BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 367–375

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

A novel role for the metabotropic glutamate receptor-7: modulation of faecal water content and colonic electrolyte transport in the mouse

M Julio-Pieper[1], NP Hyland[1,2], JA Bravo[1], TG Dinan[1,3] and JF Cryan[1,2,4]

[1]*Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland,* [2]*Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland,* [3]*Department of Psychiatry, University College Cork, Cork, Ireland, and* [4]*School of Pharmacy, University College Cork, Cork, Ireland*

Background and purpose: Increasing evidence implicates metabotropic glutamate receptor $mGlu_7$ in the pathophysiology of stress-related disorders such as depression and anxiety. Mood disorders are frequently associated with gastrointestinal (GI) dysfunction; however, the role of $mGlu_7$ receptors outside the CNS is unknown. This present study investigated the expression and possible functional role of $mGlu_7$ receptors in the mouse colon.
Experimental approach: Expression of $mGlu_7$ receptor mRNA and protein was studied in mouse colon by *in situ* hybridization and Western blotting. Effects of the selective $mGlu_7$ receptor agonist AMN082 on defecation and faecal parameters were studied in an isolation-induced stress model. AMN082 effects on ion transport and neuronal intracellular signalling were examined via Ussing chambers and calcium imaging.
Key results: $mGlu_7$ receptor mRNA and protein were highly expressed in colon mucosa. Stress-induced faecal output was unaffected by AMN082, although faecal water content was increased. In mucosa/submucosa preparations, 100 nM and 1 μM AMN082 increased bethanechol-induced changes in short-circuit current in the Ussing chamber. This was sensitive to tetrodotoxin. Also, 100 nM AMN082 significantly increased calcium signalling in a subset of submucosal neurons.
Conclusions and implications: Activating $mGlu_7$ receptors increased colonic secretory function *in vivo* and *ex vivo*. In a group of submucosal neurons, AMN082 strongly induced calcium signalling and the presence of submucosal nerves was required for the AMN082-dependent increase in secretion. These data suggest that targeting $mGlu_7$ receptors may be useful in the treatment of central components of stress disorders and also stress-associated GI dysfunction such as diarrhoea or constipation.
British Journal of Pharmacology (2010) **160,** 367–375; doi:10.1111/j.1476-5381.2010.00713.x

Keywords: AMN082; irritable bowel syndrome; stress

Abbreviations: BCh, bethanechol; ENS, enteric nervous system; GI, gastrointestinal; IBS, irritable bowel syndrome; Muc, mucosal scraping; TTX, tetrodotoxin

Introduction

L-Glutamate is the major excitatory neurotransmitter in the CNS (Robinson and Coyle, 1987; Conn, 2003; Watkins and Jane, 2006). Two types of receptors mediate the actions of glutamate: ionotropic receptors are glutamate-gated ion channels which regulate fast responses upon activation (Watkins and Jane, 2006), and metabotropic receptors (mGlu; nomenclature follows Alexander *et al.*, 2009), which are coupled to G proteins and modulate signal transduction cascades (Conn, 2003). mGlu receptors are responsible for the fine-tuning of glutamate signalling in the CNS, and, based on their sequence homologies and signal transduction mechanisms, have been further divided into three subtypes, namely group I ($mGlu_1$ and $mGlu_5$), group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) (Conn, 2003; Lavreysen and Dautzenberg, 2008).

Receptors from groups I and II have been implicated in various neuropathological disorders (Conn, 2003; O'Connor and Cryan, 2010). Group III mGlu receptors are the least investigated, mainly because of the lack of suitable pharmacological agents. However, the emergence of selective compounds and the development of transgenic animal models have shed some light into the potential role of group III mGlu receptors in the pathophysiology of mood disorders

Correspondence: Dr John F Cryan, School of Pharmacy, Cavanagh Pharmacy Building, University College Cork, Cork, Ireland. E-mail: j.cryan@ucc.ie
Received 2 November 2009; revised 18 December 2009; accepted 11 January 2010

(Lavreysen and Dautzenberg, 2008). Among these receptors, $mGlu_7$ is of particular interest because genetic deletion of this receptor produced reduced anxiety- and stress-related behaviours (Cryan *et al*., 2003). The recent discovery of AMN082, the first selective agonist for $mGlu_7$ receptors, which acts at an allosteric site, has been an invaluable tool in furthering our understanding of $mGlu_7$ receptors in stress-related physiology and behaviour (Mitsukawa *et al*., 2005; O'Connor *et al*., 2010). Together, such studies demonstrate that $mGlu_7$ receptors are an important component in the molecular mechanism underlying depression and anxiety. Moreover, they suggest that targeting $mGlu_7$ receptors may be useful in the treatment of conditions associated with chronic stress. Nevertheless, little is known about the role of $mGlu_7$ receptors outside the CNS.

Mood disorders are frequently associated with gastrointestinal (GI) dysfunction (Gros *et al*., 2009), and irritable bowel syndrome (IBS) is a clear example of a disorder because of an alteration of the brain–gut axis (Quigley, 2006; Clarke *et al*., 2009). High levels of anxiety, sleep disturbance and psychosomatic problems are risk factors for the development of IBS (Clarke *et al*., 2009), and a high frequency of IBS symptoms is found in patients with panic disorder, generalized anxiety disorder and major depressive disorder (Gros *et al*., 2009). In addition, stress also induces exacerbation of symptoms and visceral hypersensitivity in patients with IBS (Whitehead *et al*., 1992; Posserud *et al*., 2004). IBS is classified as either diarrhoea-predominant, constipation-predominant or alternating (Clarke *et al*., 2009). As such, therapies addressing both central and GI components of chronic stress are highly desirable.

In addition to being a key central neurotransmitter, glutamate is also present in the enteric nervous system (ENS). Functional ionotropic and metabotropic glutamate receptors are found in the ENS (Jankovic *et al*., 1999; Larzabal *et al*., 1999; Giaroni *et al*., 2000; Tong and Kirchgessner, 2003); however, the contribution of $mGlu_7$ receptors to colonic function remains unexplored. Therefore, this study aimed to investigate colonic $mGlu_7$ receptor expression and to determine a possible functional role of these receptors in the colon by assessing agonist-induced alterations in secretory function. We found that activation of $mGlu_7$ receptors increased faecal water content *in vivo* and colonic secretory function *ex vivo* and thus confirmed the functional relevance of the expression of $mGlu_7$ receptors in the colon.

Methods

Animals

All animal care and experimental procedures were carried out in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation and Ethics Committee of University College Cork (Animal Ethical Review Request #2007/14). Adult male C57/BL6 mice weighing 25–30 g were obtained from the Biological Services Unit, University College Cork and from Harlan, UK. Animal rooms remained temperature controlled (21 ± 1°C) and with a 12 h light/dark cycle (lights on at 07:00h). Animals were housed 3–4 per cage for *in vivo* experiments or 8 per cage for *ex vivo* experiments. Cages were cleaned once a week as part of the animal room routine.

In situ *hybridization*

Distal colon samples obtained from experimentally naïve mice were rinsed in cold phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde and dehydrated in 30% sucrose. The 10-μm cryostat sections were mounted on superfrost slides and subjected to *in situ* hybridization, as described previously (Bravo *et al*., 2006). Briefly, slides were treated with proteinase K (Sigma-Aldrich, Dorset, UK, 0.5 $mg \cdot mL^{-1}$), acetylated and dehydrated in a series of ethanol (70%, 95% and 100%). Hybridization was performed overnight in a solution containing 50% formamide and sodium citrate buffer. The $mGlu_7$ receptor cDNA probe used, complementary to bases 1292–1336 of the mouse mRNA sequence (accession number NM_177328.3), had a digoxigenin label at the 3′-OH end. For detection, an anti-digoxigenin antibody conjugated to alkaline phosphatase was used and finally, the substrate NBT/BCIP (Sigma) was added. The reaction was stopped when a violet precipitate was present on the tissues. Slides were photographed with a digital camera (Olympus DP71, Olympus, Hamburg, Germany) attached to a microscope (Olympus BX51).

Western blotting

Two different types of sample were obtained from mouse distal colon; these consisted of mucosal scrapings (Muc) or the remaining tissue left after scraping, containing the enteric nervous system together with the muscles (ENS + muscle). Forty micrograms of total protein from Muc or ENS + muscle samples was run in 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was boiled for 40 min in sodium citrate buffer pH 1.2, blocked with 1% milk and incubated overnight with an anti-$mGlu_7$ receptor antibody (1:300, Abnova, Heidelberg, Germany). After treating with the secondary antibody (1:3000, 2 h), the blots were incubated with ECL reagent (Pierce, Rockford, IL, USA) and then exposed for 1 h and photographed in a luminescent image analyser (Fujifilm, Bedford, UK).

Stress-induced defecation and faecal water content

The selective $mGlu_7$ receptor agonist (AMN082, Ascent Scientific, Bristol, UK) was dispersed in a suspension of 0.5% methylcellulose (Sigma) and administered orally (0, 3 and 6 $mg \cdot kg^{-1}$) 1 h before stress. Doses were chosen based on previous experiments showing behavioural and physiological changes in this range (Mitsukawa *et al*., 2005; Stachowicz *et al*., 2008). A constant volume of 10 $mL \cdot kg^{-1}$ was given to avoid differences in gastric emptying time. The stress-induced defecation protocol was based on previously published protocols (Miyata *et al*., 1992; Banner *et al*., 1996; Wang *et al*., 2007). Briefly, mice were transferred to individual cages (19 × 31 cm, lined with bright non-absorbent white paper, under bright light) and monitored constantly over 90 min. To prevent water absorption, no bedding was included in the observation cages; also, to minimize the risk for water evaporation and coprophagia, faecal pellets were collected at 15 min intervals. Faeces were then weighed (wet weight, in mg), desiccated in an oven (50°C, 16 h), and weighed again (dry weight, in mg). Faecal water content was calculated according to the equation: water content (%) = 100 (wet

weight – dry weight)/wet weight. All *in vivo* experiments were performed between 9 a.m. and 12 a.m.

Ussing chamber experiments

Mouse colon samples were placed in cold Krebs buffer (1.2 mM NaH_2PO_4, 117 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 11 mM glucose), opened along the mesenteric line and carefully rinsed. Up to six preparations were obtained, after stripping away the muscle layers, thereby leaving only mucosa and submucosa, from each mouse. Preparations were then placed in Ussing chambers (Harvard Apparatus, Kent, UK, exposed area of 0.12 cm^2) as described previously (Hyland and Cox, 2005) with oxygenated (95% O_2, 5% CO_2) Krebs buffer maintained at 37°C, and voltage-clamped at 0 mV (World Precision Instruments, Sarasota, FL, USA). Subsequent changes in short-circuit current, observed in response to various experimental protocols, were continuously monitored using DataTrax 2 software (World Precision Instruments). Treatments were assigned in a randomized manner. For protocol 1, after stabilization, tissues were treated basolaterally for 30 min with AMN082 (0, 30 nM, 100 nM and 1 μM), and subsequently stimulated with bethanechol (Sigma, 100 μM). For protocol 2, tissues were pretreated basolaterally for 15 min with tetrodotoxin (TTX; Sigma, 300 nM), then subsequently for 30 min with AMN082 (0, 100 nM and 1 μM), followed by bethanechol (100 μM). Results were expressed as changes in short-circuit current ($\mu A \cdot cm^{-2}$). Tissue resistance was calculated at the beginning and the end of each experiment, to assess the effect of AMN082 on transepithelial permeability.

Calcium imaging

Mouse colon samples were placed in oxygenated (95% O_2, 5% CO_2) Krebs buffer containing 1 μM nifedipine (Sigma). Muscle layers and mucosa were stripped away, leaving only the layer containing the submucosal plexus. This submucosal preparation was pinned flat in a small Sylgard-coated dish (Dow Corning Co., Midland, MI, USA), loaded with the cell-permeant dye fura-2AM (Invitrogen, Carlsbad, CA, USA, 7 μM, 1 h), and rinsed with Krebs buffer. A superfusion system allowed addition and subsequent wash of drugs at a rate of 1.5 $mL \cdot min^{-1}$ with a lag time of 1 min. To prevent tissue movement and subsequent loss of focus, 1 μM nifedipine was present during the entire experiment. After a 3 min equilibration time, 3 mL of each drug was consecutively added at 15 min intervals, in the following order: vehicle (DMSO, Sigma, 0.1% v/v), 30 nM AMN082, 100 nM AMN082, 1 μM AMN082 and 100 nM AMN082. At the end of the experiment, tissues were exposed to 75 mM KCl. The entire protocol was conducted at room temperature.

Changes in intracellular calcium (i.e. ratio of fluorescence at 340 and 380 nm) were captured by a fluorescent microscope (MT20 illumination system, Olympus) coupled to a digital epifluorescence system (Cell R, Olympus). Only cells showing a calcium influx upon addition of the positive control KCl were used for further analyses. Cells showing a response to AMN082 greater than the mean +2 standard deviations of the vehicle were considered 'responders' (Gulbransen *et al.*, 2008). Results were expressed as % of the vehicle response and all experiments were performed in duplicate (i.e. two submucosal preparations were analysed per mouse).

Statistical analysis

Values are expressed as mean ± s.e.mean. Changes in faecal water content and intracellular calcium were evaluated using one-way ANOVA, and changes in short circuit current were analysed using two-way ANOVA. Results were further analysed with either Newman-Keuls' or Bonferroni's post-tests, where appropriate. Statistical analyses were performed using GraphPad Prism4 software (GraphPad Software Inc., La Jolla, CA, USA).

Materials

AMN082 (N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride) was purchased from Ascent Scientific Ltd. Nifedipine, bethanechol and tetrodotoxin were purchased from Sigma-Aldrich.

Results

Expression of $mGlu_7$ receptors in the mouse colon

In situ hybridization of colon sections showed an intense signal for $mGlu_7$ receptor mRNA in the mucosa (Figure 1A and B), whereas staining in the circular and longitudinal muscle layers was not noticeably higher than the background signal observed in the negative control (see insert, Figure 1A). Within the mucosa, particularly strong staining was observed in some cells of the surface epithelium as well as in the bottom of crypts (Figure 1A). Goblet cells also appeared stained, as shown in Figure 1B. Subsequent Western blot analysis indicated the presence of $mGlu_7$ receptor protein in colon mucosa. These receptors were also present in preparations containing ENS together with the muscle layers (Figure 1C). Levels of $mGlu_7$ receptor protein in the colon were comparable to those found in the hippocampus of mouse brain (Figure 1C).

Effects of AMN082 on stress-induced defecation and faecal water content

The presence of $mGlu_7$ receptors in preparations of colon containing ENS + muscle as well as in the mucosa suggested that this receptor could have a potential role in modulating colonic motility and/or liquid absorption or secretion respectively. A time-course study showed that stress-induced defecation had a rapid onset and occurred predominantly within 15 min of stress. By 60 min of stress, most of the mice had stopped defecating. We noticed that when faeces were collected into glass vials with the purpose of weighing them, faeces produced by AMN082-treated mice tended to stick to the bottom of the vial, whereas faeces from vehicle-treated mice did not stick and moved freely when the vial was gently shaken. Faeces were then weighed, desiccated and weighed again to determine the percentage of water content. AMN082 (3 $mg \cdot kg^{-1}$) significantly increased the faecal water content in a time-dependent manner ($F(2,15) = 4.304$; $P < 0.05$, Figure 2A). As very few animals defecated after 45 min stress,

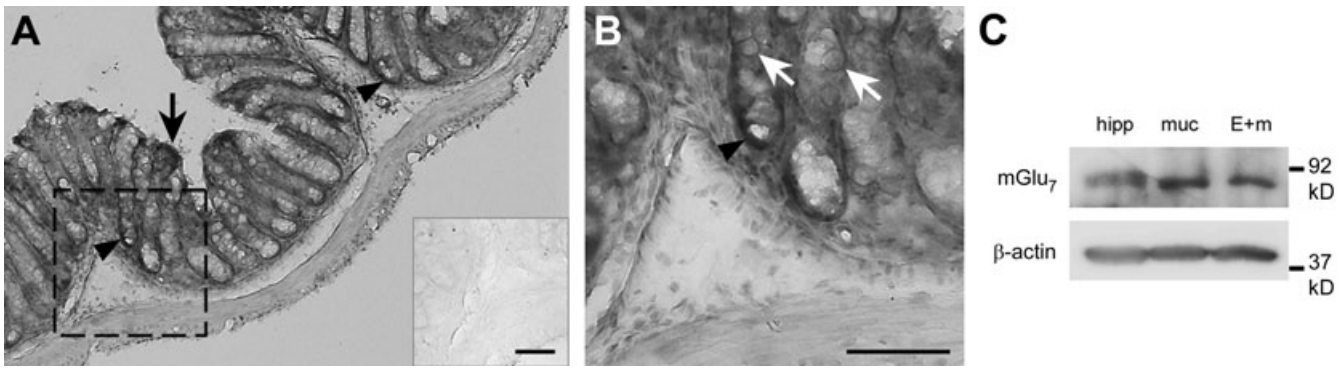

Figure 1 Expression of $mGlu_7$ receptors in the mouse colon. (A and B) *In situ* hybridization of $mGlu_7$ receptor mRNA in mouse colon. Staining in the surface epithelium (black arrow), bottom of crypts (black arrowheads) and goblet cells (white arrows) is shown. Negative control of a consecutive section incubated without probe is shown in the insert, representing a region equivalent to the one depicted in B. Bar: 100 μm. (C) Western blot of $mGlu_7$ receptors in mouse colon. Representative samples of mouse hippocampus (hipp), colon mucosa (muc) and enteric nerves + muscle (E+m) are shown.

only faeces collected during the first 45 min were considered for the statistical analysis of water content. This was also significantly increased by 3 and 6 $mg \cdot kg^{-1}$ AMN082 ($F(2,11) = 6.201$; $P < 0.05$, Figure 2B).

Faecal output expressed either as the number of faecal pellets (Figure 2C and D) or faecal wet weight (Figure 2E and F) was not affected by AMN082 at any interval. The 90 min cumulative faecal output also showed no change.

Effects of AMN082 on electrolyte transport ex vivo

As AMN082 crosses the blood brain barrier and can affect the CNS (Mitsukawa *et al*., 2005), it was important to study the isolated colon to determine if the effects of the selective mGlu7 receptor agonist on increasing faecal water content were, in part, mediated via a peripheral pathway. Colon mucosa regulates the passage of electrolytes and water between the intestinal lumen and the body, a process that is also regulated by enteric nerves located in the submucosal plexus (Goyal and Hirano, 1996). For this reason, colonic preparations containing mucosa and submucosa were analysed in Ussing chambers and changes in short-circuit current (I_{SC}) recorded. AMN082 had no effect on basal I_{SC} *per se*, as shown in Figure 3A and B. However, when the acetylcholine analogue, bethanechol 100 μM was added to stimulate the Ca^{2+}-dependent ion secretion, AMN082 significantly amplified the bethanechol-induced increase in I_{SC} ($F(1,62) = 163.4$, $P < 0.001$). *Post hoc* tests showed a significant effect of 30 nM AMN082 ($P < 0.01$), 100 nM AMN082 ($P < 0.01$) and 1 μM AMN082 ($P < 0.005$) versus vehicle when followed by bethanechol addition (Figure 3B), indicating that pre-incubation with this mGlu7 receptor agonist has a positive effect on Ca^{2+}-mediated secretion. AMN082 had no effect on transepithelial permeability at any dose (data not shown).

Figure 3C demonstrates the dependence of the AMN082-induced increase in the response to bethanechol on submucosal nerves, as pre-incubation with the neuronal blocker TTX ablated this response. TTX alone also induced an increase in the response to bethanechol, suggesting the presence of tonically active, inhibitory, neurons in our colon preparations ($P < 0.05$, Figure 3C).

Intracellular calcium mobilization induced by AMN082

Ussing chamber data showed that activation of $mGlu_7$ receptors induced an increase in a calcium-mediated response that was sensitive to TTX, suggesting that neurons are one of the components in this response. As a consequence of these findings, we decided to analyse the effects of AMN082 on calcium signalling in the submucosal neurons. As shown in Figure 4A and B, AMN082 stimulated strong intracellular calcium mobilization ($F(4,15) = 3.540$; $P < 0.05$), but only in a small subset of submucosal neurons (19%), when 100 nM AMN082 was used ($P < 0.05$, Figure 4B). Desensitization of $mGlu_7$ receptor signalling appears to have occurred as a second application of the same dose of AMN082 (100 nM) on the same submucosal preparation induced a response that was not statistically different from the vehicle (Figure 4B). Figure 4C illustrates a representative submucosal ganglion where only one cell shows a change in fluorescence (i.e. calcium mobilization) in response to the first addition of 100 nM AMN082.

Discussion

In the present work we demonstrated that $mGlu_7$ receptors were expressed in the mouse colon, and that selective activation of the receptor with AMN082 increased faecal water content upon stress, and amplified electrolyte secretion induced by a cholinomimetic agent in the isolated mouse colon. In addition, AMN082 induced intracellular calcium mobilization in a subset of submucosal neurons.

Glutamate is largely known as a key participant of intestinal metabolism (Blachier *et al*., 2009). It is an important oxidative substrate for the intestinal mucosa, which has a high rate of epithelial growth and cell turnover. Glutamate is also a precursor for the biosynthesis of glutathione, which in turn protects proliferating cells from oxidative damage (Reeds *et al*., 2000; Blachier *et al*., 2009). Unlike the small intestine, the source of colonic glutamate is mainly the arterial blood and not luminal contents (Blachier *et al*., 2009). In addition, glutamate is an important activator of vagal afferents that innervate the GI tract (Partosoedarso and Blackshaw, 2000)

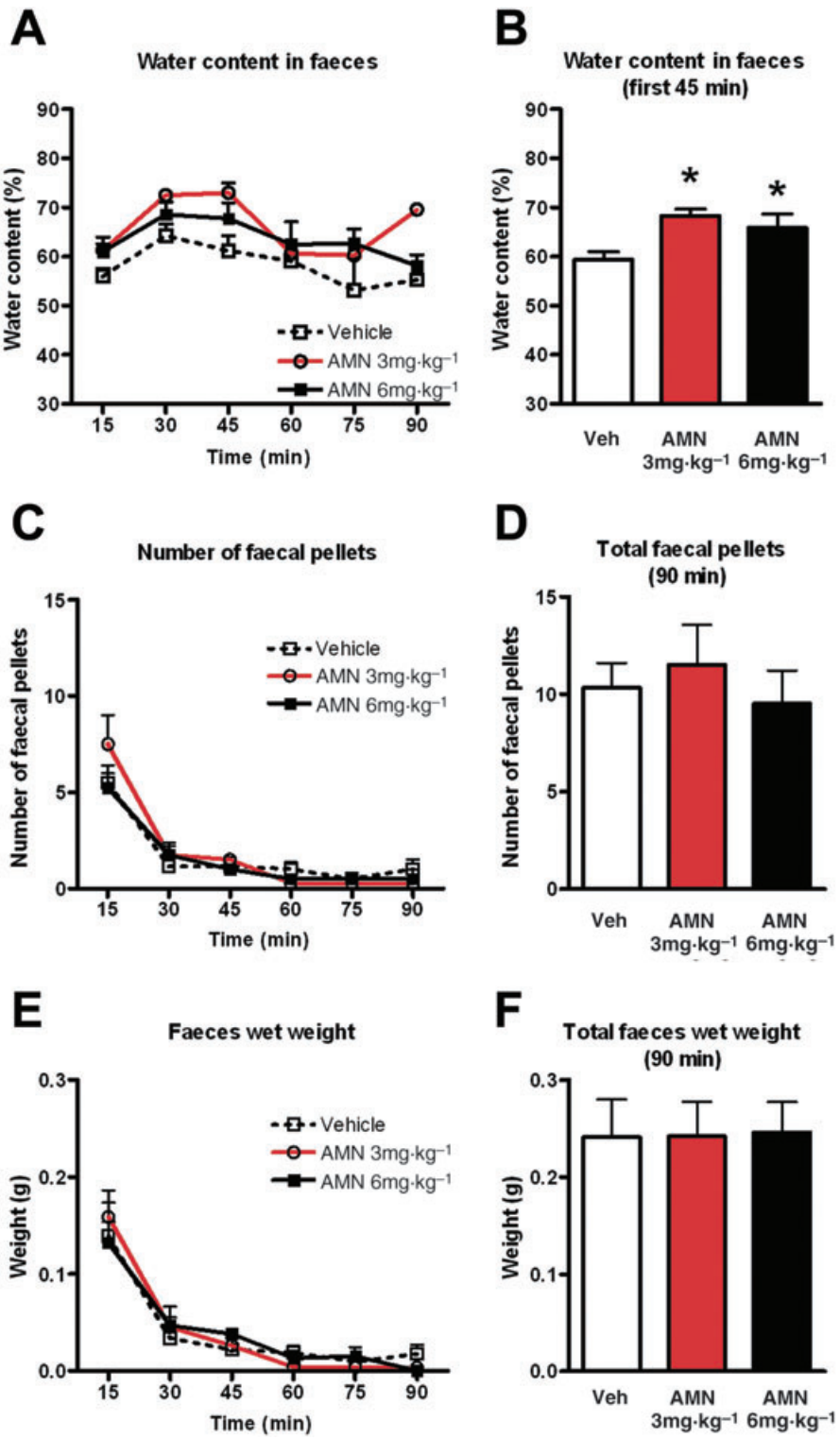

Figure 2 Effect of AMN082 on faecal water content and stress-induced defecation. AMN082 (0, 3 and 6 mg·kg^{-1}) was given orally 1 h before stress. Faeces from mice exposed to the stress-induced defecation protocol were desiccated and % water content determined. (A) Water content determined in a time-course fashion. (B) Water content of the total of faeces from the first 45 min of the experiment. C and D show the number of faeces produced in a time and 90 min cumulative fashion respectively. (E and F) Time-course and 90 min cumulative faeces wet weight. Values are expressed as mean ± s.e.mean. *$P < 0.05$, $n = 4$–6.

and it is a component of the ENS. Glutamatergic neurons have been identified in the submucosal and myenteric plexi along the GI tract of the rat (Liu *et al.*, 1997) and glutamate induces enteric muscle contraction (Jankovic *et al.*, 1999).

Functional ionotropic and metabotropic glutamate receptors have been identified in the ENS (Jankovic *et al.*, 1999; Larzabal *et al.*, 1999; Giaroni *et al.*, 2000; Tong and Kirchgessner, 2003). However, to our knowledge, there is only one

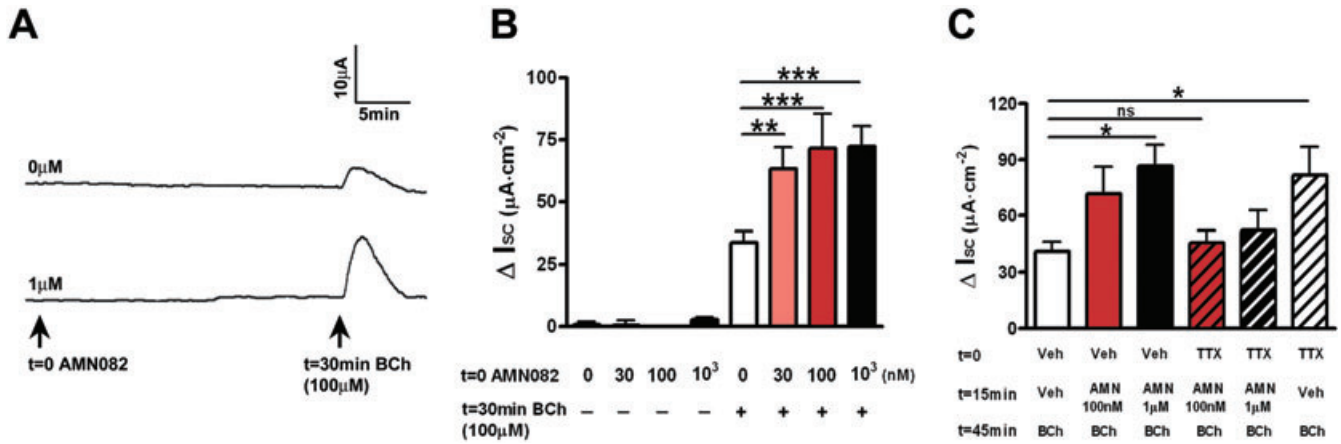

Figure 3 AMN082-induced changes in ion transport in mucosal preparations from mouse colon. (A) Representative recording of short-circuit current (I_{SC}) in submucosal preparations where vehicle or the $mGlu_7$ receptor agonist AMN082 (1 μM) were added to the basolateral side 30 min before bethanechol (BCh, 100 μM). (B) Dose–response study: vehicle or AMN082 (30 nM, 100 nM or 1 μM) were added to the basolateral side 30 min before bethanechol (BCh, 100 μM) and changes on I_{SC} were recorded and plotted. (C) Tetrodotoxin study: tissues were treated for 15 min with vehicle or tetrodotoxin (TTX, 300 nM) in the basolateral side, after which vehicle or AMN082 (100 nM or 1 μM) were added. Thirty minutes later BCh (100 μM) was added and changes on I_{SC} were recorded and plotted. Values are expressed as mean ± s.e.mean. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $n = 7–9$.

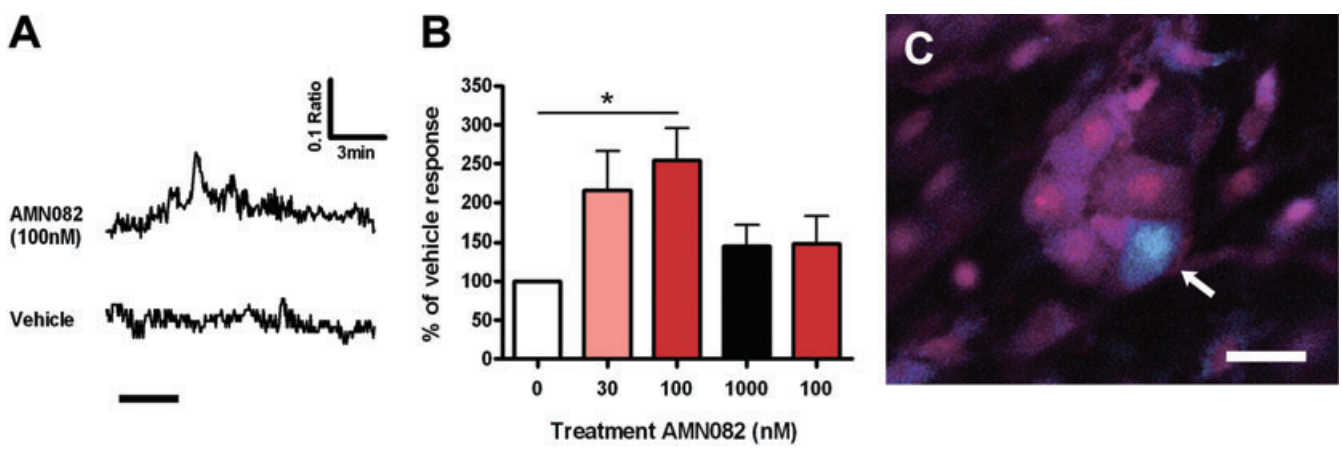

Figure 4 Intracellular calcium mobilization induced by AMN082 in neurons from the colon submucosa. In (A) an original record of the calcium mobilization is depicted. The horizontal bar indicates the duration of the stimulus. B shows the calcium signalling induced by AMN082 in responding neurons, which correspond to 19% of the submucosal neurons. (C) Change in fluorescence in a group of neurons treated with 100 nM AMN082. Bar: 20 μm. Values are expressed as mean ± s.e.mean, $^*P < 0.05$, $n = 5$.

previous piece of work showing that $mGlu_7$ receptors are present in the gut (Tong and Kirchgessner, 2003). In that study, $mGlu_7$ receptors were detected by PCR in the longitudinal muscle with adherent myenteric plexus of the rat intestine; however, this observation appears not to have been investigated any further and was only peripheral to the main focus of that paper (Tong and Kirchgessner, 2003). Our *in situ* hybridization data initially suggested that the main site of $mGlu_7$ receptor expression in the mouse colon was the mucosa; however, later Western blot results indicated that the protein was also present in the enteric nerves and colon muscle layers. This is consistent with other reports which have also shown low levels of mRNA and high expression of the protein of a given gene using similar techniques (Bravo *et al*., 2009). This very low mRNA expression and high protein levels, which are, in our study, as high as those found in mouse hippocampus (Figure 1C), suggest a high rate of $mGlu_7$ receptor translation in the colon nerves and muscles and it is an intriguing finding that warrants further investigation.

In our *in vivo* and *ex vivo* experiments, we used AMN082, the only selective agonist available for $mGlu_7$ receptors (Lavreysen and Dautzenberg, 2008). The recent development of $mGlu_7$ receptor antagonists such as 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-*c*]pyridin-4(5*H*)-one (Suzuki *et al*., 2007) will hopefully help shed further light on the role of tonic glutamate-induced activation of $mGlu_7$ receptors. Further, the use of genetic techniques such as knockouts and siRNA-induced knockdown (Mitsukawa *et al*., 2006; Fendt *et al*., 2008) has been a fruitful area of investigation in the $mGlu_7$ receptor field and should be applied to colonic function in the future.

Activation of $mGlu_8$ receptors, also included in the group III mGlu receptors, produced an accelerating effect on motility in isolated segments of guinea pig colon (Tong and

Kirchgessner, 2003). Although both $mGlu_8$ and $mGlu_7$ receptors are presynaptic group III mGlu receptors, we found that stress-induced faecal output was not altered by selective activation of $mGlu_7$ receptors, suggesting no effect on colon motility *per se*. However, one drawback of the stress-induced defecation test is that it promotes a substantial faecal output which could have masked small changes in colon motility induced by AMN082 in the absence of stress.

Faeces produced by AMN082-treated mice had higher water content than vehicle-treated mice. It should be noted that the faecal water content of vehicle-treated mice in this study is consistent with previously reported data for vehicle-treated, individually housed mice (Wang *et al*., 2007). We believe the effect induced by AMN082 treatment is physiologically relevant. For example, mice infected with *Citrobacter rodentium*, a pathogen commonly used to model *Escherichia coli* infections, have faecal water content of 89.3%, compared with 71.5% in faeces from PBS-treated mice (Cheng *et al*., 2009). In humans, water content increases are within a similar range: in the study performed by Jensen *et al*. (1976), normal human faeces had an average water content of 77%. In the same study, faeces from patients suffering from diverse types of diarrhoea had a water content ranging from 83% to 90%. A newer study (Fischer *et al*., 2001) gives values of 75.4% for normal stool and 88.8% water content for stool samples from diarrhoea patients. These increments are close to the 10–15% increase in faecal water content we observed upon AMN082 treatment in our experiments.

Secretory function and motility are two different aspects of colonic physiology and both can contribute to the manifestation of diarrhoea. Here, AMN082 does not have any major effect on colonic motility *per se* but increases water content, indicative of augmented secretory activity within the colon. However, AMN082 can cross the blood brain barrier and several central effects have been reported for this agonist including modulation of stress parameters and expression of neurotrophic factors (Mitsukawa *et al*., 2005; 2006). Consequently, we cannot exclude the participation of the CNS in the production of faeces with higher water content in AMN082-treated mice. It was interesting to confirm, by *ex vivo* experiments, that in the absence of the central influence, AMN082 could still increase electrolyte secretion. This not only shows that targeting $mGlu_7$ receptors can modify electrolyte secretion in the isolated colon, but also provides the first evidence of $mGlu_7$ receptors playing a non-sensory role in the periphery. Interestingly, although tangential to our studies, genomic analyses indicate that single nucleotide polymorphisms in GRM7, the gene encoding the $mGlu_7$ receptor, confer susceptibility to age-related hearing impairment (Friedman *et al*., 2009).

We also investigated if the presence of submucosal nerves was required for the AMN082-induced increase in the response to the muscarinic cholinergic receptor agonist bethanechol. TTX almost completely abolished the effect, strongly suggesting that aneural component is required for the effects of AMN082 on ion transport (Kao, 1986). However, we cannot rule out that the modulation of bethanechol-induced secretion may be a result of AMN082 acting on both submucosal nerves and epithelial cells. One hypothesis is that there is a population of AMN082-sensitive submucosal neurons which have a tonic inhibitory effect on the secretory activity of the epithelium. In neurons isolated from the CNS, activation of $mGlu_7$ receptors can inhibit neurotransmitter release (Lavreysen and Dautzenberg, 2008). We believe the $mGlu_7$ receptor agonist would also have an inhibitory effect in the submucosal neurons, and this would liberate the epithelium from the negative neuronal modulation, to finally generate a greater response to bethanechol. Treatment with TTX alone would have a similar effect, also inducing a higher response to bethanechol in our preparations by removal of the tonically negative neuronal activity. It is possible that, in TTX-treated tissues, as the neuronal component is silenced, the direct effects of AMN082 on the epithelial cells are more evident. If we believe that $mGlu_7$ receptor activation has an inhibitory effect also on epithelial cells, this could explain why the bethanechol-induced response was blunted in tissues treated with TTX plus AMN082.

Altogether, our functional data suggest that glutamatergic activation of $mGlu_7$ receptors could be a component in the pathophysiology of secretory disorders such as diarrhoea, and also an important factor underlying stress-induced diarrhoea. These data also support the concept that modulation of $mGlu_7$ receptor activity may have relevance to a subset of IBS patients who are diarrhoea-predominant and offer some relief to constipation-predominant IBS patients.

Activation of group III mGlu receptors is generally associated with a decrease in the production of cAMP *in vitro* (Lavreysen and Dautzenberg, 2008). Interestingly, our results show that the secretory response to bethanechol, which is dependent on the increase of intracellular Ca^{2+} (Zimmerman *et al*., 1982), is enhanced by pretreatment with the selective $mGlu_7$ receptor agonist AMN082. In rodents, bethanechol acts not only in the enterocytes but also in submucosal nerves (Hirota and McKay, 2006), and as the AMN082-induced effects on ion transport were nerve-dependent, we decided to investigate if specifically activating $mGlu_7$ receptors could induce Ca^{2+} mobilization in the submucosal neurons. Additionally, the calcium imaging assay provided us with a tool to explore baseline effects of AMN082, that is, in the absence of stress or an exogenous muscarinic agent. Our data show that AMN082 is able to increase intracellular Ca^{2+} levels in a small subset of submucosal neurons. However, some features of the experimental design are worthy of comment and which may influence the overall interpretation. First, the presence of nifedipine, a calcium channel blocker (Alexander *et al*., 2009), which was key to prevent movement of the tissue while recording calcium mobilization, could also have prevented some of the neurons from responding to the $mGlu_7$ receptor agonist. Second, the low percentage of responding neurons and the fact that they seem to rapidly desensitise upon agonist binding precluded us from performing a more comprehensive analysis on the nature of the response, such as establishing whether the calcium comes from intracellular stores or from the extracellular space. Although our experimental design could not determine if AMN082 could induce a decrease in Ca^{2+} mobilization, the results confirmed the presence of functional $mGlu_7$ receptors in the submucosal plexus. In addition, activation of $mGlu_7$ receptors is accompanied by internalization of the receptor in cultured neurons (Pelkey *et al*., 2007). In our hands, a second application of the

same dose of AMN082 on the tissue failed to induce the same increase in intracellular Ca^{2+}. This observation indicates that the system was indeed desensitized and suggests that the results reported here may well be a consequence of exclusion of the receptor from the cell surface. Whether endogenous activation of $mGlu_7$ receptors is accompanied by receptor internalization and, as such, acts as a regulatory mechanism *in vivo* is not yet known. Therefore, further studies need to be conducted to reveal if agonist-induced desensitization is a component regulating $mGlu_7$ receptor signalling in colon secretory function and can therefore be pharmacologically targeted to address diarrhoea or constipation. In this context, an interesting approach would be the use of electron microscopy to determine if stress can induce internalization of $mGlu_7$ receptors in submucosal neurons and other cell types within the colon.

Functional intestinal disorders such as IBS are co-morbid with chronic stress (Quigley, 2006; Clarke *et al*., 2009; Gros *et al*., 2009). Stressful life events and experimental stress also induce exacerbation of symptoms and visceral hypersensitivity in patients with functional GI disorders (Whitehead *et al*., 1992; Posserud *et al*., 2004). The above evidence supports the idea of a complex interaction between the GI tract and the brain, namely the brain–gut axis. The data presented suggest that $mGlu_7$ receptors may not only regulate the central components of chronic stress (Cryan *et al*., 2003; Mitsukawa *et al*., 2006), but may also have a role to play peripherally in the regulation of fluid and electrolyte transport which is significantly disrupted in diseases such as IBS, by enhancing colonic secretory activity.

Acknowledgements

The authors wish to thank Mr Patrick Fitzgerald and Mrs Colette Manley for their expert technical assistance, Ms Celine Bongiovanni and Dr Dervla O'Malley, for assistance with Ussing chamber and calcium imaging techniques respectively. The Alimentary Pharmabiotic Centre is a Centre for Science and Technology funded by Science Foundation Ireland and GlaxoSmithKline.

Conflict of interest

The authors disclose no competing interests.

References

Alexander SP, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). 4th edn. *Br J Pharmacol* **158** (Suppl. 1): S1–254.

Banner SE, Smith MI, Bywater D, Gaster LM, Sanger GJ (1996). Increased defaecation caused by 5-HT4 receptor activation in the mouse. *Eur J Pharmacol* **308**: 181–186.

Blachier F, Boutry C, Bos C, Tome D (2009). Metabolism and functions of l-glutamate in the epithelial cells of the small and large intestines. *Am J Clin Nutr* **90**: 814S–821S.

Bravo JA, Parra CS, Arancibia S, Andres S, Morales P, Herrera-Marschitz M *et al*. (2006). Adrenalectomy promotes a permanent decrease of plasma corticoid levels and a transient increase of apoptosis and the expression of Transforming Growth Factor beta1 (TGF-beta1) in hippocampus: effect of a TGF-beta1 oligo-antisense. *BMC Neurosci* **7**: 40.

Bravo JA, Diaz-Veliz G, Mora S, Ulloa JL, Berthoud VM, Morales P *et al*. (2009). Desipramine prevents stress-induced changes in depressive-like behavior and hippocampal markers of neuroprotection. *Behav Pharmacol* **20**: 273–285.

Cheng C, Tennant SM, Azzopardi KI, Bennett-Wood V, Hartland EL, Robins-Browne RM *et al*. (2009). Contribution of the pst-phoU operon to cell adherence by atypical enteropathogenic Escherichia coli and virulence of Citrobacter rodentium. *Infect Immun* **77**: 1936–1944.

Clarke G, Quigley EM, Cryan JF, Dinan TG (2009). Irritable bowel syndrome: towards biomarker identification. *Trends Mol Med* **15**: 478–489.

Conn PJ (2003). Physiological roles and therapeutic potential of metabotropic glutamate receptors. *Ann N Y Acad Sci* **1003**: 12–21.

Cryan JF, Kelly PH, Neijt HC, Sansig G, Flor PJ, van Der Putten H (2003). Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. *Eur J Neurosci* **17**: 2409–2417.

Fendt M, Schmid S, Thakker DR, Jacobson LH, Yamamoto R, Mitsukawa K *et al*. (2008). mGluR7 facilitates extinction of aversive memories and controls amygdala plasticity. *Mol Psychiat* **13**: 970–979.

Fischer B, Hoh S, Wehler M, Hahn EG, Schneider HT (2001). Faecal elastase-1: lyophilization of stool samples prevents false low results in diarrhoea. *Scand J Gastroenterol* **36**: 771–774.

Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, Corneveaux JJ *et al*. (2009). GRM7 variants confer susceptibility to age-related hearing impairment. *Hum Mol Genet* **18**: 785–796.

Giaroni C, Zanetti E, Marino F, Cosentino M, Senaldi A, Somaini L *et al*. (2000). Glutamate receptors of the AMPA type modulate neurotransmitter release and peristalsis in the guinea-pig isolated colon. *Life Sci* **67**: 1747–1757.

Goyal RK, Hirano I (1996). The enteric nervous system. *N Engl J Med* **334**: 1106–1115.

Gros DF, Antony MM, McCabe RE, Swinson RP (2009). Frequency and severity of the symptoms of irritable bowel syndrome across the anxiety disorders and depression. *J Anxiety Disord* **23**: 290–296.

Gulbransen BD, Clapp TR, Finger TE, Kinnamon SC (2008). Nasal solitary chemoreceptor cell responses to bitter and trigeminal stimulants in vitro. *J Neurophysiol* **99**: 2929–2937.

Hirota CL, McKay DM (2006). Cholinergic regulation of epithelial ion transport in the mammalian intestine. *Br J Pharmacol* **149**: 463–479.

Hyland NP, Cox HM (2005). The regulation of veratridine-stimulated electrogenic ion transport in mouse colon by neuropeptide Y (NPY), Y1 and Y2 receptors. *Br J Pharmacol* **146**: 712–722.

Jankovic SM, Milovanovic D, Matovic M, Iric-Cupic V (1999). The effects of excitatory amino acids on isolated gut segments of the rat. *Pharmacol Res* **39**: 143–148.

Jensen R, Buffangeix D, Covi G (1976). Measuring water content of feces by the Karl Fischer method. *Clin Chem* **22**: 1351–1354.

Kao CY (1986). Structure-activity relations of tetrodotoxin, saxitoxin, and analogues. *Ann N Y Acad Sci* **479**: 52–67.

Larzabal A, Losada J, Mateos JM, Benitez R, Garmilla IJ, Kuhn R *et al*. (1999). Distribution of the group II metabotropic glutamate receptors (mGluR2/3) in the enteric nervous system of the rat. *Neurosci Lett* **276**: 91–94.

Lavreysen H, Dautzenberg FM (2008). Therapeutic potential of group III metabotropic glutamate receptors. *Curr Med Chem* **15**: 671–684.

Liu MT, Rothstein JD, Gershon MD, Kirchgessner AL (1997). Glutamatergic enteric neurons. *J Neurosci* **17**: 4764–4784.

Mitsukawa K, Mombereau C, Lotscher E, Uzunov DP, van der Putten H, Flor PJ *et al*. (2006). Metabotropic glutamate receptor subtype 7 ablation causes dysregulation of the HPA axis and increases

hippocampal BDNF protein levels: implications for stress-related psychiatric disorders. *Neuropsychopharmacology* **31**: 1112–1122.

Mitsukawa K, Yamamoto R, Ofner S, Nozulak J, Pescott O, Lukic S *et al*. (2005). A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. *Proc Natl Acad Sci USA* **102**: 18712–18717.

Miyata K, Kamato T, Nishida A, Ito H, Yuki H, Yamano M *et al*. (1992). Role of the serotonin3 receptor in stress-induced defecation. *J Pharmacol Exp Ther* **261**: 297–303.

O'Connor RM, Cryan JF (2010). Role of metabotropic glutamate receptors in CNS disorders. In: Milligan G, Siehler S (eds). *G Protein Coupled Receptors: Structure, Signaling and Physiology*. Cambridge University Press: Cambridge, UK. In press.

O'Connor RM, Finger BC, Flor PJ, Cryan JF (2010). mGluR7: at the interface of cognition and emotion. *Eur J Pharmacol* In press.

Partosoedarso ER, Blackshaw LA (2000). Roles of central glutamate, acetylcholine and CGRP receptors in gastrointestinal afferent inputs to vagal preganglionic neurones. *Auton Neurosci* **83**: 37–48.

Pelkey KA, Yuan X, Lavezzari G, Roche KW, McBain CJ (2007). mGluR7 undergoes rapid internalization in response to activation by the allosteric agonist AMN082. *Neuropharmacology* **52**: 108–117.

Posserud I, Agerforz P, Ekman R, Bjornsson ES, Abrahamsson H, Simren M (2004). Altered visceral perceptual and neuroendocrine response in patients with irritable bowel syndrome during mental stress. *Gut* **53**: 1102–1108.

Quigley EM (2006). Changing face of irritable bowel syndrome. *World J Gastroenterol* **12**: 1–5.

Reeds PJ, Burrin DG, Stoll B, Jahoor F (2000). Intestinal glutamate metabolism. *J Nutr* **130**: 978S–982S.

Robinson MB, Coyle JT (1987). Glutamate and related acidic excitatory neurotransmitters: from basic science to clinical application. *FASEB J* **1**: 446–455.

Stachowicz K, Branski P, Klak K, van der Putten H, Cryan JF, Flor PJ *et al*. (2008). Selective activation of metabotropic G-protein-coupled glutamate 7 receptor elicits anxiolytic-like effects in mice by modulating GABAergic neurotransmission. *Behav Pharmacol* **19**: 597–603.

Suzuki G, Tsukamoto N, Fushiki H, Kawagishi A, Nakamura M, Kurihara H *et al*. (2007). In vitro pharmacological characterization of novel isoxazolopyridone derivatives as allosteric metabotropic glutamate receptor 7 antagonists. *J Pharmacol Exp Ther* **323**: 147–156.

Tong Q, Kirchgessner AL (2003). Localization and function of metabotropic glutamate receptor 8 in the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* **285**: G992–G1003.

Wang L, Martinez V, Kimura H, Tache Y (2007). 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT4 receptors in conscious mice. *Am J Physiol Gastrointest Liver Physiol* **292**: G419–G428.

Watkins JC, Jane DE (2006). The glutamate story. *Br J Pharmacol* **147** (Suppl. 1): S100–S108.

Whitehead WE, Crowell MD, Robinson JC, Heller BR, Schuster MM (1992). Effects of stressful life events on bowel symptoms: subjects with irritable bowel syndrome compared with subjects without bowel dysfunction. *Gut* **33**: 825–830.

Zimmerman TW, Dobbins JW, Binder HJ (1982). Mechanism of cholinergic regulation of electrolyte transport in rat colon *in vitro*. *Am J Physiol* **242**: G116–G123.