Localisation and Activation of the Neurokinin 1 Receptor in the Enteric Nervous System of the Mouse Distal Colon

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ABSTRACT

Background & Aims. The substance P neurokinin 1 receptor (NK₁R) regulates motility, secretion, inflammation and pain in the intestine. The distribution of the NK₁R is a key determinant of the functional effects of substance P in the gut. Information regarding the distribution of NK₁R in subtypes of mouse enteric neurons is lacking, and is the focus of the present study.

Methods. NK₁R immunoreactivity (NK₁R-IR) was examined in wholemount preparations of the mouse distal colon by indirect immunofluorescence and confocal microscopy. The distribution of NK₁R-IR expression within key functional neuronal subclasses was determined using established neurochemical markers.

Results. NK₁R-IR was expressed by a subpopulation of myenteric and submucosal neurons. NK₁R-IR was mainly detected in large, multipolar myenteric neurons and was colocalized with calcitonin gene-related peptide, neurofilament M, choline acetyltransferase and calretinin. The remaining NK₁R-IR neurons were positive for nitric oxide synthase. The NK₁R was expressed by the majority of submucosal neurons and was exclusively co-expressed with vasoactive intestinal peptide, with no overlap with choline acetyltransferase. Treatment with substance P resulted in the concentration-dependent internalisation of NK₁R from the cell surface into endosome-like structures.

Discussion. Myenteric NK₁R is mainly expressed by intrinsic primary afferent neurons, with minor expression by descending interneurons and inhibitory motor neurons. Submucosal NK₁R is restricted to non-cholinergic secretomotor neurons. These findings highlight key differences in

the neuronal distribution of NK₁R-IR between the mouse, rat and guinea pig, with important implications for the functional role of NK₁R in regulating intestinal motility and secretion.

Keywords: 5

Enteric Nervous System, tachykinin, neurokinin 1 receptor, neurochemical coding, receptor trafficking

INTRODUCTION

The tachykinin family of neuropeptides control motility, secretion, inflammation and pain in the intestine (Steinhoff *et al.*, *in press*). Tachykinins in the intestine, which mainly include substance P (SP) and neurokinin A, derive from intrinsic neurons of the myenteric and submucosal plexuses, as well as from extrinsic neurons of the dorsal root ganglia. Tachykinins from enteric neurons promote peristalsis and secretion of electrolytes and fluid in the intestine (Holzer & Holzer-Petsche, 1997a, b). Tachykinins that are released from the peripheral projections of primary spinal afferent neurons induce neurogenic inflammation of the intestine, and tachykinins that are released from the central projections of these neurons in the dorsal horn of the spinal cord promote visceral nociception (Steinhoff *et al.*, *in press*). These actions of tachykinins are mediated by three neurokinin receptors (NK₁R, NK₂R and NK₃R). These receptors are all expressed within the gastrointestinal tract, with distributions best characterised for the guinea pig ileum (Portbury *et al.*, 1996; Lomax *et al.*, 1998). Both NK₁R and NK₃R are primarily expressed by neurons of the enteric nervous system (ENS), whereas NK₂R is predominantly localized to gastrointestinal smooth muscle, nerve varicosities and the epithelium

(Holzer & Holzer-Petsche, 2001). Within the ENS, tachykinins acting via NK₃R stimulate slow excitatory post-synaptic potentials (Bertrand & Galligan, 1994) and enhance Na(v)1.9 currents in intrinsic primary afferent neurons (IPANs) of the myenteric plexus (Copel *et al.*, 2009). Recent evidence suggests a functional role for neuronal NK₂R, particularly in mediating contractile responses by the human intestine to electrical field stimulation (Lecci *et al.*, 2006). The role of neuronal NK₁R is less well-defined, but SP evokes NK₁R-dependent contractions of the intestine from a number of different species and plays a role in peristalsis (Nieuwmeyer *et al.*, 2006). NK₁R is also functionally expressed by intramuscular interstitial cells of Cajal (ICC) and may be involved in mediating the neurogenic effects of SP on gastrointestinal motility (Iino *et al.*, 2004). The distribution and neurochemical coding of NK₁R immunoreactive (-IR) enteric neurons has been characterized extensively in the small intestine of the guinea pig and rat (Portbury *et al.*, 1996; Mann *et al.*, 1999b), and to a lesser extent in that of the mouse and human (Smith *et al.*, 1998; Vannucchi & Faussone-Pellegrini, 2000). Comparatively fewer studies have examined NK₁R expression in the colon (Bian *et al.*, 2000; Harrington *et al.*, 2005), despite its role in controlling spontaneous colonic motility (Lecci *et al.*, 1997; Brierley *et al.*, 2001) and water and electrolyte balance (Cooke *et al.*, 1997), and in mediating the proinflammatory effects of SP (Steinhoff *et al.*, *in press*). Moreover, there is limited information about the neuron-specific distribution of the NK₁R in the intestine of the mouse (Vannucchi & Faussone-Pellegrini, 2000), despite the wealth of information about the regulation of intestinal motility, secretion, inflammation and pain in this species. Knowledge of the expression of the NK₁R by subtypes of enteric neurons can provide a mechanistic understanding of the actions of tachykinins within the intestine and can aid in predicting the functional consequences of NK₁R activation or antagonism. In this study we have used a well-characterised antibody to NK₁R combined with

established neurochemical markers to characterise the distribution and activation of NK₁R in the myenteric and submucosal plexuses of the mouse distal colon. We demonstrate NK₁R expression by myenteric IPANs and submucosal non-cholinergic secretomotor neurons and identify significant interspecies differences in the distribution of NK₁R. These results provide, for the first time, an anatomical link with the existing literature on the role of the NK₁R in regulating murine gastrointestinal function.

MATERIALS AND METHODS

Ethical Approval. All procedures on experimental animals were approved by The Institutional Animal Care and Use Committee of the University of California, San Francisco and the Monash University Animal Ethics Committee.

Animals. C57BL/6J mice (6-8 weeks, 20-25 g male and female) were from Charles River Laboratories (Hollister, CA) or from the Monash Animal Research Platform (Clayton, Victoria, Australia).

Tissue collection. Mice were killed by cervical dislocation and the distal colon was collected into cold phosphate buffered saline (PBS) containing nicardipine (10 µM). Tissues were cleared of contents, opened along the mesenteric border and pinned tautly, mucosa-downwards onto sylgard-lined 35mm dishes. Preparations were then fixed in 4% paraformaldehyde (in PBS, pH 7.4) overnight at 4°C. To facilitate detection of neuropeptides in the soma of neurons, mice were treated with colchicine (2.5 mg/kg, i.p.) 6 hours prior to tissue collection (Poole *et al.*, 2011).

NK₁R internalisation assays. Agonist-induced endocytosis of endogenous NK₁R and Alexa594-conjugated SP (Alexa-SP) uptake was examined in organotypic preparations of the distal colon as described in detail (Pelayo *et al.*, 2011).

Reagents. The following compounds were obtained from the suppliers listed: nicardipine hydrochloride (Sigma-Aldrich, St Louis, MO, USA), SP (Bachem, Torrance, CA, USA), RP-67580 (Tocris Bioscience, Ellisville, MO, USA), tetrodotoxin citrate (Alomone, Jerusalem, Israel). SP was labelled with Alexa594 dye and purified by HPLC as described (Bunnett *et al.*, 1995).

Antibodies: A full list of primary antibodies used in this study is provided in **Table 1**. Secondary antibodies raised in donkeys were conjugated to Alexa Fluor 488, 594 or 647 (Invitrogen and Jackson ImmunoResearch) and used at final dilutions of 1:500 or 1:1000.

Immunofluorescence. Myenteric and submucosal wholemounts and tissue sections of the distal colon (12 μ m thickness cryosections) were prepared as described (Poole *et al.*, 2011) and processed for immunofluorescence (Pelayo *et al.*, 2011). Tissues were incubated with primary antisera for 48 hours at 4°C in blocking buffer (10% normal horse serum, 0.1% Triton X-100 in PBS containing 0.1% sodium azide). Preparations were mounted in Prolong Gold (Invitrogen) and imaged using either Zeiss LSM510 META or Leica TCS SP8 confocal microscope systems.

Image analysis. Co-expression of NK₁R with established neurochemical markers was determined manually from Z-stacked confocal images (Poole *et al.*, 2011) and expressed as percentage overlap between the different neuronal populations. At least 3 mice were used for the analysis of each marker. The relative distribution of NK₁R-IR at the cell surface and within the cytosol was determined from captured images using ImageJ software (NIH) as described (Pelayo *et al.*, 2011).

RESULTS

SP is detected in nerve fibres of extrinsic and intrinsic origin. The ENS is the major source of intestinal tachykinins, which derive from cholinergic excitatory motor neurons, ascending interneurons and intrinsic primary afferent neurons (IPANS) of the myenteric plexus and from submucosal IPANS (Furness, 2000). The colon also receives input from tachykinin-expressing primary spinal afferent neurons (Holzer & Holzer-Petsche, 1997a), and SP is expressed by immune cells including macrophages and T lymphocytes (Koon & Pothoulakis, 2006).

The distribution of SP-IR nerve fibres in the distal colon was examined in sections and in myenteric and submucosal wholemount preparations using a well-characterised antibody that detects tachykinins (Cuello *et al.*, 1979). In tissue sections, SP-IR was detected in varicose nerve fibres running throughout the inner circular muscle of the *muscularis externa* and within the myenteric and submucosal ganglia (Fig. 1a, 1b). SP-IR nerve fibres innervating the mucosa were rarely observed (Ekblad *et al.*, 1988; Sang & Young, 1996). SP positive cells were also present throughout the *lamina propria*, suggesting expression by immune cells. In addition to its expression in varicose nerve fibres innervating submucosal ganglia, SP-IR was detected in nerve fibres running parallel to submucosal vessels (Fig. 1c). These fibres were also immunoreactive for calcitonin gene-related peptide (CGRP) and transient receptor potential vanilloid 1 (TRPV1) (Fig. 1d), an observation that is consistent with denervation and anatomical studies that have identified these as being of extrinsic origin (Costa *et al.*, 1981; Ekblad *et al.*, 1988; Galligan *et al.*, 1988). SP-IR fibres within submucosal ganglia were often detected independently of CGRP-IR. In contrast, the majority of CGRP-positive fibres were also SP-IR. SP-IR is a marker of submucosal IPANs (Bornstein *et al.*, 1989; Kirchgessner *et al.*, 1992). SP-IR was not detected in submucosal neurons, consistent with evidence that there are no submucosal IPANs in the mouse ileum and colon (Figs. 1e, f) (Sang & Young, 1996; Wong *et al.*, 2008; Mongardi Fantaguzzi *et*

al., 2009) and with studies demonstrating that SP-IR fibres innervating submucosal ganglia are of myenteric origin (Jessen *et al.*, 1980; Costa *et al.*, 1981).

In myenteric wholemount preparations, SP-positive fibres were dense throughout myenteric ganglia and also ran within and parallel to the circular muscle layer. There was no overlap between SP- and CGRP-positive varicosities in either region (Figs. 1g, h). As SP and CGRP are expressed by distinct functional subpopulations of myenteric neuron (Qu *et al.*, 2008), this suggests that these fibres are of intrinsic origin. SP-positive fibres were also detected in wholemounts of submucosal ganglia, where they were less numerous than within myenteric ganglia.

These results confirm that the endogenous NK₁R agonist SP is expressed in discrete regions of the colon wall and further extends these studies to define the distribution of SP-positive nerve fibres within enteric ganglia of the mouse colon.

NK₁R is expressed by enteric neurons of the distal colon. In sections of the distal colon, NK₁R-IR was detected in enteric ganglia (Fig. 1b) and in colonocytes (not shown). There was also weak NK₁R-IR in smooth muscle of the *muscularis externa*. Within enteric neurons NK₁R-IR was localised to the plasma membrane of the soma and neurites, with minimal intracellular stores. This was particularly evident for submucosal neurons where the majority of NK₁R-IR was cell surface associated. In submucosal wholemount preparations NK₁R-IR was expressed by the majority of all neurons in all ganglia examined. No NK₁R-IR was detected in fibres that were associated with the submucosal vasculature or lymphatics (Figs. 1c-f). NK₁R-IR was localised to a subset of large diameter, multi- and unipolar myenteric neurons (Fig. 1g). NK₁R-IR was also present in ICCs within the myenteric and intramuscular levels, although this staining was weak

relative to that of neurons and to other regions of the gut (not shown). There was no evidence for NK₁R staining of enteric glial cells, as identified by double labelling against the glial marker glial fibrillary acidic protein (GFAP) (not shown).

NK₁R-IR is expressed by non-cholinergic submucosal secretomotor neurons. Although there is good physiological evidence that activation of NK₁R-IR on submucosal neurons leads to their depolarization and to enhanced secretomotor activity (MacNaughton *et al.*, 1997), the neuronal subtypes that express NK₁R in these ganglia remain largely undefined. We examined the distribution and neurochemical coding of NK₁R-IR neurons in submucosal ganglia of the mouse distal colon. Prominent NK₁R-IR was detected in the majority of PGP9.5-IR submucosal neurons (86%, 248/ 288 neurons), where it was localised to the plasma membrane of the soma and neurites (Fig. 2a-2a''). Where neurites were clearly visible, these neurons were generally unipolar and had smooth cell bodies. Neurons with very weak intensity staining or no detectable staining were considered to be negative for NK₁R-IR for the purposes of this study. The NK₁R-IR expressing population was further characterized using established neurochemical markers for different neuronal subtypes present in the murine submucosal plexus (Mongardi Fantaguzzi *et al.*, 2009). All NK₁R-IR neurons were calretinin-IR (100%, 143/143 neurons), whereas 84% of calretinin positive neurons (143/170 neurons) were also NK₁R-IR (Fig. 2b-b''). All NK₁R-IR neurons were VIP-IR (99%, 170/171 neurons) (Fig. 2c-c''). Most NK₁R-IR neurons were NPY-IR (Fig. 2d-d''), indicating that, as in the ileum, extensive overlap between the VIP-IR and NPY-IR subpopulations of neurons also occurs in the distal colon of the mouse. The extensive colocalisation between neurofilament M (NFM) and NK₁R-IR (not quantified) (Fig. 3d-d'') suggests that NFM is mainly expressed by non-cholinergic secretomotor neurons. In contrast,

there was minimal overlap between NK₁R-IR and either ChAT-IR (1%, 2/275 neurons; Fig. 2e-e'') or somatostatin (0%, 1/225 neurons; Fig. 2f-f''), which are specifically localised to cholinergic secretomotor neurons (De Jonge *et al.*, 2003; Mongardi Fantaguzzi *et al.*, 2009). These neurons were relatively lower in abundance than those positive for VIP or NPY. Similarly, there was almost no overlap between NK₁R and CGRP, with only 2% of the NK₁R population also immunopositive for CGRP (1/54 neurons; Fig. 3a-a''). CGRP is an established marker of AH neurons in other species, and is expressed by submucosal cholinergic secretomotor neurons of the mouse (Mongardi Fantaguzzi *et al.*, 2009). SP, another potential IPAN marker was not detected within the somata of submucosal neurons, although there was an extensive network of SP-IR varicosities closely associated with NK₁R-IR neurons (Fig. 1e, f). NK₁R-IR and NOS-IR were often present in the same neurons, and 29% (56/196 neurons) of all NK₁R-IR neurons were also NOS-IR (Fig. 3b-b''). In contrast to the ileum (Mongardi Fantaguzzi *et al.*, 2009), no tyrosine hydroxylase positive neurons were detected in submucosal ganglia using the same antibody (0/64 neurons, 3 mice), and immunoreactivity was restricted to nerve fibres associated with ganglia and submucosal vasculature (Wong *et al.*, 2008) (Fig 3c-c''). All counts are summarized in **Table 2**. In summary, NK₁R-IR is expressed by most submucosal neurons of the mouse distal colon. NK₁R-IR neurons have the neurochemical composition of non-cholinergic secretomotor neurons.

NK₁R-IR is expressed by myenteric intrinsic primary afferent neurons. Tachykinins released from SP-positive circular muscle motor neurons regulate migrating motor complexes in the colon (Foxx-Orenstein & Grider, 1996; Brierley *et al.*, 2001; Mule *et al.*, 2007) and mediate a non-cholinergic component of the peristaltic reflex (Holzer & Holzer-Petsche, 1997a; Lecci *et al.*,

1997). Although these studies predict that NK₁R is expressed by cholinergic and nitrergic neurons of the colon (Mule *et al.*, 2007), the in-depth analysis of NK₁R expression by myenteric neurons in this region is lacking.

NK₁R-IR was present in a subset of PGP9.5-IR myenteric neurons of the distal colon (22%, 152/684, Fig. 4a-a''). These neurons were large, multipolar and had prominent neurites. NK₁R-IR was detected at the plasma membrane of the soma and neurites, with comparatively little cytosolic staining. Double labelling using markers of IPANs (Furness *et al.*, 2004b) indicated that 76 % (59/78 neurons) of NK₁R-IR neurons were also CGRP-IR (Fig. 4b-b'') and that many of these were also immunoreactive for neurofilament M (not quantified), which enables observation of neuronal morphology (Fig. 4c-c'') (Qu *et al.*, 2008). Nearly all NK₁R-IR neurons coexpressed calretinin-IR (95%, 124/130 neurons, Fig. 4d-d''), a putative marker of IPANs and other neurons in the mouse (Furness *et al.*, 2004a; Qu *et al.*, 2008) and rat (Mann *et al.*, 1997). Moreover, there was also extensive colocalization with ChAT-IR (88%, 118/ 134 neurons; Fig. 5a-a''). The remaining NK₁R-IR neurons were NOS-IR (9%, 21/ 230 neurons; Fig. 5b-b''). No overlap between NK₁R-IR and SP was observed (0%, 0/ 71 neurons; Fig. 5c-c''). Internalisation of Alexa-SP was used to identify NK₁R-expressing neurons (Jenkinson *et al.*, 1999), allowing examination of overlap between NK₁R and markers for which only rabbit antibodies were available. There was little colocalization between NK₁R (detected by internalization of Alexa-SP, 100 nM, 30 min treatment) and the somatostatin 2A receptor (SSTR2A), a mediator of the descending inhibitory component of the peristaltic reflex (Grider, 2003) (not shown). The majority of SSTR2A-IR neurons were Alexa-SP negative (88 %, 21/24 neurons), consistent with the localization of SSTR2A to NOS-IR neurons (Zhao *et al.*, 2013). Similarly, most Alexa-SP-positive neurons were negative for SSTR2A-IR. In summary, our data

indicate that NK₁R-IR in the myenteric plexus of the mouse distal colon is largely restricted to IPANs.

NK₁R is functionally expressed by submucosal neurons. To assess the activation of the NK₁R in submucosal neurons, we examined agonist-stimulated endocytosis of the NK₁R. Previous studies have utilized endocytosis as an indicator of receptor activation in endothelial cells and neurons (Bowden *et al.*, 1994; Marvizon *et al.*, 1997). Treatment with Alexa-SP (100 nM, 30 min) resulted in the presence of Alexa-SP positive endosome-like structures throughout the soma and neurites of both submucosal and myenteric neurons (Fig. 6a, a'). These possible endosomes were also NK₁R-IR, indicating that internalization of both receptor and agonist had occurred (Fig. 6b-b'). Alexa-SP labelling was restricted to the cell surface of the soma and neurites following Alexa-SP binding at 4°C without warming, a temperature at which endocytosis is significantly inhibited (Grady *et al.*, 1996). Both endocytosis of NK₁R-IR and uptake of Alexa-SP were not observed following prior treatment with the NK₁R antagonist RP-67580 (10 µM, not shown), a concentration that effectively abolishes NK₁R-dependent signalling in enteric neurons (Pelayo *et al.*, 2011). Neurons with Alexa-SP labelled endosome-like structures were NK₁R-IR and were also immunoreactive for NPY or ChAT (SMP and MP, respectively, not shown). Internalisation of submucosal NK₁R-IR in response to unlabelled SP was concentration dependent (100 pM - 1 µM). Significant receptor endocytosis relative to untreated controls was detected at concentrations of 1 nM and greater (Fig. 6c-c'', d). Thus, we conclude that NK₁R is functionally expressed by submucosal neurons of the mouse distal colon and that the antibody that we have used specifically detects NK₁R-IR in mouse tissues.

Discussion

The functional subtypes of enteric neurons can be readily identified by their neurochemical content, morphology and projections. These neurons have been comprehensively categorized in the guinea pig ileum (Costa *et al.*, 1996). However, despite the wealth of information about patho-physiological processes in the intestine of the mouse, the neurochemical characterization of murine enteric neurons is incomplete (Sang & Young, 1996; Sang *et al.*, 1997; Qu *et al.*, 2008; Mongardi Fantaguzzi *et al.*, 2009). To provide a mechanistic understanding of the functions of the NK₁R in this species, we have completed a comprehensive study of the localization of the NK₁R in enteric neurons of the mouse colon.

NK₁R is expressed by myenteric IPANs, descending interneurons and inhibitory motor neurons. Our results show that the NK₁R is expressed by myenteric IPANs, descending interneurons and inhibitory motor neurons of the mouse distal colon. Expression of NK₁R by myenteric IPANs is supported by the following observations. NK₁R-IR neurons were morphologically defined as Dogiel Type II due to their large, round cell bodies and the presence of multiple axons. Moreover, NK₁R-IR was generally coexpressed with the established IPAN marker CGRP (Grider, 1994; Furness *et al.*, 2004b). There was also extensive colocalization between NK₁R-IR and the neurochemical markers NFM and calretinin, which are expressed by IPANs and other neurons in the mouse myenteric plexus (Furness *et al.*, 2004b; Qu *et al.*, 2008). IPANs are cholinergic (Furness *et al.*, 2004a) and accordingly the majority of NK₁R-IR neurons were also ChAT positive. The remaining neurons were most likely to be inhibitory motor neurons and descending interneurons. This conclusion is based on the overlap with NOS-IR and SSTR2A-IR, and the presence of NK₁R-IR neurons that were ChAT negative. The colocalization between NK₁R-IR and somatostatin-IR is consistent with expression of NK₁R-IR by at least one

class of descending interneuron (Costa *et al.*, 1996; Grider, 2003). These are medium-sized neurons that are also ChAT-IR (De Jonge *et al.*, 2003; Qu *et al.*, 2008). The lack of apparent overlap between NK₁R-IR and SP-IR would preclude expression by ascending interneurons, as these are tachykininergic in both mouse and guinea small intestine (Qu *et al.*, 2008).

NK₁R is expressed by submucosal non-cholinergic secretomotor/vasodilator neurons. There are relatively few studies of the neurochemical composition of murine submucosal neurons (Sang & Young, 1996; Mongardi Fantaguzzi *et al.*, 2009; Poole *et al.*, 2011). Our results indicate that NK₁R-IR is exclusively expressed by non-cholinergic secretomotor neurons. Support for this conclusion is based on two lines of evidence. Firstly, all NK₁R-IR neurons of the submucosal plexus of the mouse colon also expressed VIP, a marker of non-cholinergic secretomotor submucosal neurons that is conserved across multiple species, including humans (Porter *et al.*, 1999). Thus, the NK₁R-IR is exclusively expressed by non-cholinergic secretomotor submucosal neurons. Moreover, nearly all VIP-IR neurons were also NK₁R positive. Secondly, there was no overlap between NK₁R-IR and ChAT, somatostatin or CGRP, all of which are markers of cholinergic neurons in mouse submucosal ganglia (Sang & Young, 1996; De Jonge *et al.*, 2003; Mongardi Fantaguzzi *et al.*, 2009). In the mouse ileum, the VIP-positive population can be further subdivided into secretomotor and vasodilator neurons based on their expression of TH (Mongardi Fantaguzzi *et al.*, 2009). It was not possible to undertake an equivalent characterisation of NK₁R-IR neurons in the distal colon as we were unable to detect TH-positive submucosal neurons in this region using the identical antibody. TH-IR in these preparations was restricted to nerve fibres associated with the submucosal vasculature and ganglia (Wong *et al.*, 2008). This is in accordance with the significantly lower population of TH-positive neurons in submucosal ganglia of the distal colon relative to other regions (Chalazonitis *et al.*, 2011).

The distribution of NK₁R differs markedly between species. The findings of this study highlight clear differences between NK₁R-IR distributions in the guinea pig, rat and mouse. In submucosal ganglia of the guinea pig ileum, the NK₁R-IR is primarily localised to secretomotor neurons containing NPY-IR and ChAT-IR (Portbury *et al.*, 1996; Moore *et al.*, 1997; Lomax *et al.*, 1998). In the guinea pig colon, the majority of NK₁R-IR submucosal neurons are cholinergic, with expression in other neuron types including IPANs (Harrington *et al.*, 2005). These findings are consistent with the distribution predicted by functional studies of tachykinin-mediated chloride secretion and neuron depolarization (Frieling *et al.*, 1999). In the rat distal colon, the NK₁R colocalizes with SP in Dogiel type II submucosal neurons (Mitsui, 2010). The present results clearly demonstrate that the NK₁R is expressed by non-cholinergic secretomotor neurons in the submucosal plexus of the mouse distal colon. Previous studies (Wong *et al.*, 2008; Mongardi Fantaguzzi *et al.*, 2009; Poole *et al.*, 2011) and the present results show that, in marked contrast to the guinea pig and rat, there is extensive overlap between NPY and VIP positive neuronal populations and little evidence for submucosal IPANs in the mouse colon. This relative structural simplicity of the mouse submucosal plexus may underlie the differences in NK₁R distribution that we observe. Whether these differences translate to species-specific differences in SP-mediated neurogenic control of fluid and electrolyte secretion in the intestine remain to be determined.

In the myenteric plexus of the guinea pig ileum, the NK₁R is mainly expressed by NOS-IR inhibitory motor neurons, with excitatory motor neurons, secretomotor neurons and a minor proportion of IPANs making up the remaining NK₁R-positive neuronal population (Lomax *et al.*, 1998). In the guinea pig distal colon, there are comparatively fewer NK₁R-IR myenteric neurons, and these co-express NOS, ChAT or calbindin (Bian *et al.*, 2000; Harrington *et al.*, 2005). The

distribution of the NK₁R in the rat ileum differs from the guinea pig, with the NK₁R localized to myenteric IPANs (Mann *et al.*, 1997) and often coexpressed with SP (Mann *et al.*, 1999a; Mitsui, 2011). Based on these studies our data suggest that the distribution of NK₁R in the myenteric plexus of the mouse colon is most similar to that in the rat.

Few studies have examined the distribution of NK₁R in the human ENS, probably due to a lack of suitable means of detection. Immunohistochemical and *in situ* hybridisation studies show that the NK₁R is expressed by enteric neurons of the human antrum, duodenum and colon (Smith *et al.*, 1998; Goode *et al.*, 2000; Smith *et al.*, 2000; Boutaghou-Cherid *et al.*, 2006). Functional studies demonstrate that the NK₁R mediates non-adrenergic non-cholinergic neuromuscular transmission to the circular muscle of the human ileum (Zagorodnyuk *et al.*, 1997) and regulates neurogenic contractions of the isolated colon (Cellek *et al.*, 2006; Guagnini *et al.*, 2006).

Functional implications of NK₁R distribution in inflammatory bowel disease. Myenteric NK₁R is unlikely to play a role in enhanced nociceptive signalling from the colon, such as occurs in irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD). The neurons identified as NK₁R-positive in the present study do not project centrally and are therefore not involved in the transduction of noxious stimuli to the spinal cord. Although NK₁R is expressed by dorsal root ganglion neurons (Zhang *et al.*, 2007), whether colonic primary spinal afferents are NK₁R positive is presently unknown.

There is an increased capsaicin-evoked SP release from extrinsic nerve fibres innervating the colon in experimental colitis. Mice deficient in SP have reduced inflammation following dextran sulphate sodium treatment relative to wild type mice (Engel *et al.*, 2012). Furthermore, extrinsic denervation or pharmacological inhibition of NK₁R also significantly attenuates

experimental colitis (Castagliuolo *et al.*, 2002; Landau *et al.*, 2007; Gad *et al.*, 2009). NK₁R expression is upregulated in the colon of patients with IBD (Goode *et al.*, 2000) and in animal models of colitis (Kimball *et al.*, 2007). The internalization of NK₁R is indicative of localised release of SP at sites of inflammation (Bowden *et al.*, 1994). The increased release of SP in IBD is supported by our own preliminary data which shows that myenteric NK₁R is largely internalized in a model of chronic colitis (Cattaruzza *et al.*, 2013). This would effectively prevent NK₁R from signalling at the cell surface and is consistent with a loss of neuronal NK₁R function in IBD (Smith & Smid, 2005) and experimental colitis (Hosseini *et al.*, 1999), and to a reduction in gastrointestinal tachykininergic signalling in general (Al-Saffar & Hellstrom, 2001). We report that myenteric NK₁R-IR is mostly expressed by neurochemically-defined IPANs. These neurons determine the magnitude and type of reflexes that are initiated by different stimuli, and intestinal inflammation leads to their hyperexcitability (Furness *et al.*, 2004a). The functional consequences of inflammation on NK₁R-dependent signalling by these neurons are presently unknown. As IPANs are key integrators of reflex activity, effective block of NK₁R signalling through internalization could potentially affect all physiological reflexes mediated by the myenteric plexus, including coordinated motility patterns.

The NK₁R is involved in secretory reflex pathways in the intestine and has a potential role in toxin-induced hypersecretion. NK₁R has a pro-secretory role in the intestine, determined pharmacologically using NK₁R-selective antagonists (Frieling *et al.*, 1999; Riegler *et al.*, 1999). In the present study we have identified VIP-positive non-cholinergic secretomotor/ vasodilator neurons as the major NK₁R-IR population. Activation of NK₁R expressed by these neurons would be expected to promote secretion. NK₁R antagonists attenuate cholera-associated hypersecretion (Turvill *et al.*, 2000) and suppress cholera toxin-induced hyperexcitability of

submucosal neurons (Gwynne *et al.*, 2009). Internalization of NK₁R in enteric neurons occurs following exposure to Toxin A, the causative agent of *Clostridium difficile*-induced ileitis. Toxin A stimulates release of SP from afferent fibres and inhibition of NK₁R or genetic deletion of NK₁R prevents Toxin A induced tissue damage (Mantyh *et al.*, 1996).

In summary, we have described the neurochemical coding of NK₁R-IR neurons in the mouse distal colon. We have discussed the neuronal subtypes by which this receptor is expressed and the differences in NK₁R distribution across commonly used laboratory species. The findings of this work will directly relate to physiological studies of the role of NK₁R in the gut and the effects that drugs targeting NK₁R activity have on colonic motility and secretion.

Figure Descriptions

Figure 1. *Distribution of NK₁R and its endogenous agonist substance P in the mouse distal colon.* **a, b:** SP-IR was detected in enteric ganglia and in nerve fibres within the inner circular muscle layer (arrowheads). SP-positive cells were also detected throughout the mucosa (arrows). NK₁R-IR was mainly localised to submucosal (**c, e, f**) and myenteric neurons (**b, g, h**). **c, d:** SP-IR was detected in nerve fibres associated with submucosal arterioles (asterisk) and to submucosal ganglia (box). **d:** SP-IR innervating arterioles (asterisk) were also CGRP- and TRPV1-IR (arrowheads). **e, f:** SP-IR (arrowheads with asterisks) was often closely associated with NK₁R positive submucosal neurons, and expression was largely distinct to CGRP-IR varicosities (arrows). Coexpression of SP-IR and CGRP-IR are indicated with arrowheads. **g:** NK₁R-IR myenteric neurons were generally large and often multipolar. SP-IR (arrowheads with asterisks) and CGRP-IR (arrows) were in distinct fibres and varicosities. Scale: **a, b:** 50 µm, **c-h:** 20 µm. *lm*: longitudinal muscle layer, *cm*: circular muscle layer, *mp*: myenteric plexus.

Figure 2. *NK₁R-IR is localized to non-cholinergic secretomotor/vasodilator neurons of the submucosal plexus.* **a-a'':** NK₁R-IR was expressed by the majority of PGP9.5-IR neurons of the distal colon (arrowheads). All NK₁R-IR neurons were also calretinin-IR (**b-b''**) and were identified as non-cholinergic secretomotor neurons based on extensive colocalization with VIP-IR (**c-c''**) and NPY-IR (**d-d''**) (arrowheads) and the lack of overlap between NK₁R-IR and ChAT (**e-e''**) or somatostatin-IR (**f-f''**) populations (arrowheads with asterisks). Scale: 20 µm.

Figure 3. *Neurochemical coding of NK₁R-IR submucosal neurons.* **a-a'':** NK₁R and CGRP immunoreactivities were detected in distinct populations of neurons (arrowheads with asterisks). In contrast, the majority of NOS (**b-b''**) and NFM (**d-d''**) positive neurons expressed NK₁R-IR

(arrowheads). TH-IR was restricted to nerve fibres, with no evidence for TH-positive submucosal neurons (**c-c''**). Scale: 20 μ m.

Figure 4. *NK₁R-IR is primarily localized to myenteric intrinsic primary afferent neurons of the colon.* **a-a''**: NK₁R-IR was expressed by relatively large diameter PGP9.5-IR neurons which were often multipolar (arrowheads). There was extensive overlap between NK₁R-IR and the putative IPAN markers CGRP (**b-b''**), NFM (**c-c''**) and calretinin (**d-d''**) (arrowheads). Scale: 20 μ m

Figure 5. *NK₁R-IR is coexpressed with NOS and ChAT in the myenteric plexus.* The majority of NK₁R-IR neurons were ChAT-IR (**a-a''**) (arrowheads). Conversely, a minority of NK₁R-IR neurons were also NOS-IR (**b-b''**) (arrowheads). There was no detectable overlap between NK₁R and SP-expressing neuronal populations (**c-c''**) (arrowheads with asterisks). Scale: 20 μ m.

Figure 6. *SP-evoked NK₁R endocytosis and recycling in submucosal neurons.* **a-a'**: Treatment with Alexa594-conjugated SP (Alexa-SP, 100 nM, 30 min) resulted in endosomal labelling of a subset of submucosal and myenteric neurons (arrowheads). **b-b''**: Endosomal Alexa-SP labelling was coincident with NK₁R-IR (arrows). **c-c'''**, **d**: In untreated neurons NK₁R-IR was mainly localized to the plasma membrane of the soma and neurites (arrowheads). Treatment with SP (0.1-1000 nM, 30 min) resulted in the concentration-dependent trafficking of NK₁R-IR to endosome-like structures (arrows). *** $p \leq 0.001$. Scale: 20 μ m.

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Table 1. Sources and dilutions of primary antibodies.

Antigen	Species	Dilution	Source
Calretinin	Goat	1:500	SWANT, Bellinzona, Switzerland
ChAT	Goat	1:200	Millipore, Temecula, CA
CGRP	Goat	1:1,000	Abcam Inc., Cambridge, MA
GFAP	Goat	1:400	Novus Biologicals, Littleton, CO
NFM	Chicken	1:500	Gene Tex Inc., Irvine, CA
Neurokinin 1 Receptor	Rabbit	1:1,000	CURE, UCLA, #94168
NOS	Goat	1:500	Novus
NPY	Rabbit	1:400	CURE, UCLA #8711
NPY	Sheep	1:200	Millipore, AB1583
PGP9.5	Chicken	1:500	Abcam
Somatostatin	Sheep	1:800	Gift from Dr G.W. Aponte, UC Berkeley
Somatostatin 2A receptor	Rabbit	1:2,000	CURE, UCLA #9431
Substance P	Guinea Pig	1:200	Neuromics, Edina, MN
Substance P	Rat	1:800	Novus, clone NC1/34
TRPV1	Rabbit	1:2000	Neuromics
Tyrosine Hydroxylase	Sheep	1:500	Millipore, AB1542
VIP	Sheep	1:200	Abcam

Table 2. Summary of NK₁R distribution in neurochemically-defined subclasses of submucosal neurons.

	PGP9.5	VIP	NPY	CGRP	ChAT	Calretinin	SOM	NOS
NK ₁ R/+	100% ^a (248/248)	99% (170/171)	88% (175/200)	2% (1/54)	1% (2/275)	100% (143/143)	0% (1/225)	29% (56/196)
NK ₁ R/-	0% (0/248)	1% (1/171)	12% (25/200)	98% (53/54)	99% (273/275)	0% (0/143)	100% (224/225)	71% (140/196)
+ /NK ₁ R	86% ^b (248/288)	94% (170/181)	97% (175/181)	5% (1/20)	4% (2/50)	84% (143/170)	3% (1/36)	73% (56/77)
+/-	14% (40/288)	6% (11/181)	3% (6/181)	95% (19/20)	96% (48/50)	16% (27/170)	97% (35/36)	27% (21/77)

NK₁R/+: NK₁R positive, neurochemical marker positive

NK₁R/-: NK₁R positive, negative for neurochemical marker

+ /NK₁R: Neurochemical marker positive, NK₁R positive

+/-: Neurochemical marker positive, NK₁R negative

^a All NK₁R positive neurons are also PGP9.5 positive (248/ 248 neurons)

^b 86% of all PGP9.5 positive neurons are also NK₁R positive (248/ 288 neurons)

Table 3. Summary of NK₁R distribution in neurochemically-defined subclasses of myenteric neurons.

	PGP9.5	NOS	ChAT	Calretinin	SP	CGRP	SOM	SSTR2A
NK ₁ R/+	100% ^a (152/152)	9% (21/230)	88% (118/134)	95% (124/130)	0% (0/71)	76% (59/78)	17% (7/42)	13% (3/24)
NK ₁ R/-	0% (0/152)	91% (209/230)	12% (16/134)	5% (6/130)	100% (71/71)	24% (19/78)	83% (35/42)	87% (21/24)
+ /NK ₁ R	22% ^b (152/684)	4% (21/591)	33% (118/357)	59% (124/211)	0% (0/65)	83% (59/71)	7% (7/29)	11% (3/28)
+/-	78% (532/684)	96% (570/591)	67% (239/357)	41% (87/211)	100% (65/65)	17% (12/71)	76% (22/29)	89% (25/28)

NK₁R/+ : NK₁R positive, neurochemical marker positive

NK₁R/- : NK₁R positive, negative for neurochemical marker

+ /NK₁R : Neurochemical marker positive, NK₁R positive

+/- : Neurochemical marker positive, NK₁R negative

^a All NK₁R positive neurons are also PGP9.5 positive (152/ 152 neurons)

^b 22% of all PGP9.5 positive neurons are also NK₁R positive (152/ 684 neurons)