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RESEARCH PAPER

Differential actions of urocortins on neurons of the myenteric division of the enteric nervous system in guinea pig distal colon

S Liu[1,2], W Ren[1], M-H Qu[1]*, GA Bishop[3], G-D Wang[1], X-Y Wang[4], Y Xia[1,4] and JD Wood[1,2]

[1]*Department of Physiology and Cell Biology, College of Medicine, Ohio State University, Columbus, OH, USA,* [2]*Department of Internal Medicine, Division of Gastroenterology, Hepatology, and Nutrition, College of Medicine, Ohio State University, Columbus, OH, USA,* [3]*Department of Neuroscience, College of Medicine, Ohio State University, Columbus, OH, USA,* [4]*Department of Anesthesiology, College of Medicine, Ohio State University, Columbus, OH, USA*

Background and purpose: Urocortins (Ucns) 1, 2 and 3 are corticotropin-releasing factor (CRF)-related neuropeptides and may be involved in neural regulation of colonic motor functions. Nevertheless, details of the neural mechanism of action for Ucns have been unclear. We have, here, tested the hypothesis that Ucns act in the enteric nervous system (ENS) to influence colonic motor behaviour.
Experimental approach: We used intracellular recording with 'sharp' microelectrodes, followed by intraneuronal injection of biocytin, and immunohistochemical localization of CRF_1 and CRF_2 receptors in guinea pig colonic tissue.
Key results: Application of Ucn1 depolarized membrane potentials and elevated excitability in 58% of AH-type and 60% of S-type colonic myenteric neurons. In most of the neurons tested, depolarizing responses evoked by Ucn-1 were suppressed by the CRF_1 receptor antagonist NBI 27914, but were unaffected by the CRF_2 receptor antagonist antisauvagine-30. The selective CRF_2 receptor agonists, Ucn2 and Ucn3, evoked depolarizing responses in 12 and 8% of the AH-type myenteric neurons, respectively, and had no effect on S-type neurons. Antisauvagine-30, but not NBI 27914, suppressed these Ucn2- and Ucn3-evoked responses. Immunohistochemical staining identified CRF_1 as the predominant CRF receptor subtype expressed by ganglion cell somas, while CRF_2-immunoreactive neuronal somas were sparse. Ucns did not affect excitatory synaptic transmission in the ENS.
Conclusions and implications: The results suggest that Ucns act as neuromodulators to influence myenteric neuronal excitability. The excitatory action of Ucn1 in myenteric neurons was primarily at CRF_1 receptors, and the excitatory action of Ucn2 and Ucn3 was at CRF_2 receptors.
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Abbreviations: C, cerebellum; ChAT, choline acetyltransferase; CRF, corticotropin-releasing factor; Cy3, indocarbocyanin; ENS, enteric nervous system; EPSP, excitatory postsynaptic potential; Hypo, hypothalamus; IR, immunoreactivity; LMMP, longitudinal muscle–myenteric plexus; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; TBS, Tris–HCl-buffered saline; Ucn, urocortin

Introduction

Corticotropin-releasing factor (CRF), and urocortin-1 (Ucn1), urocortin-2 (Ucn2) and urocortin-3 (Ucn3) are structurally related neuropeptides expressed in the brain and enteric nervous system (ENS) where they have a major involvement in brain–gut interactions during stress (Bale and Vale, 2004). Ucns and CRF exert their biological actions by stimulating the G protein-coupled receptor subtypes, CRF_1 and CRF_2 (nomenclature follows Alexander *et al.*, 2008). Many studies now show that Ucns are widely distributed outside the brain, including the gastrointestinal tract. Ucn1 is expressed in the ENS of the rat (Harada *et al.*, 1999; Kimura *et al.*, 2007), and in human gastric and colonic mucosa (Muramatsu *et al.*, 2000; Chatzaki *et al.*, 2003). Ucn2 is expressed in the mucosa, immune cells of the lamina propria and neurons in the submucosal and myenteric plexuses of the mouse and rat ENS (Chen *et al.*, 2004; Chang *et al.*, 2007). Ucn3 is expressed in the muscularis mucosae of the mouse small intestine (Hsu

Correspondence: Sumei Liu, Department of Biology, University of Wisconsin-La Crosse, La Crosse, WI 54601, USA. E-mail: liu.sume@uwlax.edu
*Present address: Department of Pharmacology, Key Lab of Clinical Pharmacology of Shandong Province, Weifang Medical University, Weifang, Shandong Province, China
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and Hsueh, 2001), and in the myenteric and submucosal plexuses of the human colon (Saruta *et al.*, 2005).

CRF_1 and CRF_2 receptors are expressed abundantly in the ENS. The CRF_1 subtype is expressed in myenteric and submucosal neurons in all regions of the guinea pig and rat gastrointestinal tract (Chatzaki *et al.*, 2004a,b; Liu *et al.*, 2005; Porcher *et al.*, 2005; 2006; Bisschops *et al.*, 2006; Kimura *et al.*, 2007; Yuan *et al.*, 2007). The CRF_2 receptor is reported to be expressed by myenteric and submucosal neurons throughout the rat gastrointestinal tract (Porcher *et al.*, 2005; 2006; Chang *et al.*, 2007; Kimura *et al.*, 2007). Nevertheless, CRF_2 has not been found in guinea pig small intestine (Liu *et al.*, 2005; Bisschops *et al.*, 2006). Detailed information on the electrophysiological behaviour and neurochemical coding for the classes of neurons that express CRF_1 receptors in the ENS is available currently only for the guinea pig ileum (Liu *et al.*, 2005).

Evidence, which points to a role for CRF and Ucns in the effects of stress on gastrointestinal motility, is accumulating. CRF, Ucn1 and Ucn2, when injected into the brain, act at the CRF_2 receptor subtype to inhibit gastric emptying (Kihara *et al.*, 2001; Chen *et al.*, 2002; Martinez *et al.*, 2004; Czimmer *et al.*, 2006). On the other hand, injection of these peptides in the brain accelerates colonic transit through signalling involving the CRF_1 subtype (Martinez *et al.*, 2004). Intraperitoneal (i.p.) administration of CRF, Ucn1, Ucn2 and Ucn3 also inhibits gastric emptying by activating CRF_2 receptors (Nozu *et al.*, 1999; Wang *et al.*, 2001; Martinez *et al.*, 2002; Million *et al.*, 2002). As with intracerebral injection, injection of CRF or Ucn1 by the i.p. route stimulates colonic motility through activation of the CRF_1 receptor subtype (Maillot *et al.*, 2000; 2003; Martinez *et al.*, 2002). Injection (i.p.) of Ucn2 and Ucn3 does not alter colonic transit, while gastric emptying is suppressed by the same dose (Martinez *et al.*, 2002).

Tsukamoto *et al.* (2006) suggested that stimulation of colonic contractility by i.p. administered CRF actually reflects an action in the brain. Recent evidence suggests that this might not be the case and that stimulation of colonic motility by peripherally administered CRF peptides involves direct excitatory actions on identified classes of neurons in the ENS. We reported previously that exposure to CRF depolarizes the membrane potential and elevates excitability in single neurons in the myenteric plexus of the guinea pig ileum and colon (Hanani and Wood, 1992; Liu *et al.*, 2005). These CRF-evoked depolarizing responses are suppressed by the CRF_1/CRF_2 receptor antagonist, astressin, and the selective CRF_1 receptor antagonist, NBI 27914, and are unaffected by the selective CRF_2 receptor antagonist, antisauvagine-30 (Liu *et al.*, 2005). Others reported that excitation of enteric neurons by i.p. administration of CRF in conscious rats is demonstrated by an increased number of neurons showing c-*fos* expression in the myenteric plexus. Enhanced *c-fos* expression in these studies is suppressed by i.p. injection of astressin or the selective CRF_1 receptor antagonist CP-154526 (Miampamba *et al.*, 2002; Yuan *et al.*, 2007). These data are in general agreement with the suggestion that the CRF_1 receptor subtype is functionally expressed by neurons in the myenteric plexus of guinea pig and rat colon (Chatzaki *et al.*, 2004a; Liu *et al.*, 2005).

Peripheral mechanisms by which Ucns are involved in the neural control of colonic motor function have not been investigated to the same extent as CRF. The present study aimed to test a hypothesis that Ucn signalling is a significant factor in ENS regulation of colonic motor behaviour. We did this by investigating the direct action of Ucn1, Ucn2 and Ucn3 on the electrophysiological and synaptic behaviour of morphologically and neurochemically identified myenteric neurons in guinea pig colon. Selective antagonists and agonists were used as pharmacological tools to characterize the involvement of CRF_1 and CRF_2 receptor subtypes in the actions of Ucns on the excitability of constituents of specific classes of neurons in the ENS. Immunohistochemistry was used in conjunction with electrophysiological recording and pharmacological analysis to identify the classes of neurons that express CRF_1 and CRF_2 receptors in the ENS.

Methods

Animals

All animal care and experimental protocols were approved by the Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture inspectors. Male Hartley-strain guinea pigs (300–400 g; Charles River, Wilmington, MA, USA; 127 guinea pigs in total) and male C57BL/6 mice (26–38 g; three mice in total) were used for the studies. The animals were housed in a temperature-controlled room (22°C) under a 12-h light/dark cycle, had free access to water and a standard chow until the day of the experiment. Guinea pigs were killed by stunning and exsanguination from the cervical vessels. Mice were killed by cervical dislocation. The distal colon was removed and placed in chilled Krebs' solution containing (in mM): NaCl, 120.9; KCl, 5.9; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 14.4; $CaCl_2$, 2.5; and glucose, 11.5. The solution contained the L-type calcium channel blocker, nifedipine (1 μM), and the muscarinic receptor antagonist, scopolamine (1 μM), to minimize muscle contraction and allow for maximal stretching of the preparations for microelectrode access to the neurons. The hypothalamus and cerebellum were removed from three guinea pigs and three mice, washed with artificial cerebrospinal fluid, snap-frozen in liquid nitrogen and stored at −70°C for later comparative neuroanalysis.

Electrophysiology

Our methods for intracellular recording from enteric neurons in whole-mount preparations of guinea pig colonic myenteric plexus are described in detail elsewhere (Wade and Wood, 1988a,b). Transmembrane electrical potentials were recorded with conventional 'sharp' microelectrodes filled with 2% biocytin in 2 M KCl buffered with 0.05 M Tris at pH 7.4. Resistances of the electrodes ranged between 80 and 140 MΩ. The pre-amplifier (model M-767; World Precision Instruments, Sarasota, FL, USA) was equipped with bridge circuits for intraneuronal injection of electrical current. Constant current rectangular pulses were driven by Grass SD9 stimulators (Grass Instrument Division, Astro-Med Inc., West Warwick, RI, USA). Fast excitatory postsynaptic potentials (EPSPs) and slow EPSPs

were evoked by electrical shocks (0.1–20 Hz) applied focally to interganglionic connectives with 20 μm diameter Teflon-insulated Pt wire electrodes connected through stimulus isolation units (Grass SIN5) to Grass S48 stimulator (Astro-Med, Grass-Telefactor Division). Electrometer output was acquired and digitized using a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA), and analysed using PowerLab Chart v5.1 software.

The morphology of each of the neurons was marked by passing hyperpolarizing current (0.5 nA for 10–30 min) to inject biocytin into the neuron from the microelectrode and later histochemical development of the intraneuronal biocytin. At the end of each recording session, the position of the neuron within the ganglion, and the location of the ganglion relative to the oral and aboral orientation of the preparation were noted for later identification following immunohistochemical processing. The whole-mount preparations were then transferred to a disposable chamber filled with fixative containing 4% formaldehyde and 1.5% of a saturated solution of picric acid, and stored at 4°C overnight. The preparations were cleared in three changes of dimethyl sulphoxide (DMSO) and three 10 min washes with phosphate-buffered saline (PBS), and then reacted with fluorescein isothiocyanate streptavidin (Vector Labs, Burlingame, CA, USA) diluted 1:200 for 30 min at 37°C, and examined with a Nikon Eclipse 90i automated fluorescent microscope (Nikon Instruments, Inc., Melville, NY, USA). The preparations were further processed for immunofluorescence staining for CRF receptors according to the methods described below.

Immunohistochemistry

Segments of distal colon were opened along the mesenteric border, stretched tautly and pinned out flat with mucosa side up onto Sylgard-coated Petri dishes. Preparations were immediately fixed in Zamboni's fixative (4% formaldehyde plus 1.5% picric acid in 0.1 M PBS) for 3 h at room temperature. After fixation, tissues were washed in PBS three times, 10 min each. Whole mounts of the longitudinal muscle–myenteric plexus (LMMP) were dissected from these segments. To minimize non-specific binding and to permeabilize the tissue, the preparations were placed in PBS containing 10% normal donkey serum and 0.3% Triton X-100 for 30 min at room temperature. The preparations were incubated in primary antibodies for CRF_1 receptors (goat, sc-12383, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or CRF_2 receptors (rabbit, ab12964, 1:500, Abcam Inc., Cambridge, MA, USA) overnight at 4°C. After being washed, the tissues were incubated in indocarbocyanin (Cy3)-labelled donkey anti-goat or anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA) at room temperature for 1 h. The tissues were washed in PBS and cover slipped with VECTASHIELD mounting medium (Vector Labs). Fluorescence labelling was examined under a Nikon Eclipse 90i automated fluorescence microscope. Specificity of the CRF_1 receptor antibody was characterized previously (Liu *et al*., 2005; Porcher *et al*., 2005; 2006; Bisschops *et al*., 2006). Pre-absorbing the antibody with the corresponding blocking peptide (sc-12383p, Santa Cruz Biotechnology) blocked all specific staining (Figure $1A_2$). Specificity of the CRF_2 receptor antibody was tested by omitting either the primary or the secondary antibodies, because no blocking peptide is commercially available for the CRF_2 receptor antibody. No specific staining was observed in either case (Figure $1B_2$). Positive controls for the CRF_2 receptor antibody consisted of the guinea pig and mouse hypothalamus (Figure 1C) and cerebellum (not shown), in which CRF_2 receptors are expressed strongly. For immunohistochemistry of the guinea pig and mouse brain, the animals were anesthetized with a combination of ketamine (9 mg 100 g^{-1}) and xylazine (1 mg 100 g^{-1}), and perfused through the aorta with saline followed by 4% paraformaldehyde. The brains were carefully removed and processed for immunofluorescence staining of CRF_2 receptors as described previously (Bishop *et al*., 2006).

Double labelling of CRF_1 and CRF_2 receptors with other specific neurochemical markers was used to identify the cell types that express CRF receptor subtypes, and was performed in a sequential manner. The tissues were first incubated with the primary and secondary antibodies for CRF_1 or CRF_2 receptors as indicated above. Samples were examined under the fluorescence microscope to ensure quality of labelling. The tissues were then washed in PBS and subsequently incubated with the primary antibodies for the anti-human neuronal nuclear protein (anti-Hu; mouse, A21271, 1:50, Invitrogen, Carlsbad, CA, USA), calbindin (mouse, C9848, 1:3000, Sigma, St Louis, MO, USA), choline acetyltransferase (ChAT: goat, AB144p, Millipore, Temecula, CA, USA), nitric oxide synthase (NOS; sheep, AB1529, 1:500, Millipore) or synaptophysin (mouse, M0776, 1:200, Dako, Carpinteria, CA, USA). The tissues were then washed in PBS and incubated with the appropriate secondary antibodies for 1 h at room temperature. After a thorough rinse, the tissues were cover slipped with VECTASHIELD mounting medium and examined under a Nikon Eclipse 90i fluorescence microscope. All images were acquired with a CoolSnap HQ2 monochrome digital camera, stored on disk and analysed with MetaMorph imaging software (Molecular Devices Corporation, Sunnyvale, CA, USA). Images were minimally adjusted for brightness, contrast and intensity using MetaMorph. Immunoreactive neurons for CRF_1, CRF_2 receptors and the marker populations, as well as the number of double-labelled cells, were assessed in randomly chosen ganglia throughout the tissue preparations. Counts of double-labelled cells were assessed in at least three animals for each chemical marker. At least 30 ganglia in the myenteric plexus were counted for each preparation. Results are expressed as means ± SEM with *n* values representing the numbers of animals studied.

Western blots

Membrane proteins were extracted from the guinea pig and mouse hypothalamus, cerebellum and distal colon LMMP preparations. Frozen specimens were crushed into powder using a liquid nitrogen-cooled biopulverizer unit (Research Products International, Philadelphia, PA, USA), and were homogenized in 350 μL lysing buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate, 1% NP-40, 0.25% deoxycholate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM NaF, with Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA)].

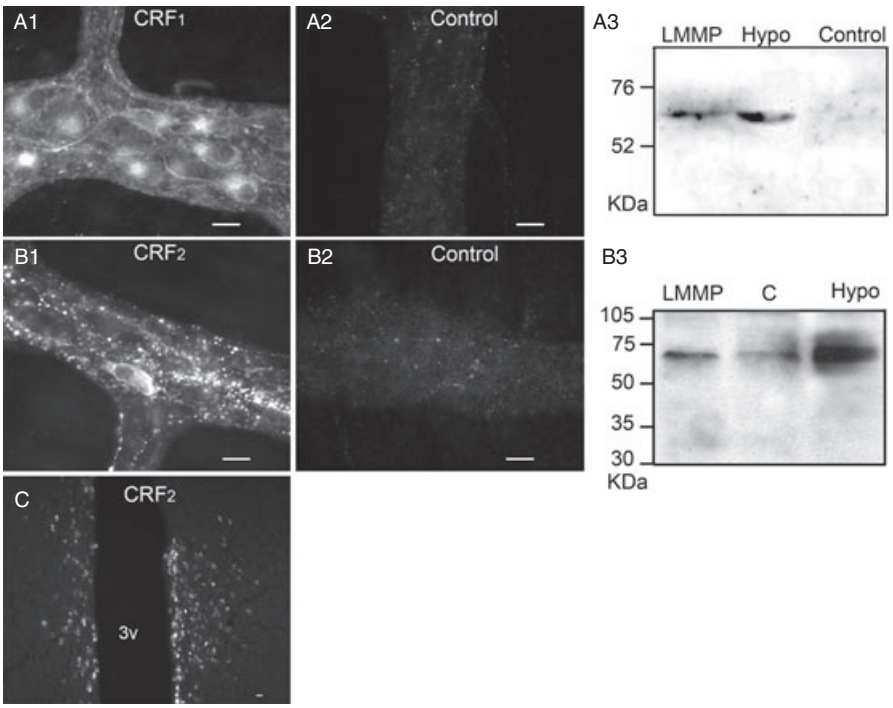

Figure 1 Expression of CRF_1 and CRF_2 receptor immunoreactivity (IR) in the myenteric plexus of the guinea pig distal colon. (A_1) CRF_1 receptor IR was expressed in neuronal cell bodies within the myenteric plexus. (A_2) Pre-absorption of the CRF_1 receptor antibody with the blocking peptide (sc-12383p) eliminated specific staining. (A_3) Western blot analysis demonstrated that the CRF_1 receptor antibody recognized a protein band around 66 kDa in samples extracted from longitudinal muscle–myenteric plexus (LMMP) and hypothalamus. Pre-absorption with the blocking peptide against which the CRF_1 receptor antibody was raised resulted in the loss of the immunoreactive band (control). (B_1) CRF_2 receptor IR was expressed predominantly in varicose nerve fibres in the myenteric plexus. CRF_2-immunoreactive neuronal cell bodies were sparse. (B_2) Omitting the primary antibody for CRF_2 receptors eliminated the immunostaining. (B_3) Western blot analysis demonstrated that the CRF_2 receptor antibody recognized a protein band around 70 kDa in samples extracted from LMMP, cerebellum and hypothalamus. (C) CRF_2 receptor IR was expressed in the guinea pig hypothalamus, which served as a positive control. LMMP, longitudinal muscle–myenteric plexus; Hypo, hypothalamus; C, cerebellum. Scale bars = 20 μm.

Homogenates were incubated in lysing buffer on ice for 1 h, and subsequently centrifuged at 10 000× *g* for 20 min at 4°C to obtain the cell membrane fraction in the supernatant. Protein levels were determined with the use of the detergent-compatible protein assay system (Bio-Rad Laboratories, Hercules, CA, USA). Protein (40 μg per lane) was resolved by gel electrophoresis followed by transfer to the nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked with 5% non-fat milk in Tris–HCl-buffered saline (TBS) for 1 h at room temperature. After washing with TBS, the membranes were incubated overnight at 4°C with CRF_1 (1:200) or CRF_2 receptor antibody (1:4000). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-goat IgG (1:5000; Santa Cruz Biotechnology, Inc.) or horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10 000; Amersham Biosciences). The immunoblots were detected with enhanced chemiluminescence reagents (Amersham Biosciences).

Data analysis

Data are expressed as means ± SEM with *n* values representing the numbers of neurons for electrophysiological studies and the numbers of animals for immunohistochemical studies. Analysis of concentration–response relationships was done with the SigmaPlot (SPSS, Inc., Chicago, IL, USA) non-linear curve-fitting program. Concentration–response curves were constructed from the pooled data by a sigmoid fit to $V = V_{max}[1 + (EC_{50}C^{-1})^{nH}]^{-1}$, where V is the observed response, V_{max} is the maximal response, C is the corresponding concentration, EC_{50} is the concentration yielding the half-maximal response and nH is the apparent Hill coefficient. Paired or unpaired Student's *t*-test was used to determine statistical significance. $P < 0.05$ was considered statistically significant.

Materials

Ucn1, Ucn2, Ucn3, astressin, antisauvagine-30, nifedipine and scopolamine were obtained from Sigma-Aldrich. NBI 27914 was purchased from Tocris Cookson (Ellisville, MO, USA). Pharmacological agents were dissolved in isotonic saline applied by addition to the bathing solution, unless otherwise indicated. Stock solutions of NBI 27914 and nifedipine were prepared in DMSO, with final DMSO concentrations less than 0.1% (v/v). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological parameters.

Results

Action of Ucn1, Ucn2 or Ucn3 on neuronal excitability

Intracellular recording with 'sharp' microelectrodes was used to study actions of Ucns in 74 AH- and 43 S-type neurons in

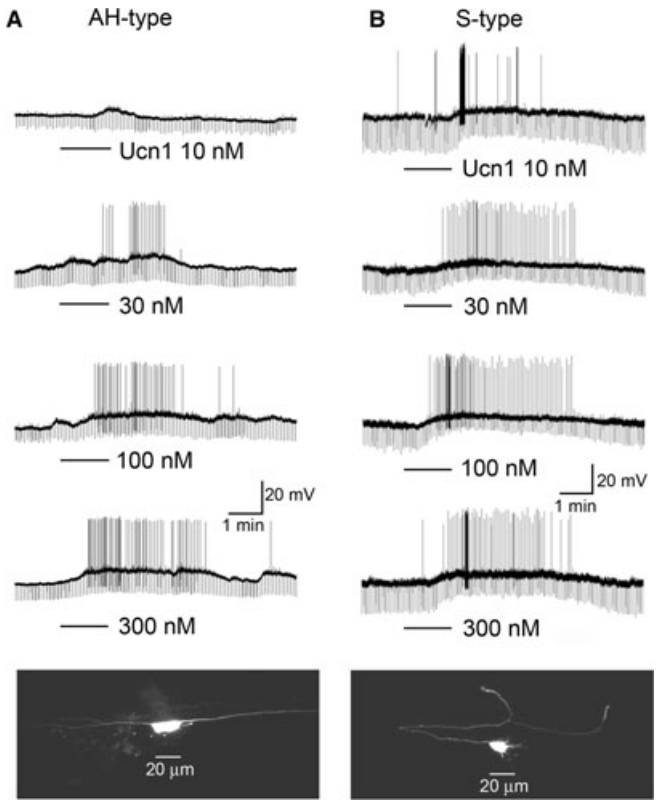

Figure 2 Depolarizing responses to urocortin-1 (Ucn1) were concentration dependent in both AH- and S-type colonic myenteric neurons. (A) Responses to Ucn1 in concentrations from 10 to 300 nM in an AH-type neuron. Downward deflections are electrotonic potentials evoked by intraneuronal injection of constant-current hyperpolarizing pulses. Increased amplitude of the downward deflections reflects increased input resistance. Upward deflections are action potentials occurring at the offset of hyperpolarizing current pulses. Occurrence of action potentials reflects elevated neuronal excitability. Morphology of the AH-type neuron from which the records in (A) were obtained appears in the inset. (B) Responses to Ucn1 (10–300 nM) in an S-type neuron. Decreased amplitude of the downward deflections reflects decreased input resistance. Morphology of the S-type neuron from which the records in B were obtained appears in the inset.

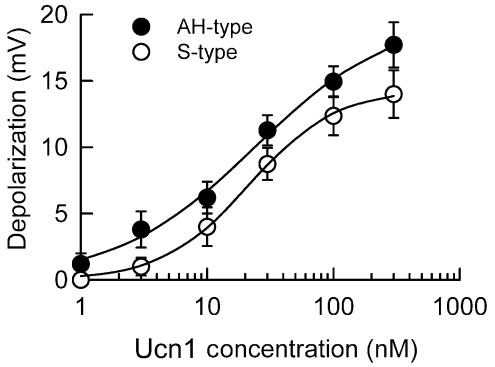

Figure 3 Concentration–response relationships for urocortin-1 (Ucn1)-evoked membrane depolarization in AH- and S-type neurons. Each data point represents six AH-type neurons and six S-type neurons. The EC_{50} value was 37.6 ± 9.8 nM for AH-type neurons, and 60.5 ± 18.9 nM for S-type neurons.

the myenteric plexus of the guinea pig distal colon. The neurons were identified as S- or AH-type according to established electrophysiological criteria (Bornstein *et al.*, 1994; Wood, 1994; 2006). The same neurons were identified morphologically by intraneuronal injection of biocytin from the microelectrode.

Application of Ucn1 (1–300 nM) in the superfusion solution depolarized the membrane potential coincident with enhanced excitability (Figure 2A,B). Enhanced excitability was reflected by an increased number of action potentials evoked by intraneuronal injection of constant depolarizing current pulses (data not shown), anodal-break excitation at the offset of hyperpolarizing current pulses (Figure 2A,B) and the occurrence of spike discharges (Figure 2B). Morphological analysis of the biocytin-filled neurons revealed that Ucn1 evoked depolarizing responses and elevated excitability in both Dogiel type II multipolar neurons (Figure 2A, inset) with AH-type electrophysiological behaviour (58%; 43/74) and uniaxonal neurons (Figure 2B, inset) with S-type electrophysiological behaviour (60%; 26/43). An increase in membrane input resistance was associated with the depolarizing responses in AH-type neurons (Figure 2A). In S-type neurons, the input resistance decreased in 58% (15/26) (Figure 2B), and was unchanged in the remainder. The effects of Ucn1 were fully reversed after a 15–20 min washout period. Time for recovery during washout was directly related to the concentration. Blockade of synaptic transmission by tetrodotoxin (TTX; 300 nM) in the bathing solution or lowering Ca^{2+} to 0.5 mM and elevating Mg^{2+} to 12 mM did not alter the depolarizing responses evoked by 100 nM Ucn1 (Ucn1: 13.7 ± 1.7 mV; Ucn1 plus TTX: 14.0 ± 1.7 mV; Ucn1 in low-Ca^{2+}/high-Mg^{2+} Krebs solution: 12.8 ± 1.6 mV; $P > 0.05$; $n = 6$). Membrane depolarization evoked by Ucn1 was concentration dependent with EC_{50} values of 37.6 ± 9.8 nM ($n = 6$) for AH-type neurons, and 60.5 ± 18.9 nM ($n = 6$) for S-type neurons (Figure 3). The maximal depolarization of the membrane potential evoked by 300 nM Ucn1 was 17.0 ± 1.9 mV ($n = 6$) for AH-type neurons, and 14.0 ± 2.4 mV ($n = 6$) for S-type neurons.

Application of Ucn2 or Ucn3 in the superfusion solution evoked slowly activating depolarizing responses similar to those evoked by Ucn1 (Figure 4). Of the 74 AH-type neurons tested, the membrane potential of nine of the neurons was depolarized by Ucn2, and membrane depolarization by Ucn3 occurred in six. Neither Ucn2 nor Ucn3 evoked membrane depolarization in any of the 34 S-type neurons tested. Ucn2 evoked membrane depolarization of 16.2 ± 1.3 mV at 100 nM ($n = 9$), and 15.0 ± 3.1 mV at 300 nM ($n = 4$). Ucn3 evoked membrane depolarization of 15.5 ± 3.1 mV at 100 nM ($n = 4$), and 17.0 ± 7.0 mV at 300 nM ($n = 2$). Full concentration–response curves for Ucn2 and Ucn3 were not obtained because the numbers of neurons responding to Ucn2 and Ucn3 were small. Blockade of synaptic transmission by TTX (300 nM) in the bathing solution, or lowering Ca^{2+} to 0.5 mM and elevating Mg^{2+} to 12 mM did not alter the depolarizing responses evoked by 100 nM Ucn2 (Ucn2: 12.7 ± 0.7 mV; Ucn2 plus TTX: 12.7 ± 0.7 mV; Ucn2 in low-Ca^{2+}/high-Mg^{2+} Krebs solution: 12.3 ± 0.3 mV; $P > 0.05$; $n = 3$) or 100 nM Ucn3 (Ucn3: 11.3 ± 3.5 mV; Ucn3 plus TTX: 11.3 ± 3.5 mV; Ucn3 in low-Ca^{2+}/high-Mg^{2+} Krebs solution: 10.7 ± 2.9 mV; $P > 0.05$; $n = 3$).

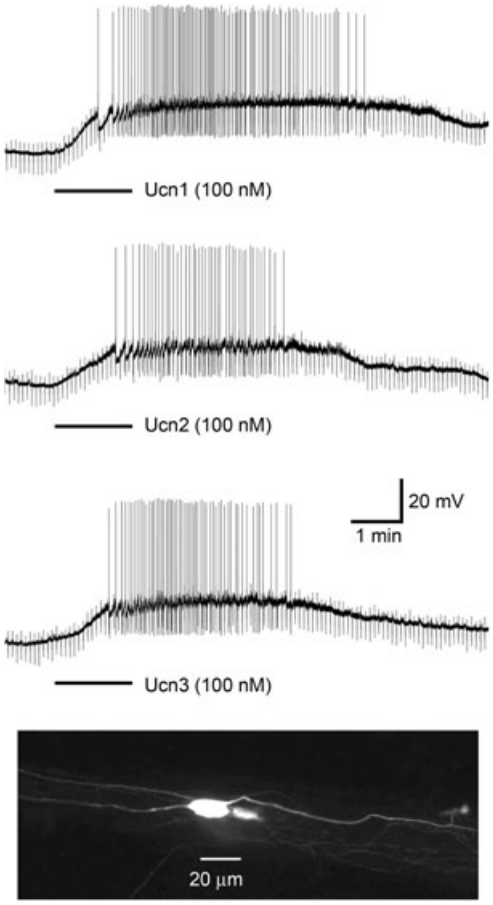

Figure 4 Urocortin-1 (Ucn1), urocortin-2 (Ucn2) and urocortin-3 (Ucn3) evoked depolarizing responses in an AH-type colonic myenteric neuron. Bath application of Ucn1, Ucn2 or Ucn3 evoked slowly activating membrane depolarization. The depolarizing responses to Ucn1, Ucn2 and Ucn3 were associated with increased input resistance. Downward deflections are electrotonic potentials evoked by intraneuronal injection of constant-current hyperpolarizing pulses. Increased amplitude of the downward deflections reflects increased input resistance. Enhanced excitability is reflected by the occurrence of anodal-break excitation at the offset of hyperpolarizing current pulses. Morphology of the AH-type neuron from which the records were obtained was shown in the inset.

CRF_1 and CRF_2 receptor antagonists

Involvement of the CRF receptor subtypes in Ucn1-, Ucn2- and Ucn3-evoked excitatory responses was investigated with the CRF_1/CRF_2 receptor antagonist astressin, the selective CRF_1 receptor antagonist NBI 27914 and the selective CRF_2 receptor antagonist antisauvagine-30. In these experiments, neurons were pretreated with each antagonist for 5 min, and the responses to applications of Ucn1, Ucn2 or Ucn3 in the presence of each antagonist were obtained. The presence of astressin, NBI 27914 or antisauvagine-30 in the bathing solution did not change the membrane potential, input resistance or neuronal excitability. The non-selective CRF receptor antagonist astressin (1 μM) suppressed the depolarizing responses to 30 nM Ucn1 in all five (two AH- and three S-type) neurons studied (Figure 5B,G). The selective CRF_1 receptor antagonist NBI 27914 (10 μM) suppressed the depolarizing responses to 30 nM Ucn1 in four S-type (Figure 5C) and four AH-type neurons. NBI 27914 (1–100 μM) concentration-dependently suppressed the depolarizing action of 30 nM Ucn1 with an IC_{50} value of 8.0 ± 2.5 μM (n = 4; including three AH- and one S-type neurons) (Figure 5H). The selective CRF_2 receptor antagonist antisauvagine-30 (1 μM) did not suppress or otherwise alter the depolarizing responses to 30 nM Ucn1 in six out of six S-type neurons (Figure 5D), and seven out of nine AH-type neurons studied. However, in two out of nine AH-type neurons, antisauvagine-30 (1 μM) suppressed the responses to 30 nM Ucn1 by 46 ± 4% (Figure 6B). The residual Ucn1 responses were further diminished by the addition of 10 μM NBI 27914 in the bathing solution (Figure 6C). The depolarizing responses evoked by 30 nM Ucn2 and 30 nM Ucn3 were suppressed by 1 μM antisauvagine-30, but were not influenced by 10 μM NBI 27914 in three AH-type neurons (Figure 7).

Immunohistochemical localization of CRF_1 and CRF_2 receptors

Immunoreactivity (IR) for CRF_1 receptors was expressed in 38 ± 3% (n = 9) of ganglion cell somas in the colonic myenteric plexus of the guinea pig (Figure $1A_1$). CRF_1 receptor IR appeared in the cytoplasm and surface membranes, and in some cases, in processes projecting from the cell soma. None of the nerve fibres with varicosities showed IR, nor was there any staining of ganglion cell nuclei or of muscle in any of the preparations. Pre-equilibration with the peptide against which the CRF_1 receptor antibody was raised resulted in the loss of all CRF_1 receptor IR (Figure $1A_2$). Specificity of the CRF_1 receptor antibody was assessed also by Western blot analysis. The CRF_1 receptor antibody recognized an approximately 66 kDa protein band in samples extracted from colonic LMMP preparations and hypothalamus of the guinea pig (Figure $1A_3$). This band corresponded to the published molecular weight for the human and rat CRF_{1a} receptor (Pisarchik and Slominski, 2005; Yuan *et al.*, 2007). Pre-absorption with the blocking peptide against which the CRF_1 receptor antibody was raised resulted in the loss of the immunoreactive band (Figure $1A_3$).

CRF_2 receptor IR was predominantly stronger in varicose nerve fibres relative to myenteric neuronal cell bodies (Figure $1B_1$). A dense network of CRF_2 receptor-immunoreactive varicose nerve fibres was present in the ganglia (Figure $1B_1$), interconnecting nerve strands and tertiary plexus. Ganglion cell bodies with CRF_2 receptor IR were sparse and represented only 0.4 ± 0.1% (n = 3) of the total in the myenteric plexus. Omission of the primary antibody for CRF_2 receptors resulted in no immunostaining (Figure $1B_2$). Specificity of the CRF_2 receptor antibody was assessed by Western blot analysis. The CRF_2 antibody recognized a 70 kDa protein band in LMMP, cerebellum and hypothalamus of the guinea pig (Figure $1B_3$) and mouse (not shown). This band corresponded to the published molecular weight for the human and rat CRF_2 receptors (Chatzaki *et al.*, 2004a; Bishop *et al.*, 2006), and was additional evidence for specificity of the antibody. Specificity of the CRF_2 receptor antibody was also tested in the guinea pig and mouse hypothalamus where expression of the CRF_2 receptor subtype is well documented (Lovenberg *et al.*, 1995). The antibody-labelled neurons in the paraventricular nucleus of the guinea pig (Figure 1C) and mouse (not shown) hypothalamus, which increased

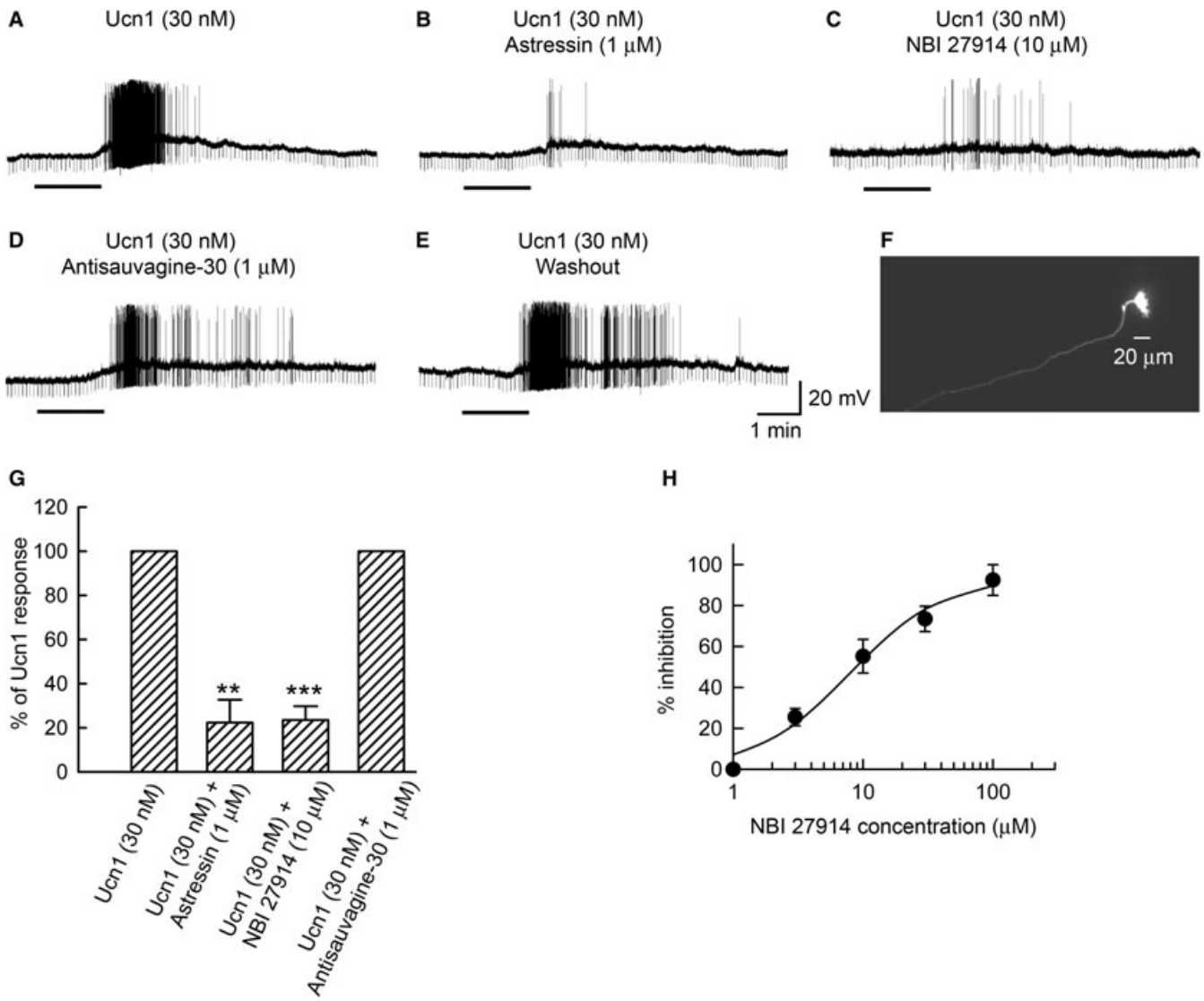

Figure 5 Effect of corticotropin-releasing factor (CRF) receptor antagonists on urocortin-1 (Ucn1)-evoked depolarizing responses. (A) Bath application of 30 nM Ucn1 evoked slowly developing membrane depolarization that was associated with enhanced excitability in an S-type myenteric neuron. (B) The non-selective CRF receptor antagonist, astressin (1 μM), suppressed the Ucn1-evoked excitatory responses. (C) The selective CRF_1 receptor antagonist, NBI 27914 (10 μM), suppressed the Ucn1-evoked excitatory responses. (D) The selective CRF_2 receptor antagonist, antisauvagine-30 (1 μM), did not suppress the actions of Ucn1. (E) Washout of the antagonists restored the excitatory action of Ucn1. (F) Morphology of the neuron from which the records in (A) to (E) were obtained. (G) Pooled data for the effect of the CRF receptor antagonists on Ucn1-evoked membrane depolarization. Paired Student's *t*-test was used to determine statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$. (H) Concentration–response relation for inhibition by NBI 27914 (1–100 μM) of membrane depolarization evoked by Ucn1 (30 nM). Each data point represents four neurons. The IC_{50} value for NBI 27914 was 8.0 ± 2.5 μM.

confidence in the specificity of the CRF_2 receptor antibody we were using.

In order to determine if the neurons that responded to Ucn1 express CRF_1 receptor IR, the neurons were filled with biocytin and later immunostained with the CRF_1 receptor antibody. Of the 69 Ucn1-responsive neurons, 39 were characterized immunohistochemically for CRF_1 receptor IR. Of the 39 neurons, 25 displayed Dogiel type II multipolar morphology, and 14 displayed Dogiel type I uniaxonal morphology. Ucn1-responsive neurons with AH-type electrophysiological behaviour and Dogiel type II morphology (Figure 8A), and Ucn1-responsive neurons with S-type electrophysiological behaviour and uniaxonal morphology (Figure 8B) all expressed CRF_1 receptor IR. Expression of CRF_2 receptor IR on Ucn1-, Ucn2- or Ucn3-responsive neurons was not determined because the number of neurons with CRF_2 receptor-mediated responses was small.

Double-labelling studies were done to identify the classes of myenteric neurons that expressed CRF_1 or CRF_2 receptor IR. Neurons expressing calbindin, ChAT and NOS as neurochemical codes were examined. Calbindin is a marker protein, which identifies most guinea pig enteric neurons with AH-type electrophysiological behaviour and Dogiel type II multipolar morphology (Iyer *et al.*, 1988). CRF_1 receptor IR was detected in 98 ± 1% ($n = 4$) of calbindin-immunoreactive ganglion cells; conversely, calbindin IR was expressed by 18.7

± 7.3% (n = 4) of CRF_1-immunoreactive myenteric neurons (Figure $9B_{1–3}$). ChAT is a marker for enteric cholinergic neurons, which includes musculomotor neurons, secretomotor neurons and interneurons (Furness, 2000). CRF_1 receptor IR was detected in 62 ± 2% (n = 3) of ChAT-immunoreactive neurons; conversely, ChAT IR was expressed by 98 ± 1% (n = 3) of CRF_1 receptor IR neurons (Figure $9C_{1–3}$). NOS is a marker for uniaxonal inhibitory musculomotor neurons, which project their axons in the anal direction and a population of interneurons in the guinea pig myenteric plexus (Furness, 2000). CRF_1 receptor IR was detected in 40.6 ± 2.7% (n = 5) of NOS-immunoreactive neurons, and NOS IR was expressed by 33.3 ± 1.5% (n = 5) of neurons with CRF_1 IR (Figure $9D_{1–3}$). A small population of CRF_1 receptor-immunoreactive neurons (2.0 ± 0.3; n = 5) also expressed CRF_2 receptor IR (Figure $9E_{1–3}$).

All CRF_2 receptor-immunoreactive myenteric neurons expressed calbindin IR (n = 7; Figure $10B_{1–3}$) and ChAT IR (n = 3; Figure $10C_{1–3}$). CRF_2-immunoreactive nerve cell bodies were detected in 7.4 ± 1.7% (n = 7) of calbindin-positive nerve cells, and 0.7 ± 0.0% (n = 3) of ChAT-positive nerve cells. No colocalization of CRF_2 receptors with NOS was found in the myenteric plexus (Figure $10D_{1–3}$). In varicose nerve fibres, CRF_2 receptor IR was colocalized with IR for synaptophysin (Figure $10E_{1–3}$), which suggests that the CRF_2 receptor subtype might be expressed at pre-synaptic transmitter release sites.

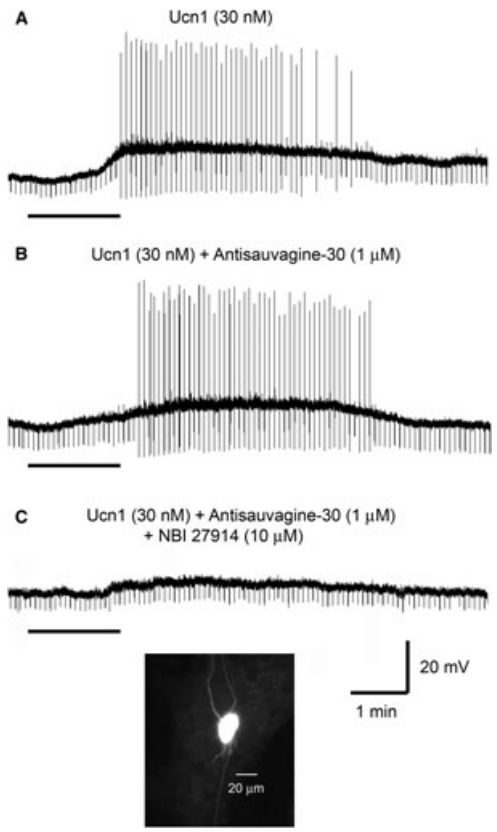

Figure 6 Responses evoked by urocortin-1 (Ucn1) were mediated by both CRF_1 and CRF_2 receptors in two out of nine AH-type myenteric neurons. (A) Bath application of Ucn1 (30 nM) evoked a slowly developing membrane-depolarizing response that was associated with enhanced excitability and elevated input resistance in an AH-type myenteric neuron. (B) The selective CRF_2 receptor antagonist, antisauvagine-30 (1 μM), suppressed the Ucn1-evoked membrane depolarization by ~50%. (C) The residual Ucn1 responses were further diminished by addition of 10 μM NBI 27914 in the bathing solution. Morphology of the neuron from which the records were obtained appears in the inset.

Action of Ucn1, Ucn2 or Ucn3 on synaptic transmission

The expression of CRF_2 receptors on varicose nerve fibres raised the question of whether activation of pre-synaptic CRF_2 receptors by Ucns altered synaptic transmission. We addressed the question by applying focal electrical stimulation to interganglionic fibre tracts, and recording stimulus-evoked EPSPs. Fast and slow EPSPs are the principal synaptic potentials evoked by fibre tract stimulation in the myenteric plexus of guinea pig colon (Wade and Wood, 1988b). None of the Ucns altered electrophysiological parameters of either fast or slow EPSPs (Figure 11A,B and Table 1).

Discussion and conclusions

Ucns are believed to be mediators in the signalling functions involved in neural control of intestinal motility (Harada *et al.*, 1999; Maillot *et al.*, 2000; 2003; Martinez *et al.*, 2002; Chen *et al.*, 2004; Saruta *et al.*, 2005; Chang *et al.*, 2007; Kimura *et al.*, 2007). The evidence for such mediation includes observations of accelerated transit after intraperitoneal administration of Ucn1 in conscious rats (Maillot *et al.*, 2000; 2003; Martinez *et al.*, 2002), and increased phasic contractions and electric field stimulation-induced off-contraction in isolated colonic muscle strips (Kimura *et al.*, 2007). Ucn2 and Ucn3 are ineffective on colonic motility (Martinez *et al.*, 2002; Kimura *et al.*, 2007). The neuronal blocker, TTX, abolishes Ucn1-evoked phasic contractions in colonic smooth muscle strips (Kimura *et al.*, 2007), suggesting an ENS-mediated event. In

Table 1 Effects of urocortins (Ucns) on synaptic transmission

	Ucn1 (100 nM)	*Ucn2 (100 nM)*	*Ucn3 (100 nM)*
Fast EPSP	Control: 12.0 ± 1.55 Ucn1: 12.0 ± 1.55 (n = 6)	Control: 12.7 ± 1.8 Ucn2: 12.7 ± 1.8 (n = 3)	Control: 12.7 ± 1.8 Ucn3: 12.7 ± 1.8 (n = 3)
Slow EPSP	Control: 14.4 ± 3.4 Ucn1: 14.4 ± 3.4 (n = 9)	Control: 8.0 ± 1.2 Ucn2: 8.0 ± 1.2 (n = 3)	Control: 8.0 ± 1.2 Ucn3: 8.0 ± 1.2 (n = 3)

These data represent the peak amplitude of the synaptic potentials in mV, and are expressed as means ± SEM; n = numbers of neurons from which data were obtained.

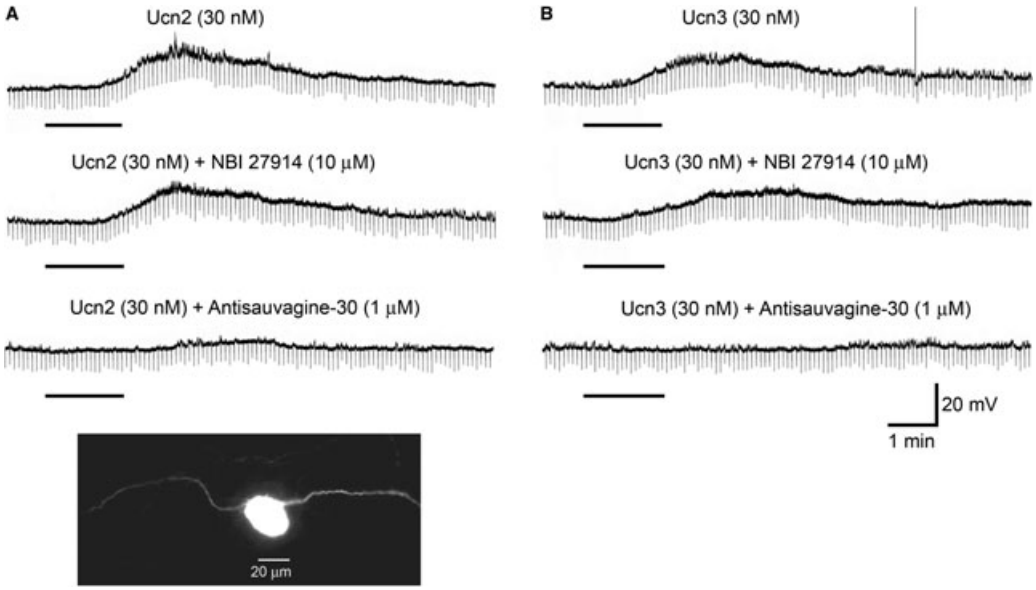

Figure 7 Depolarizing responses evoked by urocortin-2 (Ucn2) and urocortin-3 (Ucn3) were mediated by the CRF_2 receptor subtype. (A) The depolarizing responses evoked by 30 nM Ucn2 in an AH-type neuron were not influenced by 10 μM NBI 27914, but were suppressed by 1 μM antisauvagine-30. (B) Depolarizing responses evoked by 30 nM Ucn3 in the same AH-type neuron were not influenced by 10 μM NBI 27914, but were suppressed by 1 μM antisauvagine-30. Morphology of the neuron from which the records were obtained appears in the inset.

view of this, we tested the twofold hypothesis that myenteric neurons in the colon express CRF receptors and that binding of Ucns to the CRF receptors enhances neuronal excitability. Our finding that CRF receptors are expressed by myenteric neurons and that excitation of the neurons is a major action of Ucns supports the hypothesis.

The excitatory actions of Ucns mimic those evoked by CRF on myenteric neurons in intracellular microelectrode studies (Hanani and Wood, 1992; Liu *et al.*, 2005). Ucn1 was the Ucn that most often evoked excitation in the present study. Pharmacological analysis identified CRF_1 as the predominant receptor responsible for the excitatory action, and CRF_2 receptor involvement was found in a very small number of neurons. The proportion of myenteric neurons, which expressed CRF_1 receptor IR, was about 100-fold greater than the fraction showing CRF_2 receptor IR. The number of neurons that expressed CRF_1 IR was greater by far than the number found to respond to CRF_1 receptor agonists in single-neuron electrophysiological recording. This might reflect sampling bias due to immunohistochemical marking of the entire population of neurons that expressed CRF_1 receptors and the limitation of electrophysiological recording to a smaller sub-sample of the same neurons. Another plausible explanation is that the anti-CRF_1 receptor antibody labelled non-functional receptors in the neuronal cytoplasm in addition to the functional receptors in the somal membranes. The labelled cytoplasmic receptors might be newly synthesized receptors not yet integrated into the neuronal membrane. Overall, the immunohistochemical and electrophysiological results are consistent with the conclusion that CRF_1 is the predominant receptor subtype expressed in the colonic myenteric plexus of the guinea pig.

The excitatory actions of Ucn1, Ucn2 and Ucn3 on the neurons with AH-type electrophysiological behaviour and Dogiel type II morphology were typical of the excitatory actions of multiple other neurotransmitters and paracrine signal substances, which act at metabotropic G protein receptors coupled to adenyl cyclase and elevation of cAMP in this class of enteric neurons (Palmer *et al.*, 1986; Wood and Kirchgessner, 2004). Excitation evoked by Ucns like that of other mediators that mimic slow synaptic excitation (slow EPSP) in AH-type neurons was associated with increased input resistance, which reflected suppression of resting Ca^{2+}-gated K^+ conductance (Grafe *et al.*, 1980; Hanani and Wood, 1992; Wood and Kirchgessner, 2004). Ucn1 evoked more of these types of responses in AH neurons (i.e. 58%) than did Ucn2 with 12% of the neurons responding or Ucn3 with 8% responding.

The excitatory actions of Ucn1 on the neurons with S-type electrophysiological behaviour and a single long axon were typical of the excitatory actions of many other neurotransmitters and paracrine signal substances, which act at metabotropic G protein-coupled receptors to activate phospholipase C; synthesis of inositol 1,4,5-trisphosphate; mobilization of Ca^{2+} from intracellular stores; and opening of non-selective cation channels in S-type neurons (Hu *et al.*, 2003; 2004; Wood and Kirchgessner, 2004). Excitation evoked by Ucn1, like that of other mediators that mimic slow EPSPs in S-type neurons, was associated with decreased input resistance (i.e. increased membrane conductance), which reflected opening of non-selective cation channels (Wood and Kirchgessner, 2004; Qu *et al.*, 2007). Neither Ucn2 nor Ucn3 evoked responses in S-type neurons.

The slow EPSP-like action of the Ucns in AH-type neurons may be significant when the neurons are 'wired' into the ENS integrative microcircuitry in the functioning organ. Cell bodies of AH neurons in the myenteric plexus are flat coin-like disks with multiple long neurites attached around the perimeter (Hanani *et al.*, 1998). One or more neurites of the AH neurons leave the ganglion and project through the

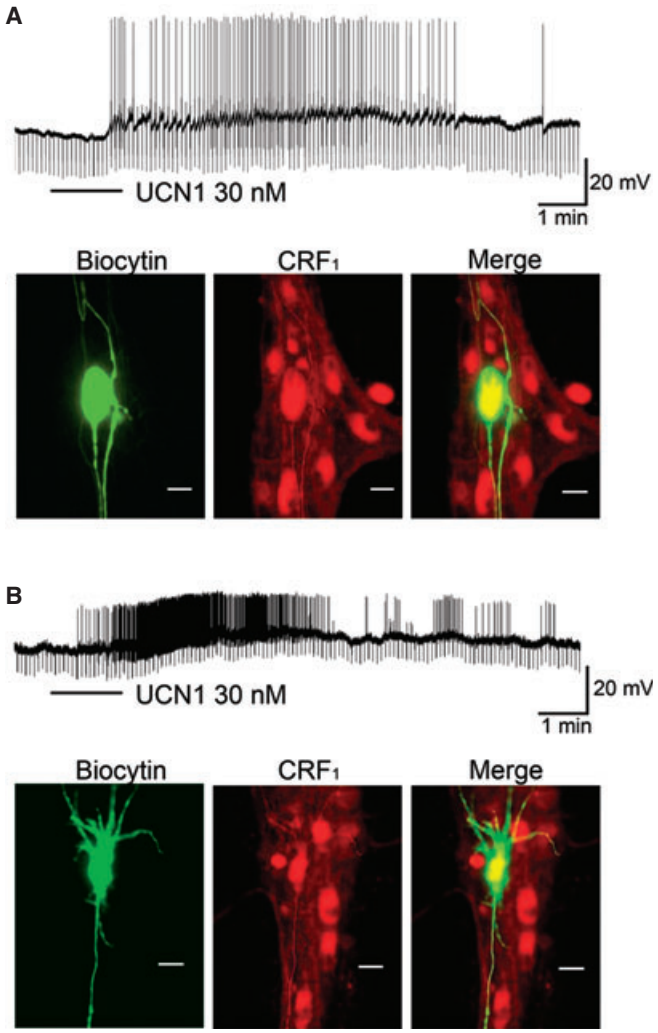

Figure 8 Expression of CRF_1 receptor immunoreactivity (IR) on urocortin-1 (Ucn1)-responsive myenteric neurons in the guinea pig distal colon. (A) Bath application of Ucn1 (30 nM) evoked slowly activating membrane depolarization and enhanced excitability in an AH-type neuron. The neuron was traced by filling with biocytin, and later staining for biocytin and the CRF_1 receptor. The Ucn1-responsive neuron with AH-type electrophysiological behaviour and Dogiel type II morphology was found to express CRF_1 IR. (B) Bath application of Ucn1 (30 nM) evoked slowly activating membrane depolarization and enhanced excitability in an S-type neuron. The Ucn1-responsive S-type neuron was found to have uniaxonal morphology and express CRF_1 receptor IR. Scale bars = 20 μm.

intestinal circular muscle and lamina propria, and terminate in the mucosal epithelium (Song *et al.*, 1994; Bertrand *et al.*, 1998). The nerve ending in the mucosa expresses 5-HT_3 receptors (Bertrand *et al.*, 2000), and 5-HT released from enterochromaffin cells by mechanical or chemical stimulation activates the receptors and stimulates the nerve ending to fire (Kirchgessner *et al.*, 1992; Kunze *et al.*, 1995; 1998; Bertrand *et al.*, 1997; 2000). This is why AH neurons have been referred to as intrinsic primary afferent neurons. However, AH neurons do not behave like classic primary afferent neurons. AH neurons receive synaptic input from other neurons, including other AH neurons, spinal afferents and parasympathetic neurons, mainly in the form of slow EPSPs (Wood and Mayer, 1979; Takaki and Nakayama, 1988; 1989; Kunze *et al.*, 1993; Tamura, 1997; Wang *et al.*, 2009), but also as nicotinic fast EPSPs (Grafe *et al.*, 1979; Tamura, 1997; Tamura *et al.*, 2001). These neurons form recurrent, excitatory networks in the ENS (Kunze *et al.*, 1993; Thomas *et al.*, 2000; Thomas and Bornstein, 2003; Chambers *et al.*, 2005; Wood, 2006), in which the neurons of the circuit make recurrent, slow excitatory, synaptic connections one with another. Neural circuits, 'wired' like this, are feedback circuits in which the interneuronal synaptic connectivity causes excitation to build rapidly to firing threshold in each of the neurons in the circuit. Rapid build-up of firing in individual neurons in such circuits would ensure simultaneous activation of an entire network of multiple AH neurons around the circumference and along the length of a segment of bowel. Output of the circuit becomes excitatory synaptic input to pools of motor neurons around the circumference and along the length of an intestinal segment (Thomas *et al.*, 2000; Thomas and Bornstein, 2003; Chambers *et al.*, 2005). Outputs from AH networks, in the myenteric plexus, are postulated to activate pools of excitatory and inhibitory musculomotor neurons during generation of propulsive motility, and to synchronize the firing of pools of secretomotor neurons to ensure uniform secretion within an intestinal segment. Our finding that AH neurons express the CRF_1 receptor subtype and that CRF_1 receptors mediate the excitatory action of Ucn1 in this class of enteric neurons suggests that Ucn1 and CRF might be another of the signals that elevate excitability in neuronal circuits with AH-type neurons.

Whereas multipolar AH-type neurons are named for their characteristic post-spike after-hyperpolarizing potentials, uniaxonal S-type neurons (i.e. synaptic-type neurons) are so named because ionotropic nicotinic fast EPSPs appear in virtually all S-type enteric neurons (Wood, 2006). All musculomotor neurons and some interneurons show S-type electrophysiological behaviour and single axon morphology. A pool of S-type neurons with ChAT IR in the guinea pig colon consists of cholinergic motor neurons, which when active evoke contraction of the intestinal musculature. Another population with NOS IR consists of inhibitory motor neurons to the musculature, and suppresses muscle contraction when active (Lomax and Furness, 2000). Activation of the CRF_1 receptors expressed by these functionally different kinds of motor neurons would be expected to energize propulsive motility in the colon and might be a neural correlate of the augmented contractile activity and propulsive motility that are reported to occur during exposure to Ucn1 (Maillot *et al.*, 2000; 2003; Martinez *et al.*, 2002; Kimura *et al.*, 2007).

Unlike enteric ganglion cell bodies, which expressed mainly CRF_1 receptor IR, axonal varicosities in both the myenteric and submucosal plexus were found to express CRF_2 IR. CRF_2 receptor IR, in these cases, was colocalized with synaptophysin IR, which suggests that the CRF_2 receptor subtype might be expressed at pre-synaptic transmitter release sites where multiple neurotransmitters and paracrine mediators act to either suppress or enhance neurotransmitter release (Wood, 2006). Hanani and Wood (1992) reported that application of CRF suppressed nicotinic neurotransmission (i.e. fast EPSPs) in the guinea pig small intestinal ENS. Nevertheless, this was interpreted as a post-synaptic action because

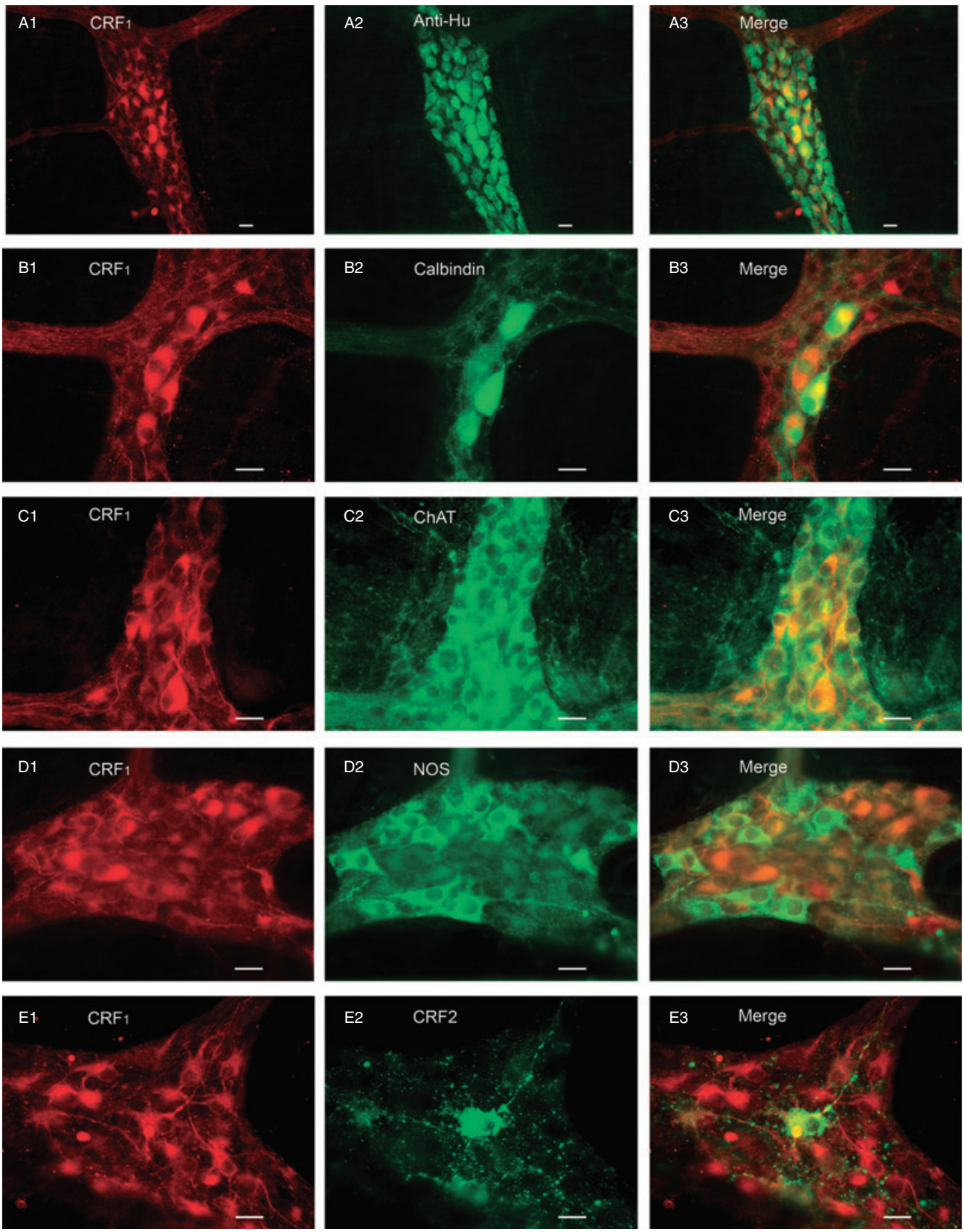

Figure 9 Colocalization of CRF_1 receptor immunoreactivity (IR) with neurochemical codes in the myenteric plexus of the guinea pig distal colon. (A_{1-3}) Double labelling of CRF_1 receptors with anti-Hu, which labels all enteric neurons, showed that the CRF_1 receptor was expressed exclusively by myenteric neurons. (B_{1-3}) CRF_1 receptor IR was colocalized with calbindin IR. (C_{1-3}) CRF_1 receptor IR was colocalized with ChAT. IR. (D_{1-3}) CRF_1 receptor IR was colocalized with nitric oxide synthase (NOS) IR. (E_{1-3}) A small subset of CRF_1 receptor-immunoreactive myenteric neurons also expressed CRF_2 receptor IR. Scale bars = 20 μm.

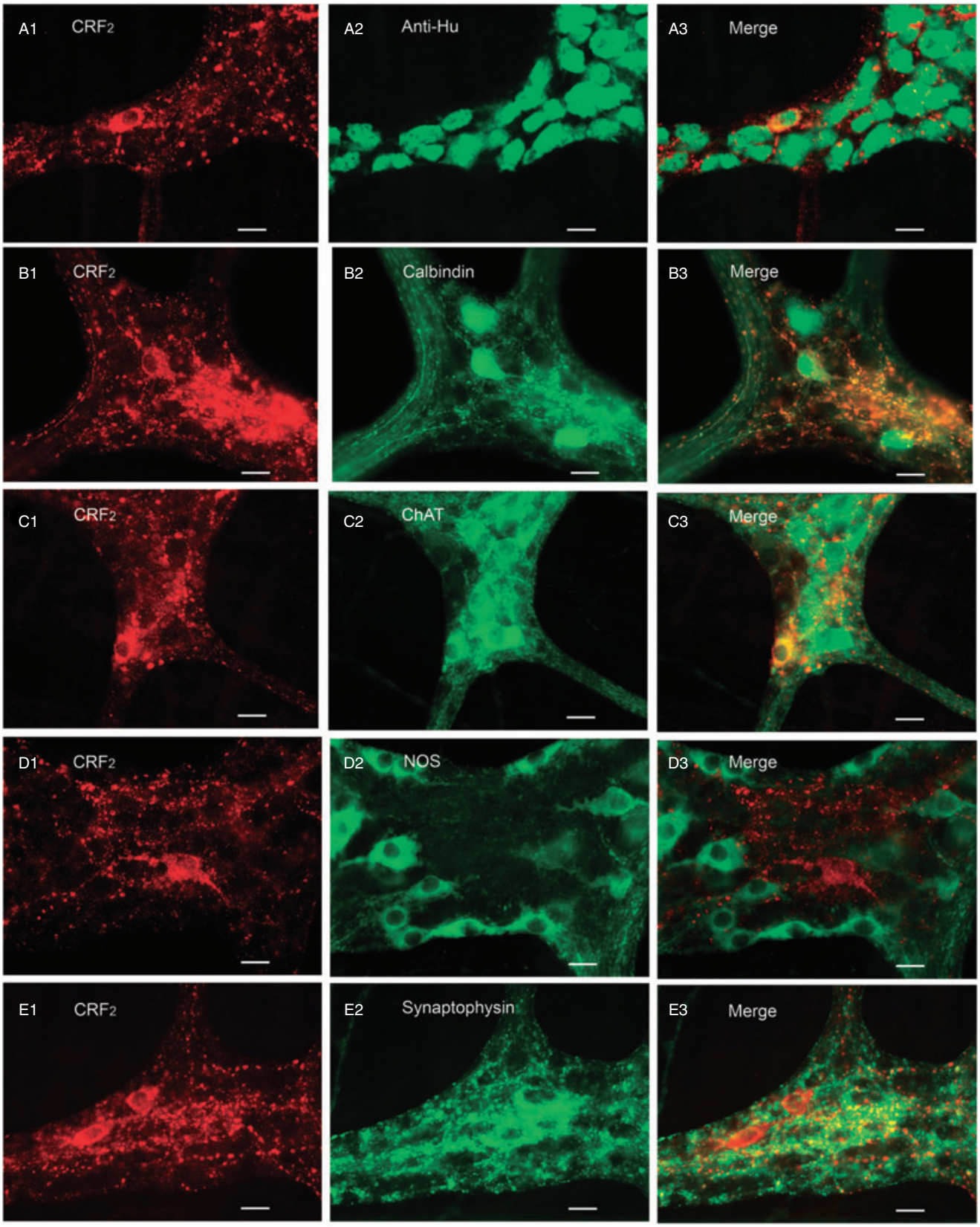

Figure 10 Colocalization of CRF_2 receptor immunoreactivity (IR) with neurochemical markers in the myenteric plexus of the guinea pig distal colon. (A_{1-3}) Double labelling of CRF_2 receptors with anti-Hu, which labels all enteric neurons, showed expression of CRF_2 receptor IR by only a small subset of neurons. (B_{1-3}) CRF_2 receptor IR was colocalized with calbindin IR. (C_{1-3}) CRF_2 receptor IR was colocalized with ChAT IR. (D_{1-3}) CRF_2 receptor IR was not colocalized with NOS IR. (E_{1-3}) CRF_2 receptor IR and synaptophysin IR were coexpressed in varicose nerve fibres. Scale bars = 20 μm.

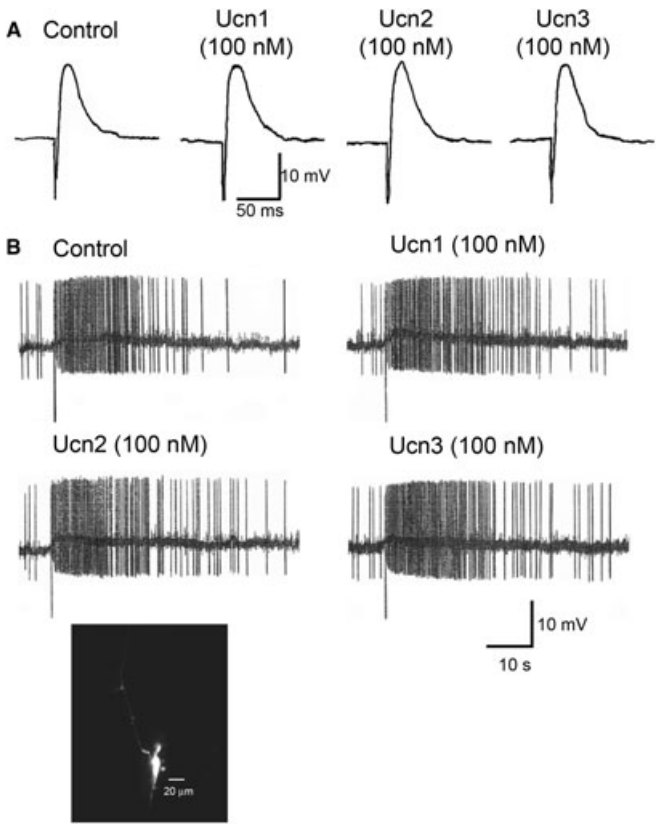

Figure 11 Exposure to urocortins (Ucns) did not alter excitatory neurotransmission in the myenteric plexus of guinea-pig colon. (A) Fast excitatory postsynaptic potentials (EPSPs) evoked in an S-type myenteric neuron were unaffected by Ucn1, Ucn2 or Ucn3. (B) Slow EPSPs evoked in the same myenteric neuron were unaffected by Ucn1, Ucn2 or Ucn3. Morphology of the neuron from which the results in A and B were obtained appears in the inset. Scale bars = 20 μm.

exposure to CRF also suppressed ionotropic depolarizing responses to ACh. Neither of the Ucns altered the electrophysiological parameters of fast or slow EPSPs evoked by electrical stimulation of interganglionic fibre tracts in the myenteric plexus, which raises a question as to the identification of the pre-synaptic varicosities that might express the CRF_2 receptor subtype. We have found that keeping the guinea pig myenteric plexus organotypic culture for 5 days resulted in significant loss of CRF_2 receptor IR nerve fibres, suggesting that the CRF_2 receptors might be primarily expressed by extrinsic nerve fibres after they enter the intestine. A likely extrinsic fibre candidate would be spinal afferents. The suggestion that spinal afferents might express CRF_2 receptors is compatible with a report that peripheral injection of Ucn2 blunts pain induced by colorectal distension, suppresses colonic afferent spike activity and reduces ERK1/2 phosphorylation evoked by CRF_2 receptor activation in rat spinal cord (Million *et al.*, 2006). Moreover, dorsal root ganglia are known to express the CRF_2 receptor gene (Million *et al.*, 2006).

In summary, this study has demonstrated that Ucn1 evokes neuronal excitation primarily via CRF_1 receptor activation in the colonic myenteric plexus, whereby it might influence colonic motility and secretion. Activation of the CRF_2 receptors on myenteric neurons might not be sufficient alone to produce measurable changes in colonic motility. Our immunohistochemical data suggest that the Ucn1-mediated effects involve activation of both cholinergic and nitrergic neuronal pathways. The predominant expression of functional CRF_1 receptors, relative to CRF_2 receptors, along with the expression of CRF_1 receptor ligands, CRF (Liu *et al.*, 2006) and Ucn1 (Harada *et al.*, 1999; Kimura *et al.*, 2007) in the ENS of the colon suggests a neuromodulator role for CRF or Ucn1 in the ENS under normal conditions and perhaps more importantly during stress.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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