Research in Veterinary Science 114 (2017) 297–307

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Substance P and the neurokinin-1 receptor expression in dog ileum with and without inflammation

Giulia Polidoro[1], Fiorella Giancola[1], Federico Fracassi, Marco Pietra, Giuliano Bettini, Martina Asti, Roberto Chiocchetti*

Department of Veterinary Medical Sciences (UNI EN ISO 9001:2008), University of Bologna, Italy

ARTICLE INFO

Keywords:
Enteric nervous system
Immunohistochemistry
NK_1
Substance P

ABSTRACT

In the gastrointestinal tract, the tachykinin Substance P (SP) is involved in motility, fluid and electrolyte secretion, and blood flow and regulation of immunoinflammatory response. SP exerts its biological activity on target cells by interacting mainly with the neurokinin-1 receptor (NK_1R). The present study aims to quantify the percentage of SP-immunoreactive (SP-IR) enteric neurons and the density of SP-IR nerve fibers in the ileum of control dogs (CTRL-dogs; $n = 7$) *vs* dogs with spontaneous ileal inflammation (INF-dogs; $n = 8$). In addition, the percentage of enteric neurons bearing NK_1R, and nitrergic neurons (nNOS-IR) expressing NK_1R immunoreactivity were evaluated in both groups. The percentages of SP-IR neurons were similar in CTRL- and INF-dogs, in either the myenteric (MP) (15 ± 8% *vs.* 16 ± 7%, respectively) and submucosal plexus (SMP) (26 ± 7% *vs.* 24 ± 14%, respectively). In INF-dogs, the density of SP-IR mucosal nerve fibers showed a trend to decrease ($P = 0.07$). Myenteric neurons of CTRL- and INF-dogs expressed similar percentages of NK_1R-immunoreactivity (39 ± 5% *vs.* 38 ± 20%, respectively). Submucosal NK_1R-IR neurons were occasionally observed in a CTRL-dog. MP nitrergic neurons bearing NK_1R showed a trend to decrease in INF-dogs *vs.* CTRL- dogs (41 ± 22% *vs.* 65 ± 10%, respectively; $P = 0.11$). In INF-dogs, muscle cells and immune cells overexpressed NK_1R immunoreactivity. These findings should be taken as a warning for possible intestinal motility disorders, which might occur during administration of NK_1R-antagonist drugs. Conversely, the strong expression of NK_1R immunoreactivity observed in muscle and mucosal immune cells of inflamed tissues may provide a rationale for the use of NK_1R antagonist drugs in the treatment of intestinal inflammation.

1. Introduction

The enteric nervous system (ENS) comprises millions of neurons and glial cells embedded within the digestive tract (from the esophagus to the inner anal sphincter) and their extrinsic connections with sympathetic, parasympathetic, and sensory fibers. In the gastrointestinal tract (GIT) enteric neurons are distributed in two ganglionated plexuses: the myenteric plexus (MP) and the submucosal plexus (SMP) (Costa and Brookes, 2008). The MP is located between the longitudinal muscle layer (LML) and the circular muscle layer (CML) (Furness, 2006), while the SMP is located between the *muscularis mucosae* and the CML.

Enteric neurons have been classified based on various criteria such as shape, immunohistochemical staining, electrophysiological, and functional properties. These cells may be divided into functional groups including motor neurons, interneurons, and intrinsic primary afferent neurons (Furness, 2006). The motor neurons include: excitatory and inhibitory muscle motor neurons; secretomotor/vasodilator neurons; secretomotor neurons; and neurons innervating entero-endocrine cells (Furness, 2000). Enteric motor neurons differ in their neurochemical code and the primary transmitter of excitatory muscle motor neurons is acetylcholine (ACh), while the main transmitter of inhibitory muscle motor neurons is ~~the~~ nitric oxide (NO). Among secondary neurotransmitters, substance P (SP) is usually co-localized with ACh in excitatory motor neurons in different species (Bornstein et al., 2004). In the submucosa, SP-immunoreactive (SP-IR) neurons innervate the *muscularis mucosae* (Hens et al., 2000; Holzer and Holzer-Petsche, 1997; Steele and Costa, 1990), the cores of the villi, intestinal glands, and muscular sheath of blood vessels (Chiocchetti et al., 2009; Costa et al., 1981; Domeneghini et al., 2004). In addition, SP stimulates secretion in intestinal mucosa (Brown et al., 1992) in the small intestine of dogs (McFadden et al., 1986; Rangachari et al., 1990).

The effects of SP are mediated by three neurokinin (NK) receptors,

* Corresponding author at: Department of Veterinary Medical Sciences, University of Bologna, Tolara di Sopra n. 50, 40064 Ozzano Emilia, Bologna, Italy.
E-mail address: roberto.chiocchetti@unibo.it (R. Chiocchetti).
[1] Authors share co-first authorship.

http://dx.doi.org/10.1016/j.rvsc.2017.06.002
Received 7 November 2016; Received in revised form 6 June 2017; Accepted 10 June 2017

belonging to the G-protein receptor superfamily: NK_1R, NK_2R, and NK_3R. The NK_1R is the high-affinity receptor for SP, while NK_2R and NK_3R bind with lower affinity to this peptide (Guard and Watson, 1991; Lomax et al., 1998; Shimizu et al., 2008). Nevertheless, SP can interact with all tachykinin receptor types with the ability to stimulate and/or inhibit propulsive motility (Holzer, 1997; Shimizu et al., 2008). Several cell types (excitatory, inhibitory, secretomotor, and intrinsic sensory neurons) express NK_1R-immunoreactivity in the guinea-pig ENS. Furthermore, NK_1R is located on interstitial cells of Cajal (ICC) (Vannucchi et al., 1997). This wide cellular distribution is congruent with a role of NK_1R in regulating motility, neuronal excitability, and mucosal water and ion transport.

It has been assumed that NK_1R-mediated inhibition of peristalsis arises from activation of inhibitory motor pathways because NK_1R in the guinea-pig intestine is largely located on inhibitory neuronal nitric oxide synthase-IR (nNOS-IR) neurons (Holzer, 1997). An inhibitory function of SP and NK_1R has been demonstrated in the stomach and ileum of a dog (Fox and Daniel, 1986; Mayer et al., 1990). In addition, the SP/NK_1R complex seems to play a role as a neurogenic mediator in gastrointestinal inflammation (Goode et al., 2000; Holzer, 1998; Mantyh et al., 1988a; Renzi et al., 2000) and in several models of visceral pain (Gazzieri et al., 2007).

The literature provides comprehensive studies on the distribution of SP-IR neurons and nerve fibers in the dog GIT (Daniel et al., 1987; Furness et al., 1990, 1991). Nevertheless, no quantitative studies are yet available regarding the density of SP-IR neurons and nerve fibers in healthy and inflamed intestine. The present *ex vivo* study aims to quantify the percentage of SP-IR neurons and evaluates the density of SP-IR fibers in the mucosa and muscular layers of the ileum in normal dogs (control dogs, CTRL-dogs) and in dogs with intestinal inflammation (inflamed dogs, INF-dogs). Furthermore, we characterized and quantified in the same groups of dogs the proportion of all enteric neurons expressing NK_1R immunoreactivity because the cellular localization of NK_1R is important to search for possible physiological and pathological roles. In addition, we evaluated the percentage of inhibitory nitrergic neurons expressing NK_1R immunoreactivity.

2. Material and methods

2.1. Animals

The entire ileum was collected from dogs that had spontaneously died or were euthanized for humane reasons due to different diseases. Seven dogs had no history of gastrointestinal disorders (control CTRL-dogs) (Table 1) and the ileal mucosa did not show any gross alteration. The dogs that were included as INF-dogs were not patients followed for gastroenterological problems, with the exception of #3 (Protein losing enteropathy, PLE) (Table 2). In these dogs, we observed gross alteration of the ileum, such as from mild to severe mucosal hyperemia and moderate diffuse thickening of the wall. The age of CTRL- and INF-dogs was 109 ± 65 months and 120 ± 18 months, respectively. Student's *t*-test did not show any differences between two groups ($P = 0.71$).

Table 1
Signalment and cause of death of the control dogs included in the present research.

Controls	Breed	Gender	Age	Cause of death
CTRL1	Chihuahua	F	8 mo	Head trauma
CRTL2	West Highland White Terrier	M	17 yr	Intracranial neoplasia
CTRL3	German Shepherd	M[a]	10 yr	Euthanasia due to progressive physical deterioration
CTRL4	German Shepherd	M	9 yr	Heart hemangiosarcoma
CTRL5	Boxer	M[a]	8 yr	Cardiovascular disease
CTRL6	German Shepherd	M	10 yr	Cardiovascular disease
CTRL7	Siberian Husky	M[a]	16 yr	Neurological (CNS) disorders
CTRL8	English Setter	F	2 yr	Car accident

Abbreviations: M, male; F, female; M[a], male neutered.

All animals died spontaneously or were euthanized, then their tissues were collected following owner permissions. According to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not require any approval by competent authorities or ethics committees, because this research did not influence any therapeutic decisions.

2.2. Tissues collection

The ileum was removed within 2 h after each animal's death and was longitudinally opened along the mesenteric border and fixed in 2% paraformaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C overnight. After washes in phosphate-buffered saline (PBS 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2), tissues were treated to obtain tangential (to the serosal surface of the tissues) (1.0 cm × 1.0 cm) and longitudinal (2.0 cm × 0.5 cm) cryosections.

2.3. Histology

Ileum samples from CTRL- and INF-dogs were deep frozen in liquid nitrogen and cryosections were stained with hematoxylin and eosin (HE) for histological examination.

2.4. Immunohistochemistry

Specimens from all the subjects were processed for immunohistochemistry as previously described (Sadeghinezhad et al., 2013). Double labeling studies were carried out by an indirect immunofluorescence method using the primary and secondary antibodies (for antisera details see Table 3). The antibody anti-human neuronal protein (HuC/HuD) was utilized as a pan-neuronal marker to identify all the enteric neurons. Cholinergic and nitrergic neurons were immunohistochemically identified using antibodies for the enzymes choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS), respectively.

2.5. Specificity of the primary antibodies

The specificity of the antibodies anti-SP (code 10-515A, Fitzgerald) and anti-ChAT (code P3YEB, Technische Universität München, Germany) was demonstrated in dog tissues by Talmage et al. (1996). The specificity of the antibodies anti-HuC/HuD (code A21271, Life Technologies) and anti-nNOS (code Ab5380, Millipore; code SC5302, Santa Cruz) was demonstrated in dog by Western blot analysis by Giancola et al. (2016). The antibody anti-NK_1 receptor (AB-N33AP, Advanced Target System) was developed in rabbits using a synthetic peptide corresponding to an amino acid sequence at the C-terminus of dog NK_1R. Furthermore, this antibody was previously used on dog spinal cord neurons (Wiese et al., 2013).

The specificity of the secondary antibodies was tested as described in a previous work (Sadeghinezhad et al., 2013).

2.6. Fluorescence microscopy

Preparations were examined on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to distinguish the fluorochromes employed. The images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).