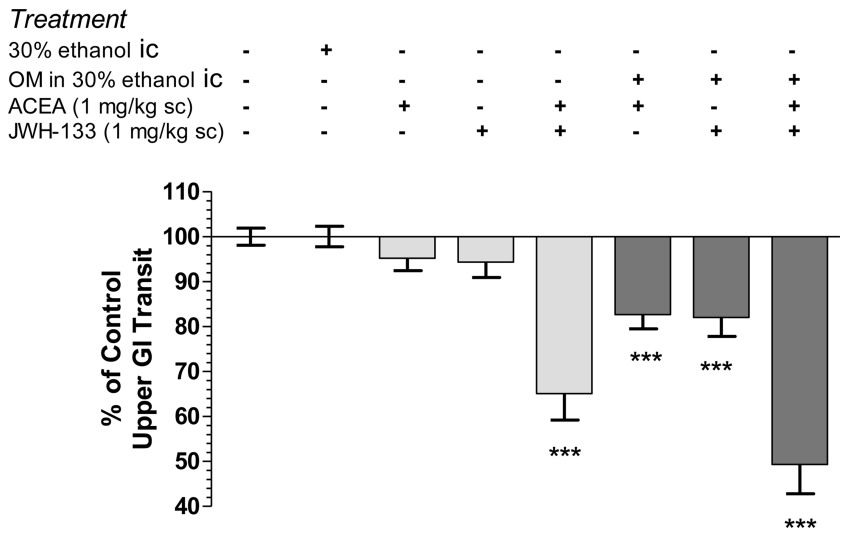

FIGURE 4 | Intracolonic 30% ethanol had no impact on upper GI transit, measured 28 days later, compared to wild-type controls. CB1R (ACEA 1 mg/kg) and CB2R (JWH-133, 1 mg/kg) agonists alone inhibited small intestinal transit in mice 28 days post-OM administration, but not in control mice. A combination of both agonists inhibited transit in both groups. All values were normalized to the appropriate control group mice (i.e., columns 1–5 were normalized to mean transit in normal mice and columns 6–8 were normalized to mean transit in OM-treated treated mice) and are represented as % of control. ***$P < 0.001$ *versus* appropriate control (normal or 30% ethanol intracolonic).

tions because OM induces physiological perturbations similar to those associated with IBS, such as altered motility (Kimball et al., 2005) and visceral hyperalgesia (Laird et al., 2001). In addition, early changes in CBR mRNA expression was noted in OM-induced colitis (Kimball et al., 2007), but this had not been confirmed by protein staining for longer time periods after the acute colitis. Finally, CBR signaling is a consequence of OM neuronal stimulation (Bereiter et al., 2002) and seems to modulate the resulting hyperlagesia. Specifically, CBR interacts with TRPV1 channels on sensory afferents to ameliorate OM-induced hyperalgesia (Jordt et al., 2004; Akopian et al., 2008; Sawyer et al., 2009). Therefore this appears to be a useful model to study the role of CBR signaling in some of the pathophysiology associated with IBS.

We did not confirm selectivity of the CBR agonists in these experiments by using selective antagonists. This is because many studies have used CB1R and CB2R agonists, including ACEA and JWH-133, and already characterized their subtype selectivity using receptor antagonists or the CB1R inverse agonist in models of inflamed gut (Izzo et al., 1999, 2000, 2001, 2003; Landi et al., 2002; Mathison et al., 2004). The doses of agonists (ACEA and JWH-133) used in the present study were similar to that shown to be selectively acting at CB1R and CB2R to reduce rat LPS increased transit (Mathison et al., 2004). We recognize that without demonstrating competitive inhibition by using selective CB1R and CB2R antagonists in this study, we cannot completely exclude non-target related effects of the agonists such as TRPV1 receptors that bind endocannabinoids, and which ACEA structurally resembles. However, ACEA and JWH-133 has not been tested against TRPV1 or other TRPs, nor has the TRP-family receptor has been implicated in controlling small intestinal motility.

Twenty-eight days after OM administration we observed an up-regulation of CB2R in the small intestine and an inhibition of enhanced upper GI transit by CB2R agonists. This is similar to the situation that occurs in acute inflammation-induced hypermotility, reviewed in (Izzo, 2004; Wright et al., 2008; Izzo and Camilleri, 2009), where CB2R are increased in sensitivity compared to little or no effect in normal tissue. For example, upregulation of CB2R contributed to the increased efficacy of non-selective CBR agonists (Izzo et al., 2000, 2001) and a CB2R agonist reduced the electrically evoked ileal twitch responses in LPS-treated, but not normal, tissue (Duncan et al., 2008) and reduced motility in ileitis, but not in control mice (Capasso et al., 2008). Furthermore, a protective role of the CB2R in inflammation is demonstrated by a study in which a CB2R-selective antagonist exacerbated colitis (Storr et al., 2009). It is possible that CB2R is limiting the extent of enhanced transit in the post-inflammatory condition, but this has not been tested yet.

Up-regulation and enhanced anti-transit effects of CB1R agonists were also noted in the present study. Previously CB1R agonists inhibited inflammation in OM-induced acute colitis (Kimball et al., 2006) and dinitrobenzene sulfonic acid-induced colonic inflammation (Massa et al., 2004) with enhanced effects of CBR agonists reducing intestinal transit, fluid accumulation or the rate of colonic expulsion in rodents who were given an inflammatory stimulus (Izzo et al., 2000, 2001, 2003; Izzo, 2004; Mathison et al., 2004). Therefore our data support this enhanced role of CB1R in pathophysiology of the gut and show that it is maintained during the post-inflammatory period. In our study in normal mice, the CB1R-selective agonist (ACEA 1 mg/kg) did not inhibit transit in normal mice, in contrast to the situation in normal rats (Mathison et al., 2004). Two differences are apparent in these studies - species (rats *versus* mice) and the method of drug administration (i.p. *versus* s.c.). Discrepancies in CBR efficacy to inhibit gastric contractility has reported previously in rat (Storr et al., 2002) and mouse (Mule et al., 2007) stomach.

Intraperitoneal drug administration in rats could introduce a higher local concentration of drug to the immediate vicinity of the intestinal tract than subcutaneous administration during a short term test (<30 min).

The increased sensitivity to either subtype of CBR agonists in OM-treated mice can be explained by the increased CB1R and CB2R expression in the small intestine in OM-treated mice tissues. Immunohistochemical data showed moderate CB1R expression in normal myenteric plexus that was reduced at 7 days post-OM colitis induction and which was restored, and appeared to be increased, by day 28. Likewise, striking changes for CB2R expression were seen in lamina propria, with dense staining in increased numbers of cells appearing at day 28, and appearing to occur mainly in monocytic cells.

Our results support a model for small intestinal motility dysfunction in which CB2R and CB1R agonists act in concert to affect transit. A review of cannabinoids and IBS concluded that the effects of the cannabinoid system on motility in humans are similar to those in rodents (Storr et al., 2008). Thus, although cannabinoids have not been studied in IBS patients to our knowledge, these data suggest that either CB1R- or CB2R-selective agonist could reduce GI motility in individuals experiencing diarrhea-predominant IBS. The utility of a CB2R agonist to reduce transit in mice with a post-inflammatory functional GI defect, coupled with a much lower CNS side effect liability, makes this an attractive concept.

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Alternative functional *in vitro* models of human intestinal epithelia

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Physiologically relevant sources of absorptive intestinal epithelial cells are crucial for human drug transport studies. Human adenocarcinoma-derived intestinal cell lines, such as Caco-2, offer conveniences of easy culture maintenance and scalability, but do not fully recapitulate *in vivo* intestinal phenotypes. Additional sources of renewable physiologically relevant human intestinal cells would provide a much needed tool for drug discovery and intestinal physiology. We compared two alternative sources of human intestinal cells, commercially available primary human intestinal epithelial cells (hInEpCs) and induced pluripotent stem cell (iPSC)-derived intestinal cells to Caco-2, for use in *in vitro* transwell monolayer intestinal transport assays. To achieve this for iPSC-derived cells, intestinal organogenesis was adapted to transwell differentiation. Intestinal cells were assessed by marker expression through immunocytochemical and mRNA expression analyses, monolayer integrity through Transepithelial Electrical Resistance (TEER) measurements and molecule permeability, and functionality by taking advantage the well-characterized intestinal transport mechanisms. In most cases, marker expression for primary hInEpCs and iPSC-derived cells appeared to be as good as or better than Caco-2. Furthermore, transwell monolayers exhibited high TEER with low permeability. Primary hInEpCs showed molecule efflux indicative of P-glycoprotein (Pgp) transport. Primary hInEpCs and iPSC-derived cells also showed neonatal Fc receptor-dependent binding of immunoglobulin G variants. Primary hInEpCs and iPSC-derived intestinal cells exhibit expected marker expression and demonstrate basic functional monolayer formation, similar to or better than Caco-2. These cells could offer an alternative source of human intestinal cells for understanding normal intestinal epithelial physiology and drug transport.

Keywords: human intestinal epithelial cell (hInEpC), induced pluripotent stem cell (iPSC), permeability, Transepithelial Electrical Resistance (TEER), neonatal Fc receptor (FcRn)

INTRODUCTION

The use of *in vitro* cell models for human drug transport studies has focused on intestinal epithelial cells, as these cultures contain primarily absorptive cells. While isolated human intestinal epithelial cells (hInEpCs) retain important *in vivo* anatomical and biochemical features, they are difficult to culture and have limited viability. As a result, immortalized human adenocarcinoma cell lines have been extensively used to study absorption mechanisms. While immortalized cells offer many advantages, extrapolation of data generated with these cell lines to *in vivo* conditions is often difficult, as these cells originated from tumors and are therefore not representative of the true physiological environment (Le Ferrec et al., 2001). In addition, these cells form monolayers that are widely used for small molecule intestinal permeation *in vitro* studies (below). But, with increasing numbers of biotechnology protein therapeutics and novel scaffolds available, which open the possibility for oral delivery, there is a need for alternatives that more closely recapitulate the physiology of the intestinal epithelial cell.

The human colorectal adenocarcinoma cell line Caco-2 is frequently used for drug absorption studies, particularly in the context of small molecules (Le Ferrec et al., 2001; Balimane and Chong, 2005). Caco-2 cells are easy to culture and have the capacity to spontaneously differentiate into cells possessing the morphology and function of enterocytes, the absorptive cells of the intestine (Balimane and Chong, 2005). Caco-2 cells are commonly cultured on semi-permeable inserts in a transwell format, where the cells form a polarized monolayer (Leonard et al., 2000; Le Ferrec et al., 2001), and the transport of molecules between the apical and basolateral chambers can be easily evaluated. While Caco-2 cells are a good model for observation of passive transcellular and paracellular permeability (Balimane and Chong, 2005), there are differences in cytokine production and cytokine receptor expression between Caco-2 cells and normal epithelial cells (Aldhous et al., 2001). In addition, Caco-2 cells under-express transporters and metabolizing enzymes relative to *in vivo* tissue, potentially excluding mechanisms crucial for drug absorption studies (Balimane and Chong, 2005).

Due to the limitations of immortalized intestinal cell lines, many studies have focused on the use of primary hInEpCs as a more physiologically relevant cell-based model (Perreault and Beaulieu, 1998; Aldhous et al., 2001; Ootani et al., 2009; Lahar et al., 2011). However, stocks of these cells are difficult to maintain due to limited donors and low viability in culture. Recently, commercial sources of primary hInEpCs were made available (Lonza; Walkersville, MD), which greatly increase the convenience of obtaining primary cell stocks. Commercial quality control data suggest that these primary hInEpCs have the capacity to form monolayers with tight junctions and express general epithelial markers, such as cytokeratins 8 and 18 (Bosch et al., 1988); however, little characterization has be done on their expression of intestinal cell type-specific markers or transport function. Other efforts to enable long-term culture of primary cells and enhance physiological conditions have led to the development of 3-dimensional (3D) models of the intestinal epithelium, which have focused on the use of primary intestinal stem cells and directed differentiation of pluripotent stem cells.

Stem cells have the capacity to self-renew and differentiate into the various cell lineages that make up specific tissue types. For example, intestinal stem cells are responsible for the self-renewal of the gut epithelium, and have been used in developing 3D intestinal models. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR-5)-positive stem cells can be isolated from primary intestinal tissue and grown as 3D intestinal organoids with crypt-villius physiology and culturing capacity up to 8 months (Sato et al., 2009). While 3D organoids derived from primary intestinal stem cells appear to possess physiologically relevant phenotypes, they cannot be used to assess classical functionality typically determined within 2-dimensional transwell cultures, such as the formation of monolayers with tight junctions and intestinal permeability and transport.

An additional source of human intestinal cells is possible through directed differentiation of pluripotent stem cells to intestinal cell lineages. The recent advent of human induced pluripotent stem cells (iPSCs) has provided a huge therapeutic potential as a tool for drug discovery, as patient-specific somatic cells can be reprogrammed into an embryonic stem cell-like state that can be directly differentiated to a specific cell type of interest for more physiologically relevant disease modeling. Induced human intestinal organoids (iHIOs) have recently been derived from iPSCs (Spence et al., 2010), which are capable of expressing epithelial and intestinal markers such as caudal type homeobox 2 (CDX2) (hindgut marker), E-Cadherin (cell-to-cell junction marker), and Villin (epithelial brush border marker). However, to our knowledge, the methods for differentiating these into a polarized epithelial monolayer similar to Caco-2 have not been reported.

In this study, we assessed expression of markers and functional activity of the newly commercially available primary hInEpCs and iPSC-derived intestinal cells compared to Caco-2 (**Figure 1**) in cell-based *in vitro* assays. We adapted our previously described 3D intestinal organogenesis to differentiation within transwells. Intestinal marker expression, formation of monolayers with tight junction formation and functional molecule transport and binding were evaluated.

MATERIALS AND METHODS

CELL CULTURE

Intestinal cells

Human primary small intestinal epithelial cells (Lonza; Walkersville, MD) from 3 donors (Donor A: Lot # 0000258132; Donor B: Lot # 0000256741; Donor C: Lot # 0000256744) were thawed and cultured in transwell inserts for 10–11 days in SmGM-2 media (Lonza), according to manufacturer's instructions. Caco-2 colorectal adenocarinoma-derived cells (ATCC; Manassas, VA) were cultured in transwell inserts for 14–21 days in Caco-2 media (10% fetal bovine serum, 1X non-essential amino acids, 1X sodium pyruvate, and 6 mM L-Glutamine in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, reagents from Life Technologies; Carlsbad, CA).

iPSCs

A1145A and B2198A (Johnson & Johnson; Spring House, PA) were produced from human kidney-derived cells using retroviral (Takahashi et al., 2007) and modified mRNA (non-viral) reprogramming methods (Yakubov et al., 2010), respectively. C2198A and C2200B (Johnson & Johnson; Spring House, PA) were produced from human umbilical tissue-derived cells by modified RNA reprogramming methods. D2043A (System Biosciences; Mountain View, CA) was derived from human foreskin fibroblasts by retroviral methods. iPSC lines were maintained in mTeSR1 culture media (STEMCELL Technologies; Vancouver, BC) on culture dishes/flasks coated with Geltrex (Life Technologies) and passaged by Dispase (STEMCELL Technologies) dissociation every 3–5 days as previously described (McCracken et al., 2011).

iPSC DIRECTED DIFFERENTIATION

Transwell differentiation

Semi-permeable transwell inserts were coated apically with Geltrex (Life Technologies) prior to plating iPSCs within 12-well transwell tissue culture plates. iPSCs were plated apically as cell clumps (5–10 cells per clump) at a density of 3000–6000 clumps per transwell insert. Two to three days after plating, iPSCs were differentiated within transwell inserts modified previously reported methods (Spence et al., 2010) into definitive endoderm using GDF8 with GSK3b inhibitor and B27 supplement then differentiated to hindgut using Keratinocyte Growth Factor and Retinoic Acid then EGF, Noggin, and R-Spondin1 for >26 days (Kauffman et al., Submitted) on both sides of the transwell insert; however, the many spheroid structures observed at Stage 2 were maintained as part of the adherent layer throughout Stage 3 differentiation (**Table 1**) for up to 31 days.

MONOLAYER ASSESSMENT

Transepithelial electrical resistance (TEER)

TEER of transwell cultures was recorded in measurements of Ohms using an Epithelial Volt Ohm Meter (EVOM)[2] and electrode set (World Precision Instruments; Sarasota, FL). Raw

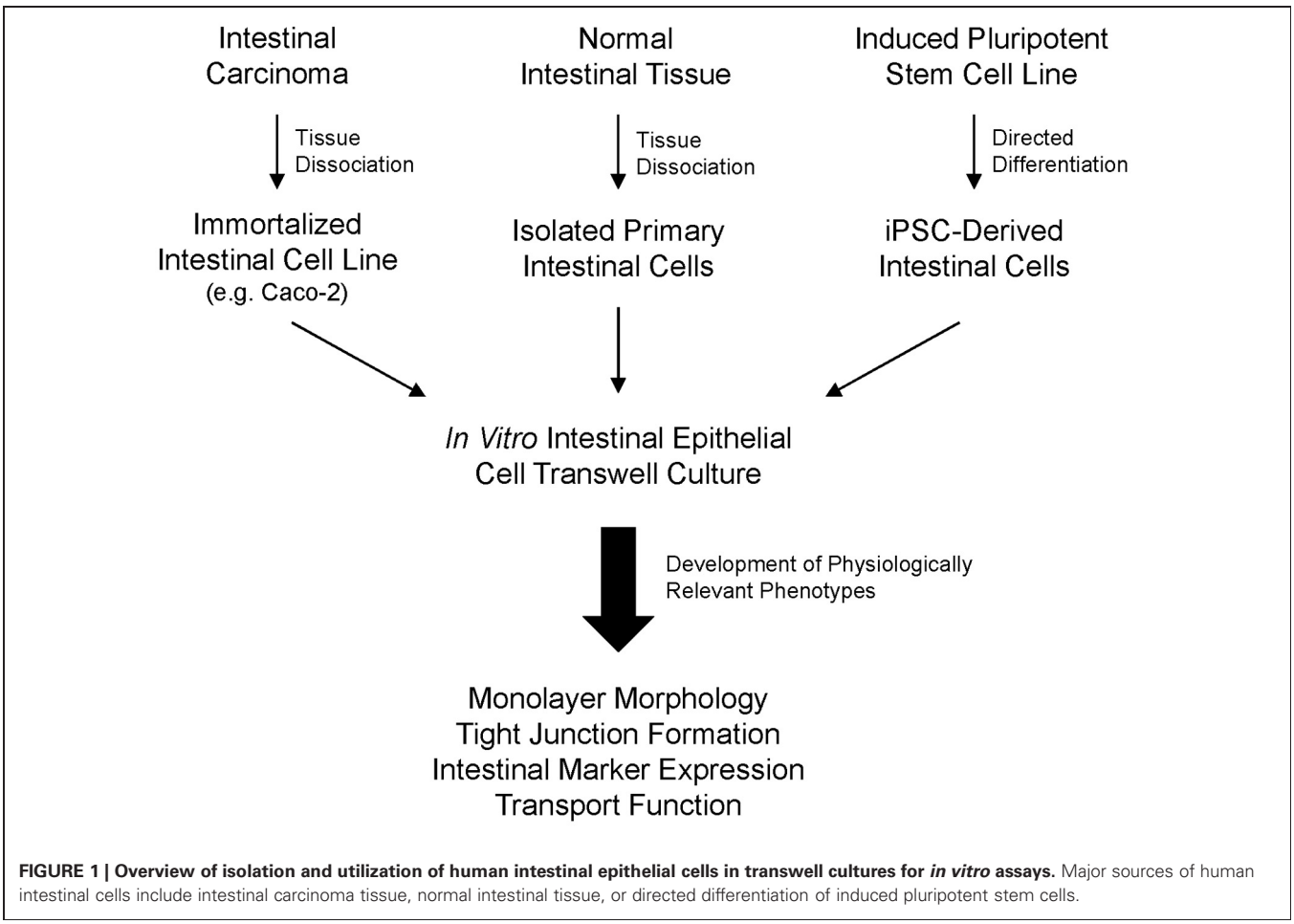

FIGURE 1 | Overview of isolation and utilization of human intestinal epithelial cells in transwell cultures for *in vitro* assays. Major sources of human intestinal cells include intestinal carcinoma tissue, normal intestinal tissue, or directed differentiation of induced pluripotent stem cells.

Table 1 | Summary of growth factors and differentiation cell culture conditions used for transwell differentiation, relative to previously established intestinal organogenesis to 3D organoids.

Stage	Growth factors	Days	Cell type	Stage markers	Organoid culture	Transwell culture
1	GDF-8, GSKi, B27	3	Definitive endoderm	SOX17, CXCR4	2D Standard tissue culture plate	2D Transwell
2	KGF, RA	7	Hindgut	CDX2	2D Excise spheroids from standard plate	2D Transwell (No Spheroid Excision)
3	EGF, Noggin, R-Spondin1	26+	Intestinal	E-Cad, CDX2, Villin	3D Intestinal matrigel	2D Transwell

data was converted to $\Omega \times cm^2$ based on area of transwell plate inserts ($1.12\,cm^2$).

FITC-dextran permeability

Cells in 24-well transwell plates were washed twice with DPBS (Life Technologies). To the apical side, 200 µ L of 12.5 mg/mL Fluorescein isothiocyanate–dextran, molecular weight 150 kDa (FD150) (Sigma–Aldrich) diluted in Caco-2 media ($pH = 6.0$) was. To the basolateral side, 500 uL of Caco-2 media ($pH = 7.4$) was added. After a 90-min incubation at 37°C, 100 uL of media was collected from the basolateral chamber and analyzed for the presence of FITC-Dextran using a SpectraMax M5 microplate reader (Molecular Devices; Sunnyvale, CA).

P-GLYCOPROTEIN TRANSPORT ASSAY

Primary hInEpCs were dosed on the apical or basolateral side with a 2 mM mix of Digoxin and Atenolol in HBSSg [2 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in Hank's Balanced Salt Solution (HBSS)] with calcium and magnesium, $pH = 7.4$) and incubated for 90 min at 37°C in the presence or absence of 10 µM Cyclosporin A (CSA). Samples were collected from both the apical/donor and basolateral/receiver chambers and analyzed by LCMS. Digoxin and Atenolol levels in each condition were used to calculate apparent permeability ($Papp = \delta Cr/\delta t) \times Vr / (A \times C_0)$) in the apical to basolateral (A–B) or basolateral to apical (B–A) direction. δCr = final receiver concentration; δt = assay time; Vr = receiver volume;

A = transwell growth area; C_0 = initial apical concentration. To ensure monolayer integrity during the assay, all wells were dosed apically with 100 μ g/mL Lucifer Yellow (LY) at the start of Digoxin and Atenolol incubation, and samples were collected from the basolateral chamber for analysis by SpectraMax M5 microplate reader at the end of the 90 min incubation. Only transwells with a LY Papp(A–B) of $< 1 \times 10^{-6}$ cm/s were used in calculating final Papp Ratios for Digoxin and Atenolol. Based on this cutoff, one of 8 wells each for hInEpC Donors A and B, and two of 8 wells for Donor C was excluded from data analysis.

mRNA EXPRESSION

RNA was harvested from Caco-2, primary hInEpCs, or iPSCs before or after differentiation to Stage 3 by RNeasy Mini kit (Qiagen; Germantown, MD), and reverse transcribed to cDNA using the RT^2 First Strand kit (Qiagen). Using 30 ng/μL starting cDNA, samples were used in reactions within Custom RT^2 Profiler PCR array containing probes supplied by the manufacturer (SABiosciences; Valencia, CA) for intestinal and control markers (**Table 2**), following reaction cycling conditions outlined in the manufacturer's protocol. Data analysis was performed using the Δ CT method, where raw *Ct* values were normalized to housekeeping gene 60S acidic ribosomal protein P0 (RPLP0) before comparing expression relative to Caco-2. Expression levels for primary hInEpCs represents the average across all three donors used in this study.

Table 2 | List of probes used in Custom RT^2 Profiler PCR array mRNA expression analyses of intestinal and control markers in intestinal epithelial cells.

Gene	NCBI reference no	SAB catalog no
E-Cadherin	NM_004360	PPH00135
CDX2	NM_001265	PPH13618
KLF5	NM_001730	PPH00434
Villin	NM_007127	PPH23365
SOX9	NM_000346	PPH02125
LGR5	NM_003667	PPH13346
ASCL2	NM_005170	PPH12852
MUC2	NM_002457	PPH06990
Chromogranin A	NM_001275	PPH01181
LYZ	NM_000239	PPH14748
VIM	NM_003380	PPH00417
FcRn	NM_004107	PPH11194
CXCR4	NM_003467	PPH00621
PDX1	NM_000209	PPH05536
OCT4	NM_002701	PPH02394
TNNT2	NM_000364	PPH02619
PAX6	NM_000280	PPH02598
TUBB3	NM_006086	PPH02607
RPLP0	NM_001002	PPH21138

Probes for the NCBI Reference sequence numbers listed were obtained from SABiosciences (SAB) using the catalog numbers provided.

PROTEIN EXPRESSION

Flow cytometry

Undifferentiated or Stage 1 iPSCs were detached by treatment with Accutase (Sigma-Aldrich; St. Louis, MO) and stained for viability by Near Infrared Live/Dead kit (Invitrogen; Carlsbad, CA) before fixation in Cytofix Buffer (BD Biosciences; San Jose, CA). For pluripotent marker analyses, cells were stained using Human Pluripotent Stem Cell Transcription Factor Analysis or Human Pluripotent Stem Cell Sorting and Analysis kits (BD Biosciences). For definitive endoderm marker analysis, cells were stained with a 1:5 dilution of PE-conjugated mouse anti-human CD184/ C-X-C chemokine receptor type 4 (CXCR4) (306506, Biolegend; San Diego, CA) before permeabilized with Phosflow Perm Buffer (BD Biosciences) and stained with a 1:5 dilution of APC-conjugated goat polyclonal anti-human Sry-related HMG box 17 (SOX17) (IC1924A, R&D Systems; Minneapolis, MN). For analysis of surface expression of neonatal Fc receptor (FcRn), Caco-2 and primary hInEpCs were dissociated from transwell culture by Accutase, incubated with 50 μg/mL affinity purified rat anti-human FcRn polyclonal antibody (generated in house), followed by incubation with 7.5 μg/mL FITC Donkey anti-rat IgG (FAb2) secondary antibody (109-006-006, Jackson Immunoresearch; West Grove, PA). Fluorescence of stained cells was measured in conjunction with appropriate compensation controls (BD Biosciences) by flow cytometry using an LSR Fortessa FACS Sorter (BD Biosciences). Raw data was analyzed by FlowJo analysis software (Tree Star; Ashland, OR), with gating parameters set based on isotype controls (**Figure A1A**).

Immunofluorescence

iPSC-derived cells Stage 2 iPSC-derived cells were fixed in 4% paraformaldehyde (Electron Microscoopy Sciences; Hatfield, PA) before permeabilization with 0.5% Triton X-100 (Electron Microscoopy Sciences). After addition of Image-iT® FX signal enhancer (Invitrogen), cells were treated with 1X Blocking Buffer (Sigma) before exposure to a 1:50 dilution of E-Cadherin, CDX2, or Villin antibodies (Dako), using manufacturer recommended concentrations. Immunoreactivity to primary antibodies was detected with a 1:500 dilution of AF568-conjucated goat anti-mouse antibody (Invitrogen). Prolong GOLD antifade with DAPI (Invitrogen) was added to wells prior to visualizing on a Nikon SMZ-1500 fluorescence dissecting microscope.

FcRn-DEPENDENT IMMUNOGLOBULIN G (IgG) BINDING

Caco-2, hInEpCs (Donor A), or iPSC-derived cells were detached from transwells by Accutase (Sigma) treatment and washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies). Cells were transferred in DPBS to MesoScale Discovery (MSD) High Bind plates (MSD; Rockville, MD) at a density of 1×10^4 or 2.5×10^4 cells per well, and incubated at room temperature for 2 h to allow attachment to the plate surface. Plates were then blocked with 20% Fetal Bovine Serum (Life Technologies) and 0.18% Sodium Azide (VWR International; Radnor, PA) for 15 min at room temperature. Wells were washed once with DPBS at *pH* 6.0 before incubation with FcRn-binding

variants (anti-RSV N434A or anti-RSV H435A in DPBS at *p*H 6.0) for 90 min at 37°C. Plates were washed 3 times with DPBS at *pH* 6.0, and cells were incubated with ruthenium-labeled goat anti-human IgG F(ab')2 (1 μg/ml in DPBS at *p*H 6.0) for 1 h at room temperature. Cells were washed 3 times with DPBS at *p*H 6.0. Tris-based Read Buffer T without surfactant (MSD) was added to wells immediately before measuring Relative Luminscent Units (RLU) using a Sector Imager 6000 reader and Discovery Workbench software (MSD). For analysis, background signal RLUs were subtracted from RLUs of samples run in triplicate.

RESULTS

MULTIPLE SOURCES OF HUMAN INTESTINAL EPITHELIAL CELLS EXHIBIT INTESTINAL MARKER EXPRESSION

Along with limited viability in culture, one of the major drawbacks of routinely using primary hInEpCs for studying intestinal physiology is that these cells must be collected from human donors. A newly available commercial source of primary hInEpCs greatly increases the convenience of obtaining primary cell stocks, but need to be further characterized for intestinal cell-type specific marker expression and functional monolayer formation. Thus, we sought to characterize these commercially available stocks to assess their physiological relevance for use in cell-based *in vitro* assays of intestinal uptake and transport.

As previously reported, a measure of intestinal cell phenotypic quality is the expression of a panel of general intestinal epithelial markers (Spence et al., 2010). We used immunocytochemistry to verify expression and expected localization of several representative intestinal epithelial markers within primary hInEpCs from three different donors (**Figure 2**, top row; **Figure A1B**), relative to the immortalized human intestinal epithelial cell line Caco-2 (**Figure 2**, bottom row). This was visualized for E-cadherin, an epithelial cell adhesion marker found within tight junctions between cells (Zbar et al., 2004), CDX2, a transcription factor that is upstream of signaling promoting intestinal cell fate (Gao et al., 2009) and Villin, an actin-binding protein associated with the intestinal brush border of absorptive enterocytes (Friederich et al., 1999). The localization of these markers within cells was appropriate in primary hInEpCs and Caco-2 (**Figure 2**). Marker expression intensity was variable between the three hInEpC donors within this study, with strongest expression evident in Donor A (**Figure 2**, top row; **Figure A1B**). Expression results for hInEpCs and Caco-2 were used to compare to iPSC-derived iHIOs grown in transwells (below).

In order to evaluate iPSC-derived intestinal cell phenotypes within classical *in vitro* monolayer conditions, we adapted intestinal organogenesis to transwell culture by performing iPSC differentiation through Stage 3 within transwell inserts (**Table 2**, **Figure 3A**). Differentiation of a panel of iPSC lines (A1145A, B2198A, C2128A, C2200B, and D2043A) on matrigel-coated transwell inserts using Myostatin, Glycogen Synthase Kinase 3β inhibitor and B27 supplement resulted in cells expressing definitive endoderm markers SOX17 and CXCR4 (**Figure 3B**), similar to iPSCs differentiated to Stage 1 in standard tissue culture plates (Kauffman et al., Submitted). Further differentiation of iPSC-derived definitive endoderm cells within transwell plates, by addition of Keratinocyte Growth Factor and Retinoic Acid, produced layers of cells with developing spheroid structures at Stage 2 in many of the iPSC lines (**Figure 3C**, top row). CDX2 expression at Stage 2 was relatively uniform within layers of cells differentiating in transwells (**Figure 3C**, bottom row). However, D2043A showed more intense expression around spheroid structures.

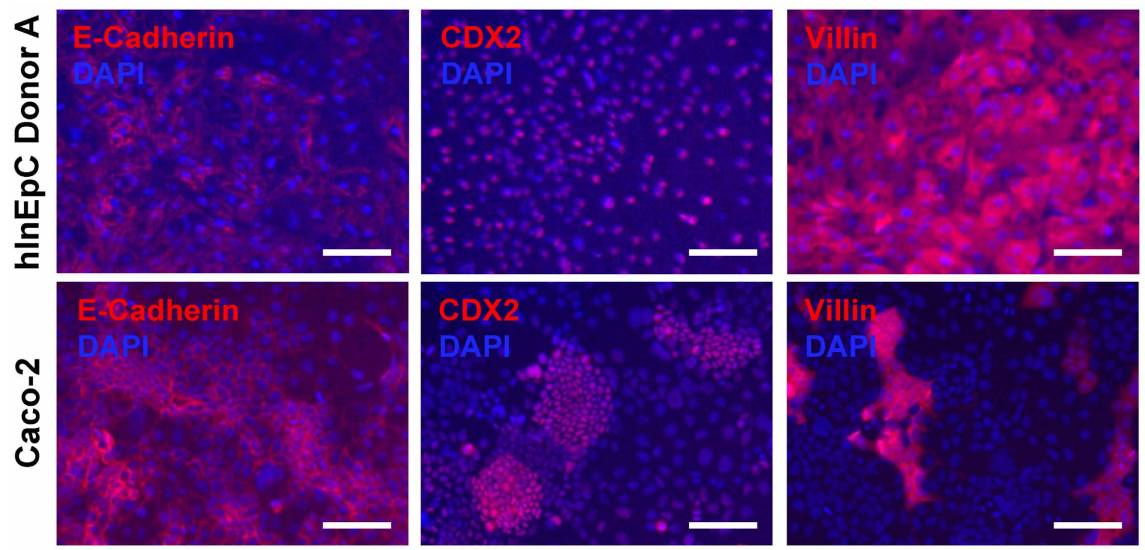

FIGURE 2 | Characterization of intestinal marker expression in commercially available (Lonza) primary human intestinal epithelial cells (hInEpCs) relative to immortalized human intestinal cell line Caco-2. Top row: primary hInEpCs uniformly express intestinal epithelial markers E-Cadherin (tight junctions), CDX2 (hindgut), and Villin (enterocytes). Primary hInEpC Donor A shown (see **Figure A1B** for Donors B and C). **Bottom row**: Caco-2 show less uniform expression of enterocyte marker Villin. Scale bar, 100 μm.

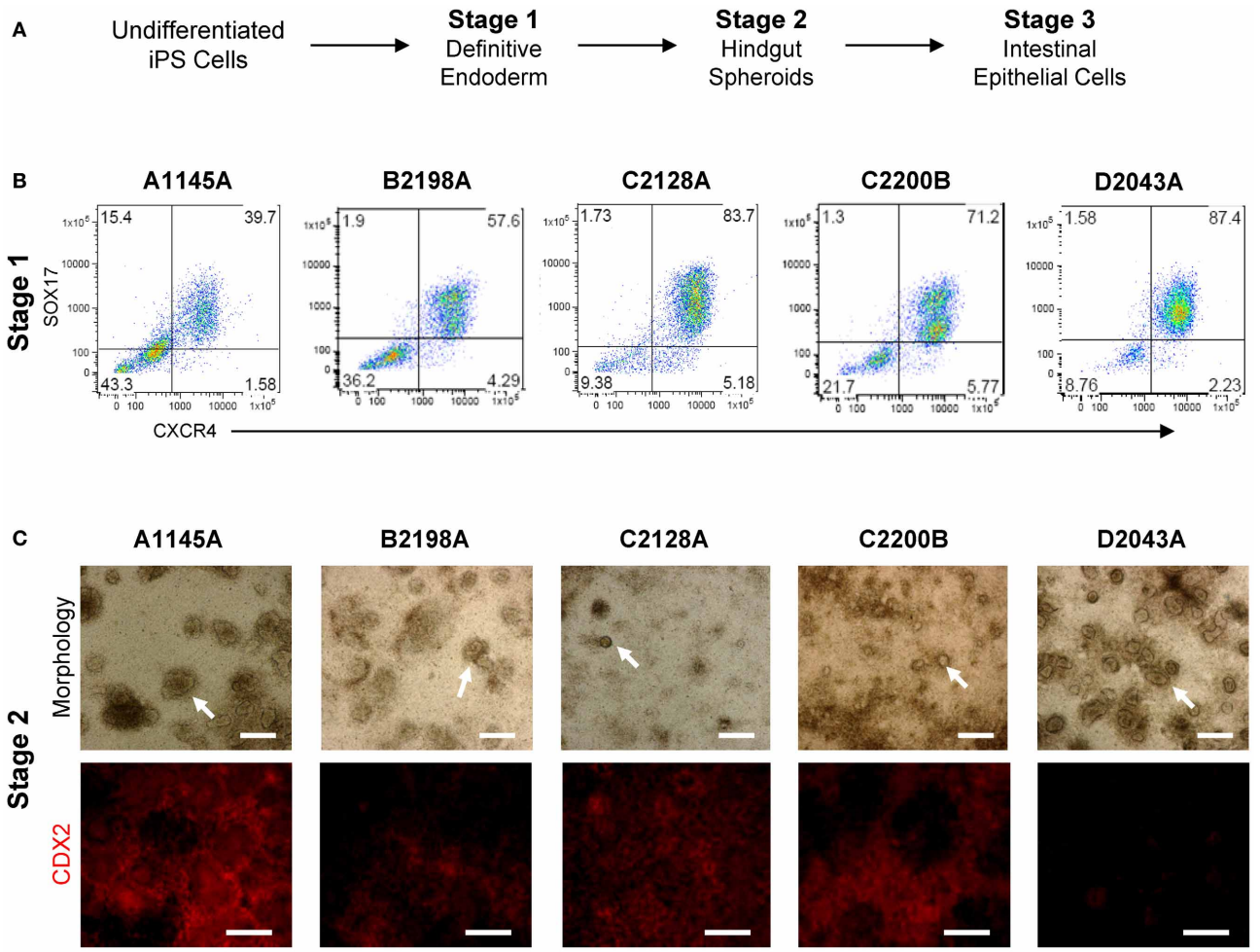

FIGURE 3 | iPSC directed intestinal differentiation in transwell culture results in cells with expected morphology and marker expression through Stage 2. (A) Directed intestinal differentiation occurs through several distinct stages. **(B)** Transwell cells derived from a panel of iPSC lines express Definitive Endoderm markers SOX17 and CXCR4 at Stage 1. **(C)** Top row: transwell iPSC panel morphology at Stage 2 differs in amount and size of budding spheroids (arrow heads). Scale bar, 250 μm. Bottom row: CDX2 expression of Stage 2 transwell iPSC-derived cells differs among cell lines in intensity and uniformity. Scale bar, 250 μm.

iPSC-derived Stage 3 3D intestinal organoids exhibit E-Cadherin, CDX2, Villin, and Chromogranin A immunoreactivity with the expected localization, (Kauffman et al., Submitted). In this study, a supply of transwell A1145A iPSC-derived Stage 3 Day 31 cells was limited, and did not allow sufficient material for extensive immunocytochemistry analyses of intestinal markers. Thus, mRNA expression analysis was used to directly compare larger panel of known intestinal marker and differentiation control genes Stage 3 A1145A iPSC-derived intestinal marker expression to Caco-2 and primary hInEpCs by RT-PCR (**Figure 4**). Differentiated Stage 3 iPSCs showed increased marker expression relative to undifferentiated cells, usually reaching a level more similar to primary hInEpCs in the case of 3D iHIOs (**Figure 4**), or more similar to Caco-2 in the case of transwell-differentiated iPSCs (**Figure 4**). Intestinal markers that followed this expression pattern included epithelial tight junction marker E-Cadherin (Zbar et al., 2004), hindgut epithelial marker CDX2 (Gao et al., 2009), enterocyte maker Villin (Friederich et al., 1999), enteroendocrine marker Chromogranin A (O'Connor et al., 1983), and Mucin-2 (MUC2), a marker for intestinal goblet cells (Gum et al., 1999) (**Figures 4A–E**).

For some intestinal makers, expression in iPSC-derived intestinal cell types was highest in iPSC-derived cells. For example, expression levels of intestinal crypt cell marker Sex determining region Y-box 9 (SOX9), paneth cell marker Lyzosyme (LYZ) (Peeters and Vantrappen, 1975), and intestinal stem cell marker LGR5 (Barker et al., 2007) were greatest in iHIOs and transwell differentiated cells compared to Caco-2 or primary hInEpCs (**Figures 4F–H**). On the other hand, an additional intestinal stem cell marker, Achaete Scute-Like 2 (ASCL2) (van der Flier et al., 2009), was most highly expressed in Caco-2 (**Figure 4I**). In the case of intestinal epithelial cell differentiation transcription factor Kruppel-like factor 5 (KLF5) (Bell et al., 2013), mRNA expression

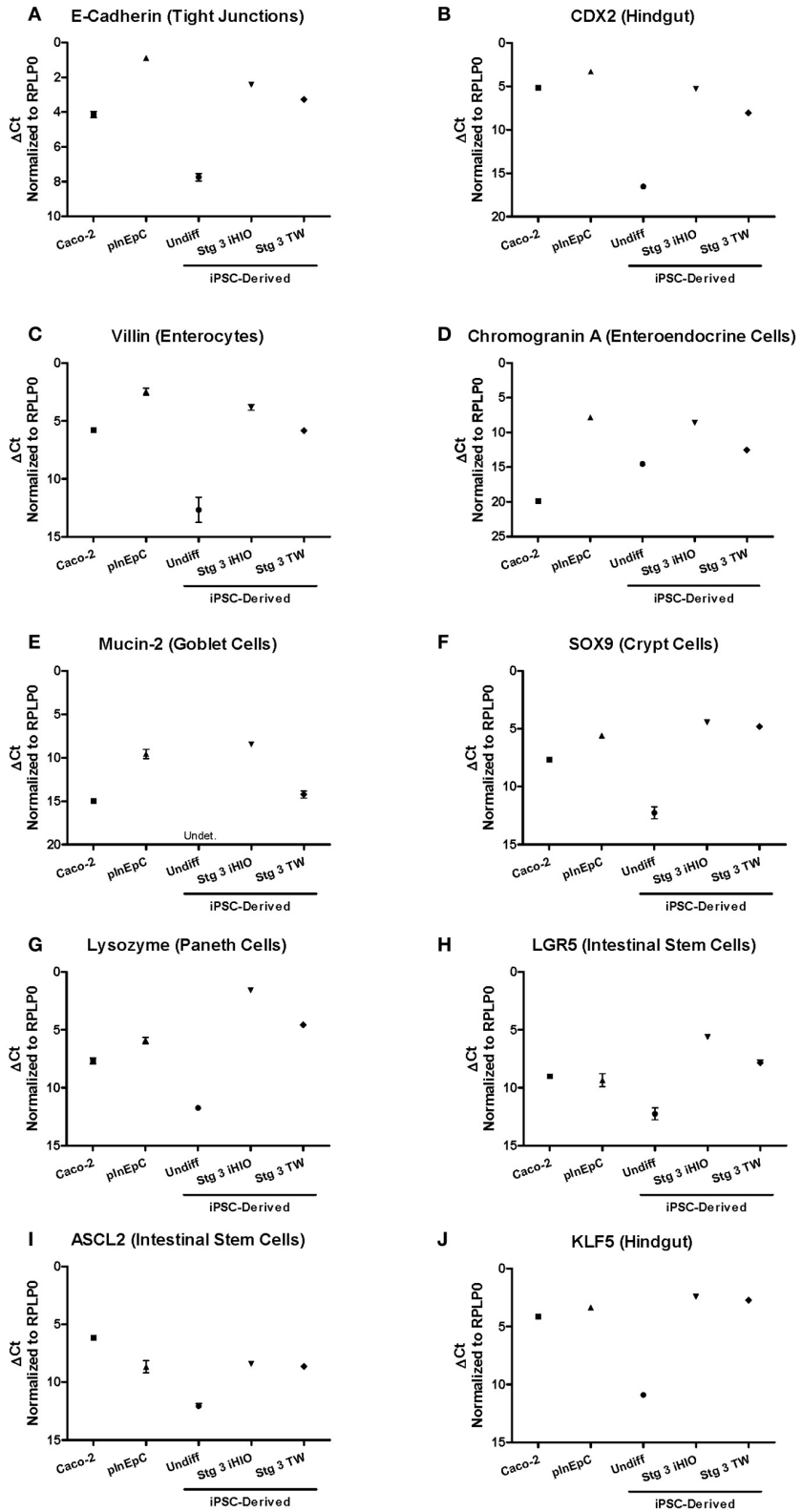

FIGURE 4 | Comparison of intestinal marker mRNA expression in multiple sources of human intestinal epithelial cells. Expression of tight junction marker **(A)** and hindgut epithelia **(B)**, as well as specific epithelial lineage markers **(C–J)** intestinal markers of epithelial intestinal lineages was evaluated by RT-PCR in Caco-2, primary hInEpCs (average of Donors A–C), and iPSC-derived undifferentiated (Undiff), Stage 3 induced human intestinal organoids (Stg 3 iHIOs), or Stage 3 transwell intestinal cells (Stg 3 TW). Using the ΔCt method, raw *Ct* values were normalized to housekeeping gene RPLP0. $N = 3$; Error bars represent SEM.

levels were similar for all intestinal cell types (**Figure 4J**). The expression of several control genes [e.g., pluripotent marker Octamer-binding Transcription factor 4 (OCT4) (Nichols et al., 1998)] confirmed the cell sample quality (**Figure A3**).

PRIMARY hInEpCs AND iPSC-DERIVED INTESTINAL CELLS FORM TRANSWELL MONOLAYERS WITH TIGHT JUNCTIONS

While all three primary hInEpCs donors appeared to be capable of forming confluent cell layers in transwell culture that expression the tight-junction marker E-Cadherin (**Figures 2**, **4A**), we assessed tight junction formation functionally through measurements of Transepithelial Electrical Resistance (TEER) and monolayer permeability. As confluent epithelial monolayers form, TEER measurements generally increase, reaching 260–420 $\Omega \times cm^2$ on average in Caco-2 cultures (Le Ferrec et al., 2001). We found that after 11 days of transwell culture, primary hInEpCs from all three donors exhibited TEER measurements of $> 1500\Omega \times cm^2$ (**Figure 5A**), providing a strong indication of tight junction formation. As changes in epithelial TEER can also be explained by changes in transcellular ion permeability (Yu and Sinko, 1997), we confirmed tight junction formation of pInEpC transwell cultures by determining the monolayer permeability to FITC-labeled Dextran at a molecular weight of 150,000 (FD150). Apical side incubation in transwell chambers, resulted in <3% FITC-labeled Dextran detected on the basolateral side, relative to control transwells with no cells for primary hInEpC Donors A and B (**Figure 5B**). Donor C, which corresponds to the cells with highly variable TEER measurements (**Figure 5B**) showed slightly more FITC-Dextran permeability (∼3% relative to control transwells).

While Stage 3 Day 31 transwell A1145A iPSC-derived cells exhibited intestinal marker expression consistent with other intestinal epithelial cell sources, it was difficult to gauge at which point during Stage 3 differentiation iPSC-derived cells may begin to take on functional phenotypes. Thus, we performed a time course experiment in which A1145A iPSCs were differentiated within transwell culture and assessed for monolayer morphology and evidence of tight junction formation throughout Stage 3 (Days 0, 7, 14, 21, 31). Morphologically, A1145A iPSC-derived cells showed 3D structures and dense patches of cells within transwells at the beginning of Stage 3 that appeared to disappear as differentiation progressed, leaving a flat monolayer-like layer of cells by Day 31 of Stage 3 (**Figure 5C**). To assess tight junction formation in confluent A1145A iPSC-derived transwell monolayers, we measured TEER and FITC-Dextran permeability during iPSC transwell differentiation. At Day 0 of Stage 3 differentiation, A1145A iPSC-derived cells within transwells exhibited low TEER measurements of less than 200 $\Omega \times cm^2$ which steadily increased to measurements reaching 937 $\Omega \times cm^2$ by Day 31 (**Figure 5D**). At Stage 3 Day 0, iPSC-derived cells showed FD150 permeability of ∼2% relative to the no cell control (**Figure 5E**), a range similar to that seen for primary hInEpCs (**Figure 5B**). Coinciding with changes in TEER measurements during differentiation (**Figure 5D**), FD150 permeability decreased for iPSC-derived cells to as low as 0.06% of the no cell control (**Figure 5E**).

INITIAL ASSESSMENT OF INTESTINAL EPITHELIAL TRANSPORT FUNCTION

To further validate these cell sources functionally, we assessed monolayers for molecule transport or binding. A previously established mechanism influencing small molecule transport by intestinal epithelial cells is efflux by membrane associated ATP-binding cassette P-glycoprotein (Pgp) transporters, which facilitate cellular efflux to prevent accumulation of their substrates (Murakami and Takano, 2008). To assess Pgp transport activity for a given substrate, the basolateral to apical (B–A) permeability is compared to apical to basolateral (A–B), where compounds with efflux ratios (B–A/A–B) greater than 2 or 3 are generally considered to be Pgp substrates (Balimane et al., 2006). Using transwell monolayers of primary hInEpCs, we found that efflux ratios for Digoxin, a compound known to be highly effluxed by Pgp (Balimane et al., 2006), were >8 for pInEpC Donors A and B (**Figure 6A**). Importantly, efflux of Digoxin was reduced when these cells were also treated with CSA, a known Pgp transporter inhibitor (Watanabe et al., 1997). Atenolol, a poorly Pgp-effluxed compound (Balimane et al., 2006) showed very low efflux ratios of <1 in all three primary hInEpC donors, which was not further reduced by CSA (**Figure 6B**). During the Pgp transport assay, transwells were also dosed apically with lucifer yellow to confirm primary hInEpC monolayer integrity based on permeability of this fluorescent molecule (**Figure A3**). While the average apparent permeability for all three primary hInEpC donors was below 1.5×10^{-6} cm/s, only transwells below this standard cutoff were used for analysis of Digoxin and Atenolol flux.

Unfortunately, as A1145A iPSC-derived transwell intestinal cells were very limited, there were insufficient cells within this study to perform a properly-controlled Pgp transport assay. Thus, to further validate iPSC-derived cells, we assessed FcRn immunoreactivity and performed a cell surface binding assay for neonatal Fc receptor (FcRn)-mediated transport, which was amenable to the limited cell supply.

Intense FcRn expression in iPSC-derived intestinal organoids was noted as intense single-cell expression within the population of cells (**Figure A4A**) similar that previously reported for human intestinal tissue (Dickinson et al., 1999). We confirmed FcRn expression immunocytochemically in transwell monolayer cultures of Caco-2 and primary hInEpCs both intracellularly (**Figure 6C**) and on the surface of cells (**Figure A4B**). Real-Time RT-PCR analysis showed that FcRn mRNA expression increases to the level close to that of Caco-2 by Stage 3 of transwell differentiation (**Figure 6D**), whereas FcRn expression was highest in primary hInEpCs and iHIOs.

We examined binding of high or low FcRn-binding IgG mAb variants to Caco-2, primary hInEpCs, or iPSC-derived transwell intestinal cells by Meso Scale Discovery assay. Use of this highly sensitive ELISA-like assay allowed us to perform FcRn-dependent binding experiments with proper controls, even with limited-supply iPSC-derived cells. Similar to Caco-2, primary hInEpCs showed significantly higher binding of the IgG variant with a high affinity to FcRn (N434A) than the low FcRn-binding variant (H435A) (**Figure 6E**). iPSC-derived intestinal cells also demonstrated significantly higher binding of a high FcRn-binding IgG variant (M428L) relative to the low FcRn-binding variant

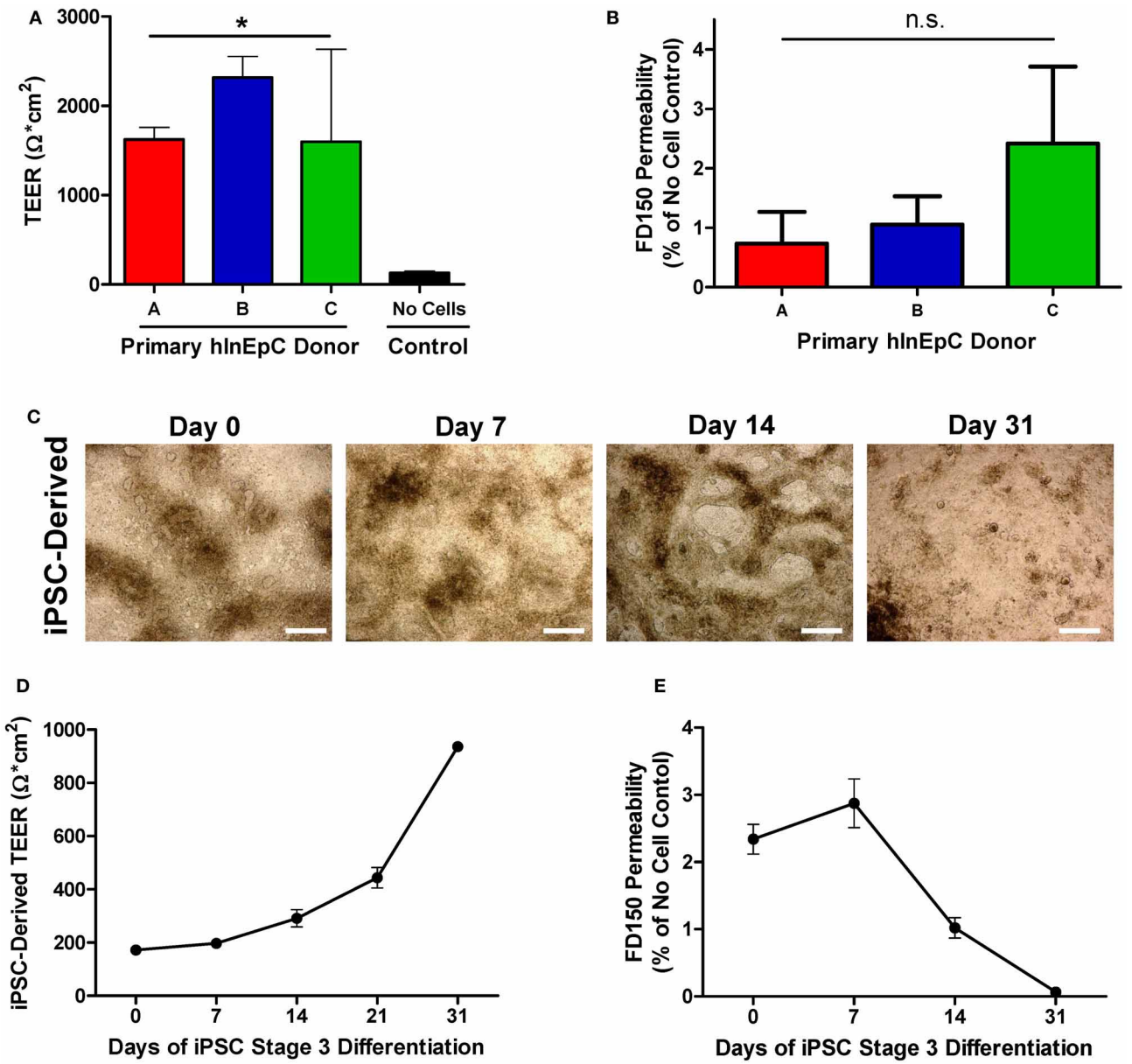

FIGURE 5 | Primary hInEpCs and iPSC-derived cells develop transwell monolayers with comparable integrity. (A) Primary hInEpC monolayers exhibited Transepithelial Electrical Resistance measurements of $> 1500\Omega \times cm^2$. **(B)** All primary hInEpC donors exhibit FITC-Dextran, molecular weight 150 kDa (FD150) permeability below 3%, relative to no cell control. **(C)** A1145A-derived cells lose dense structures during transwell differentiation in Stage 3 media, leaving behind confluent monolayer-like cells by Day 31. Scale bar, 250 μm. **(D)** Differentiating iPSCs (cell line A1145A shown) are capable of exhibiting increasing TEER during Stage 3 differentiation, reaching $> 900\Omega \times cm^2$ by Day 31. **(E)** A1145A cells exhibit low FD150 permeability with increasing days in Stage 3 culture relative to no-cell control. $N \geq 3$; Error bars represent SEM. For panels **(A)** and **(B)**, significance was determined by One-Way ANOVA, $^*p < 0.01$.

(H435A), however, maximal cell binding was not as high as Caco-2 (**Figure 6F**).

DISCUSSION

The major findings of this study are that marker expression of primary hInEpCs and iPSC-derived intestinal cells were on the same order of magnitude, or better than Caco-2, as determined by immunocytochemistry and mRNA expression analyses. The iPSC-derived intestinal cells were successfully adapted to differentiation within 2D transwell monolayer culture, and similar to primary hInEpCs, demonstrated functional tight junctions with TEER and low permeability similar to, or better than, Caco-2. Initial assessment suggested functional activity for intestinal transporters such as Pgp transport (primary hInEpCs) or FcRn-dependent binding of molecules (primary hInEpC and iPSC-derived cells). The main conclusions from our study are

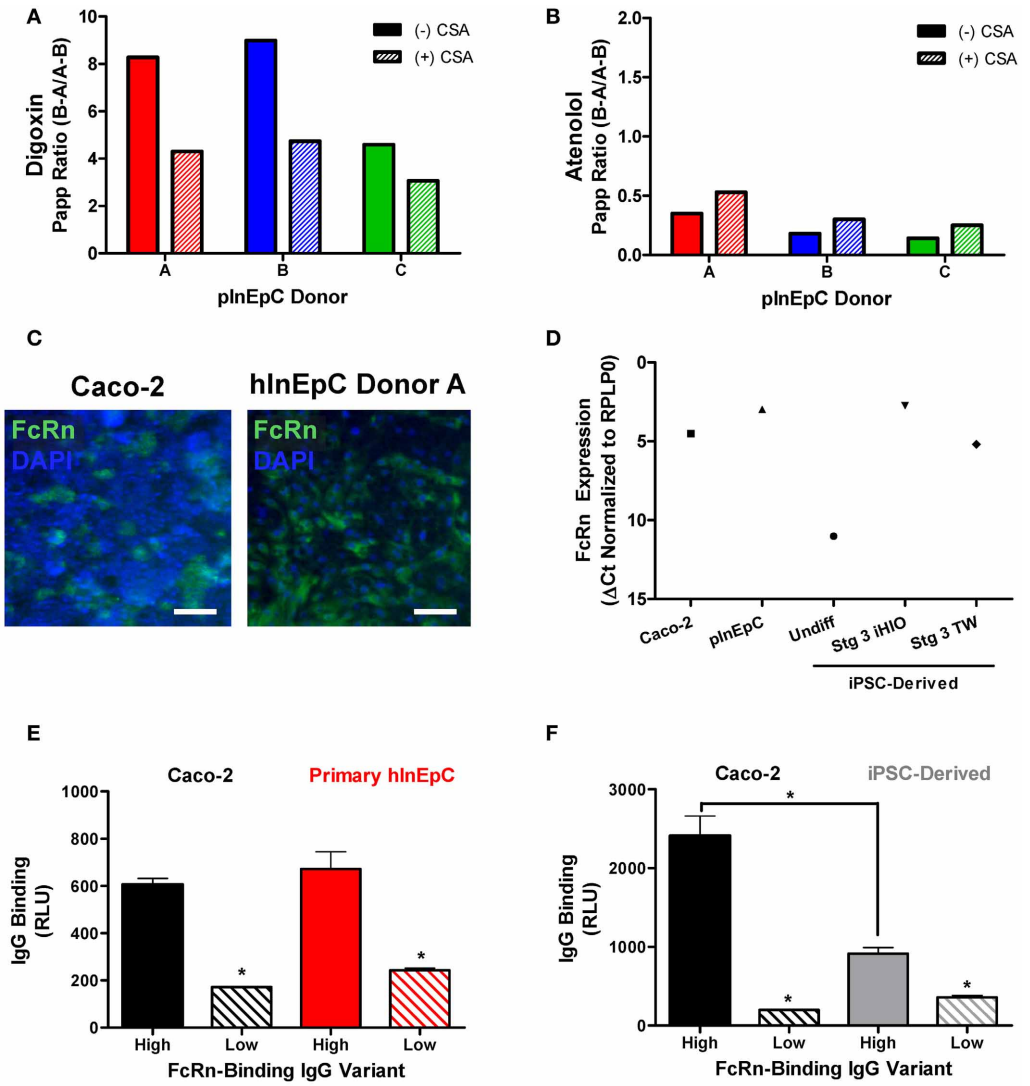

FIGURE 6 | Primary hInEpCs and iPSC-derived intestinal monolayers show functional activity in transwell culture. (A) pInEpC donors A and B show Digoxin Papp Ratios >8, which are decreased in the presence of cyclosporin A (CSA), suggesting that these cell lines have P-glycoprotein (Pgp) transport activity. **(B)** Atenolol Papp ratios for all samples fell below the expected <2 cutoff for Pgp activity. **(C)** Primary hInEpCs (right) show more uniform intracellular FcRn expression than Caco-2 (left), as shown by immunofluorescence. Scale bar, 100 μm. **(D)** iPSC-derived transwell cells express FcRn mRNA levels similar to Caco-2, but lower than primary hInEpCs and iPSC-derived 3D induced human intestinal organoids (iHIOs). Caco-2 **(E,F)**, primary hInEpCs **(E)** and iPSC-derived transwell cells **(F)** show FcRn-dependent mAb binding by MSD assay, as demonstrated by statistically greater cell binding of high FcRn-binding IgG variants [High, N434A **(E)** or M428L **(F)**] than low FcRn-binding variants (Low, H435A). $N \geq 4$; Error bars represent SEM. **(E,F)**, statistical significance determined by *t*-test, $^{*}p < 0.001$.

further discussed below in relation to previously published studies.

Before using a recently released commercially available source of primary hInEpCs for comparing iPSC-derived intestinal cells, it was necessary to more fully characterize this intestinal epithelial cell source for marker expression and barrier function. The extent to which primary hInEpCs expressed markers by immunofluorescence varied, with expression of E-Cadherin, Villin, and CDX2 strongest in Donor A; however, intestinal marker expression appeared to be more consistent between donors by RT-PCR analysis. While all three donors exhibited high TEER ($> 1500\Omega \times cm^2$), TEER and FD150 permeability showed high variability in Donor C. Moreover, Donor C showed poor efflux of Pgp-transported Digoxin, suggesting that this primary hInEpC donor is less useful for intestinal barrier function and transport than Donors A and B.

A 3D directed intestinal organogenesis protocol was successfully adapted to 2D transwell differentiation, based on intestinal marker similarity. Stage 1/Definitive endoderm markers SOX17 and CXCR4 were expressed in 40–87% of the cell population. Stage 2-marker CDX2 was also robustly expressed within

transwell cultures. Stage 3 differentiation within transwells of iPSC-line A1145A (Kauffman et al., Submitted) was similar to the development of iHIOs, except that spheroid structures that formed by Stage 2 of differentiation were not excised for 3D culture, but allowed to continue differentiation within the adherent layer of cells. Interestingly, spheroid structures found at Stage 2 were gradually lost, so that by Stage 3, Day 31, a monolayer-like morphology was found within transwell cultures, which showed TEER measurements more similar to primary hInEpCs and greater than the average TEER for Caco-2 transwell cultures (Le Ferrec et al., 2001). Consistent with this result, low permeability of FD150 for primary hInEpCs and iPSC-derived transwell cells suggests that a barrier function was present in these cell cultures. It is important to note that the TEER obtained in this study for human intestinal epithelial cell sources is several-fold higher than typically found in intestinal tissue *ex vivo*; however, high TEER values may not discount the physiological relevance of these cells *in vitro*, as the presence of several epithelial layers and cell types within intestinal tissue can add multiple impedances to intestinal transmural TEER measurements (Gitter et al., 1998, 2000).

In addition to A1145A, which shows consistent robust intestinal differentiation in 3D and 2D monolayer culture, we also performed a transwell differentiation time course on C2128A because this cell line showed uniform expression of CDX2 at Stage 2 within transwell culture, but demonstrated a distinct morphology. At Stage 2, C2128A cells appeared to already exhibit a monolayer-like morphology with little to no dense structures; therefore, we reasoned that C2128A might be more amenable to the development of iPSC-derived intestinal cells for use in functional transwell monolayer assays. However, as Stage 3 differentiation of C2128A-derived progressed, holes appeared within the layer of cells, which corresponded to low TEER measurements and high FD150 permeability at Stage 3 (**Figure A5**). Thus, differences in iPSC line intestinal differentiation capacity appear to be evident during 2D transwell culture differentiation.

mRNA expression analysis of an extensive panel of intestinal marker and control genes enabled a direct comparison of primary hInEpCs and iPSC-derived cell maker expression to Caco-2 cells. As expected, undifferentiated iPSCs showed levels of marker expression several fold lower than all intestinal samples with the exception of the pluripotent cell control marker OCT4 (Nichols et al., 1998). Differentiated iPSC-derived iHIOs and transwell cells exhibited upregulated intestinal marker expression that was more similar to primary hInEpCs than Caco-2, consistent with a better *in vitro* representation of human intestinal epithelia than Caco-2.

As might be expected, expression of definitive endoderm marker CXCR4 was highest in Stage 3 iHIOs and transwell cells, which went through a definitive endoderm intermediate at Stage 1. Similarly, expression of the mesenchymal marker Vimentin was also most highly upregulated in iPSC-derived intestinal cells, relative to Caco-2. This result is consistent with previously reported increases in expression of Vimentin and another mesenchymal marker, Forkhead Box F1 (Mahlapuu et al., 1998), in intestinal cells derived from human embryonic stem cells, any may indicate the development of intestinal subepithelial myofibroblasts (Spence et al., 2010).

The intestinal stem cell marker LGR-5 (Barker et al., 2007) was highly upregulated in iPSC-derived intestinal cells relative to Caco-2, which may reflect more active intestinal cell proliferation within these cells, particularly in iHIOs. Expression of Paired Box Gene 6 (PAX6), with known roles in brain development (Mastick et al., 1997), was also highly expressed in iHIOs cells, with less expression in Stage 3 iPSC-derived transwell cells or the other intestinal sources tested. This is consistent with the high Chromogranin A expression in the development of a more mature enteroendocrine system within these cells, as PAX6 also has a known role in the development of Glucagon-like peptide (GLP)-1 and GLP-2 secreting cells (Fujita et al., 2008; Ye and Kaestner, 2009).

While intestinal marker expression in primary of iPSC-derived cells was very encouraging, it was unclear how functional these cells are compared to other established *in vitro* intestinal models, such as Caco-2 or primary tissue. For example, in pluripotent stem-cell derived models of liver or pancreas, progenitor cells must be engrafted into whole animals to produce mature, fully-functional cell types (Liu et al., 2011; Rezania et al., 2011). We realized that adaptation of directed intestinal organogenesis to 2D transwell culture would allow a more direct comparison of iPSC-derived intestinal cell barrier and transport functions to previously characterized intestinal epithelial cell models *in vitro*. Well-studied intestinal transport mechanisms within Caco-2 and primary human intestinal tissue include efflux of small molecules by membrane associated Pgp transporters, (Murakami and Takano, 2008), and intestinal receptor-mediated antibody transcytosis by FcRn (Dickinson et al., 1999; Claypool et al., 2004). These mechanisms were used as an initial assessment of functional quality of primary hInEpCs and iPSC-derived intestinal cells.

Primary hInEpCs A and B showed cyclosporine A-dependent efflux of Digoxin, but not Atenolol, indicative of the presence of Pgp transport activity in these cells. In addition to primary hInEpCs (Donor A), Stage 3 iPSC-derived transwell cells showed FcRn expression and FcRn-dependent mAb binding using high or low FcRn-binding IgG variants; however, unlike primary hInEpCs, iPSC-derived IgG binding did not occur to as large an extent as Caco-2. It should also be noted that in all FcRn-binding experiments, the cell binding signal, while well above background noise, was relatively low for this ELISA-based assay. Furthermore, since FcRn is expressed in neonatal and adult intestinal epithelial cells in primates (Israel et al., 1997; Dickinson et al., 1999) and marker expression during differentiation of iHIOs closely resembles that of embryonic intestinal development (Spence et al., 2010), it is still unclear whether the phenotype of these cells more closely resembles embryonic or adult intestinal tissue.

In summary, our studies indicate that iPSC-derived intestinal cells and newly commercially available primary hInEpCs may provide an alternative source of physiologically relevant hInEpCs. Future studies will be needed to further evaluate the function of primary hInEpCs and iPSC-derived

intestinal cells. Additionally, as these cells are still limited by their viability (hInEpCs) and extensive differentiation time (iPSC-derived cells), large-scale studies will require additional development of methods for scale up or long-term maintenance of these transwell cultures, such as non-oncogenic immortalization strategies.

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APPENDIX

RESULTS

In addition to intestinal cell-specfic markers (**Figure 4**), other control markers were assessed by RT-PCR to evaluate sample quality of iPSC-derived cells, Caco-2, and primary intestinal cells (**Figure A2**). iPSC-derived cells and primary intestinal cells showed very low expression of pluripotent cell transcription factor POU domain, class 5 (OCT4) (Nichols et al., 1998), and about 2000-fold lower than undifferentiated cells (**Figure A2A**). To determine how successfully iPSC-derived intestinal cells differentiated away from a definitive endoderm cell type after Stage 1, expression of CXCR4 was analyzed. As somewhat expected, expression of this definitive endoderm marker was highest in iPSC-derived cells, which went through a definitive endoderm intermediate at Stage 1, with expression levels ranging about 150-fold higher relative to Caco-2 (**Figure A2B**). Expression of the pancreatic foregut marker PDX1 (Miller et al., 1994) was found to be 10–15-fold higher in iPSC-derived cells and primary hInEpCs relative to Caco-2 (**Figure A2C**). Similarly, expression of the mesenchymal marker Vimentin was also highly upregulated in primary hInEpCs and iPSC-derived intestinal cells, relative to Caco-2 (**Figure A2D**).

Neuronal marker Tubulin, beta 3 (TUBB3) (Poirier et al., 2010), expression was higher in iPSC-derived cells and primary hInEpCs relative to Caco-2 (**Figure A2E**). Expression of cardiac cell marker Troponin T, type 2 (TNNT2) (Townsend et al., 1994) was the comparable across intestinal cell types (**Figure A2F**). Expression of Paired Box Gene 6 (PAX6), with known roles in brain development (Mastick et al., 1997), was highly expressed only Stage 3 iHIOs (**Figure A2G**).

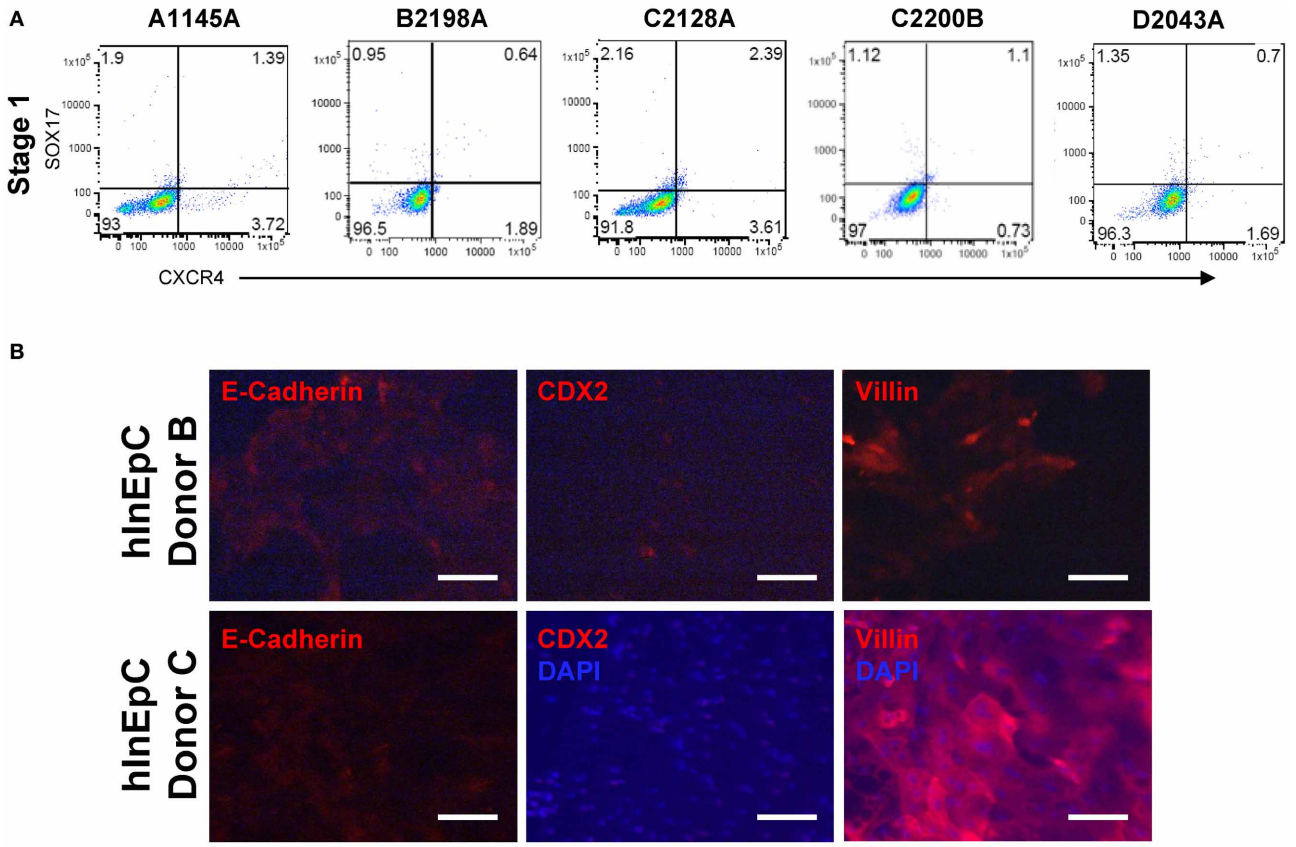

FIGURE A1 | Additional protein expression data. (A) Isotype controls used for flow cytometry plot gating in **Figure 3B**. **(B)** Expression of intestinal markers E-Cadherin, CDX2, and Villin is shown in primary hInEpC Donors B (top row) and C (bottom row).

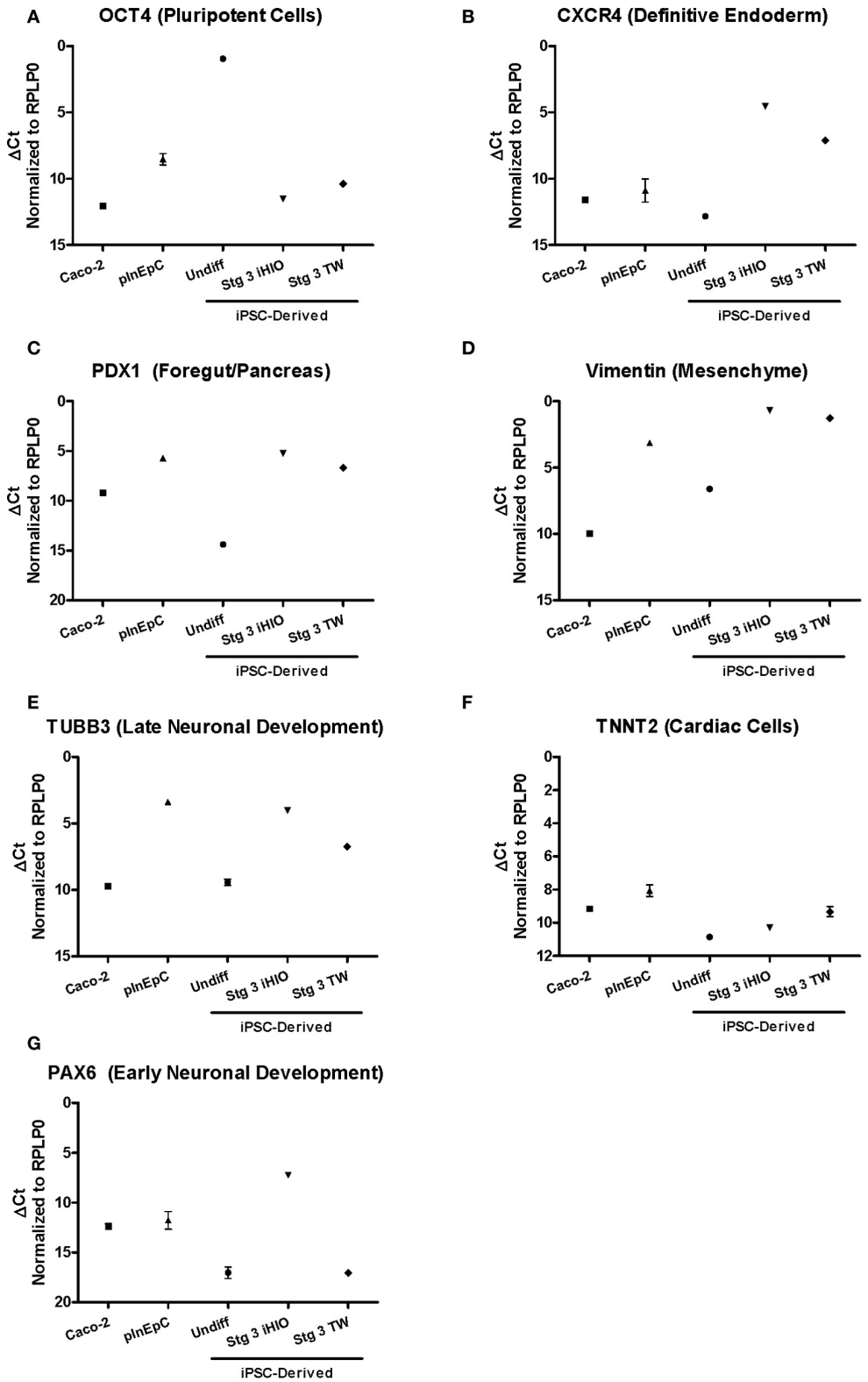

FIGURE A2 | Validation of intestinal cell RT-PCR analysis through a panel of control/non-intestinal markers. Expression of intestinal markers was evaluated by RT-PCR in Caco-2, primary hInEpCs (average of Donors A–C), and iPSC-derived undifferentiated (Undiff), Stage 3 induced human intestinal organoids (Stg 3 iHIOs), or Stage 3 transwell intestinal cells (Stg 3 TW). Using the DCt method, raw *Ct* values were normalized to housekeeping gene RPLP0. $N = 3$; Error bars represent SEM.

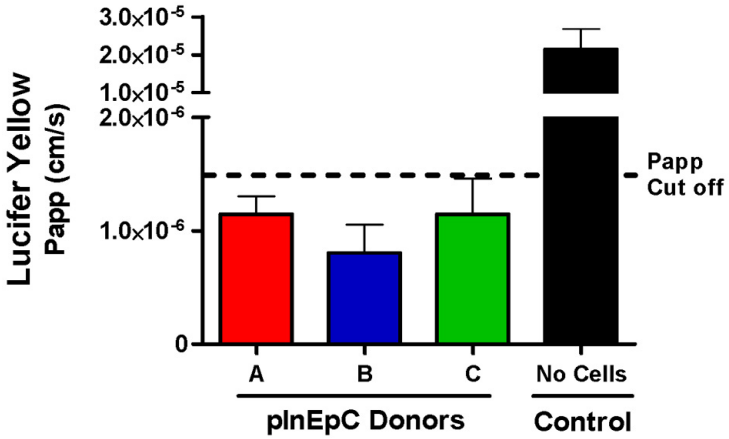

FIGURE A3 | All primary hInEpC donors used within the P-glycoprotein transport assay showed an average lucifer yellow apparent permeability (Papp) below accepted cutoff for intact monolayers **1.5 x 10^{-6} cm/s.** Only transwells that met this cutoff were used to calculate Papp ratios for Digoxin and Atenolol (**Figure 6**). $N = 3$; Error bars represent SEM.

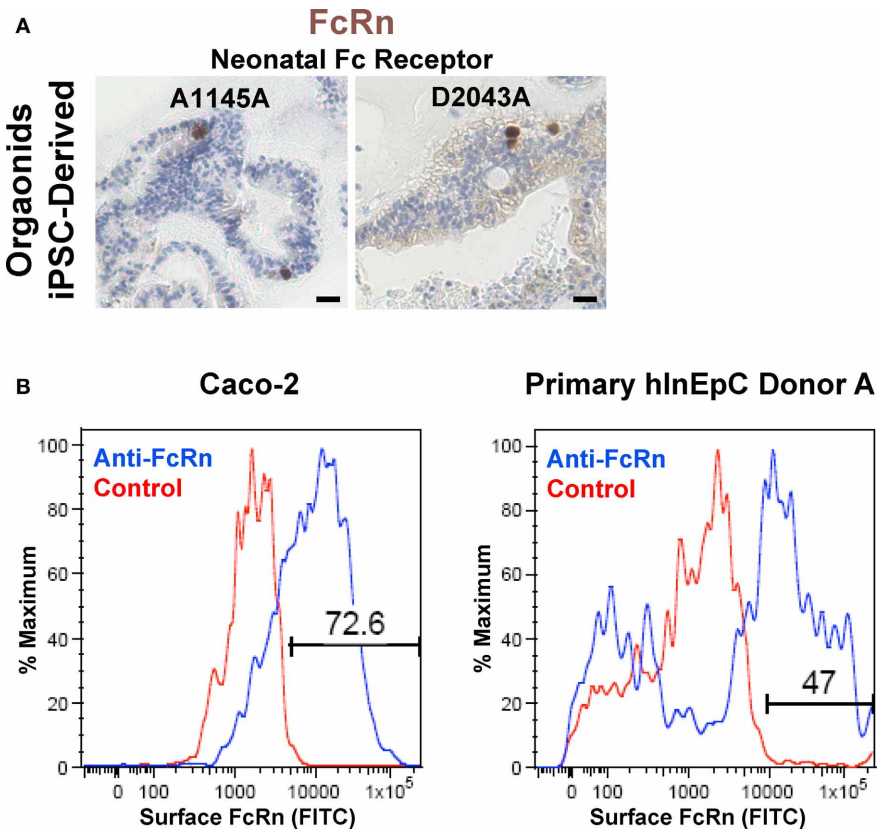

FIGURE A4 | Intestinal epithelial cell expression of FcRn. (A) Immunohistochemical analysis demonstrates iPSC-derived Stage 3 intestinal organoid expression and punctate localization of neonatal Fc Receptor expression in iPSC-derived organoids. Scale bar, 100 μm. **(B)** Flow cytometry analysis shows surface FcRn expression on Caco-2 (left) and primary hInEpCs (right, Donor A shown).

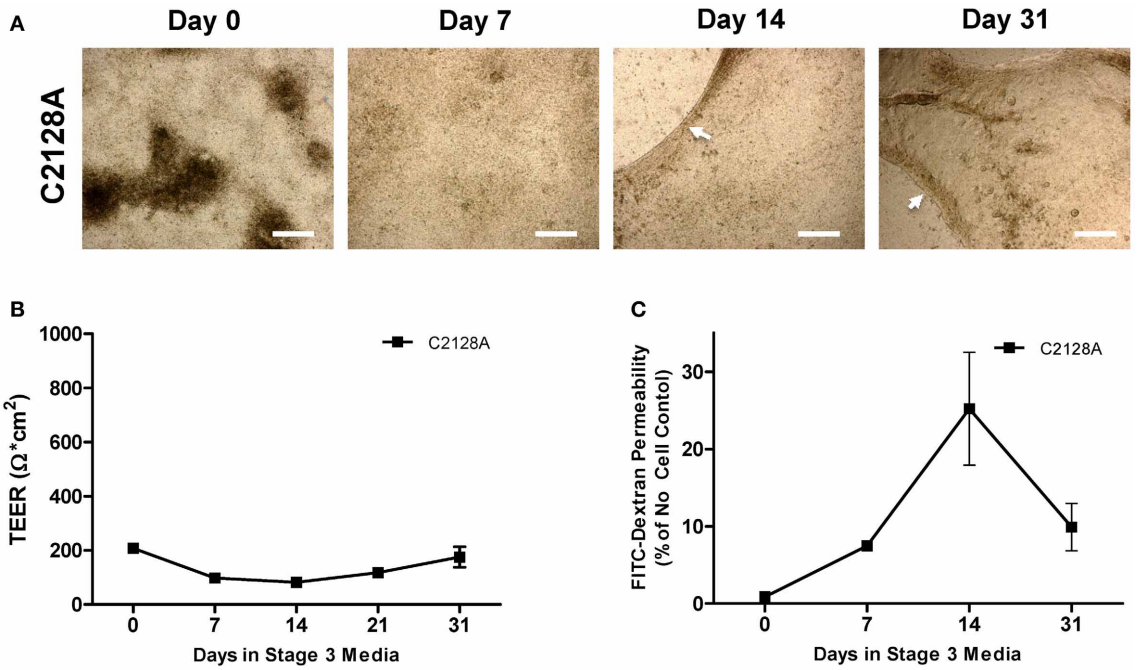

FIGURE A5 | Not all iPSC lines are capable of producing transwell intestinal cell monolayers with characteristics suggestive of tight junction formation. (A) Morphology of during C2128A transwell differentiation shows that cells lose dense structures during differentiation; however, the monolayer begins to pull into itself during late Stage 3 differentiation (arrow heads), resulting in large holes on transwell inserts. Scale bar, 250 μm. **(B)** C2128A cells do not show increasing TEER with increasing days in Stage 3 culture. **(C)** C2128A shows high FD150 permeability at later stages of differentiation. $N = 3$; Error bars represent SEM.

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