Onderstepoort Journal of Veterinary Research, 61:207–213 (1994)

Neuropeptides in the myenteric ganglia and nerve fibres of the forestomach and abomasum of grey, white and black Karakul lambs

H.B. GROENEWALD

Department of Anatomy, Faculty of Veterinary Science, University of Pretoria
Private Bag X04, Onderstepoort, 0110 South Africa

ABSTRACT

GROENEWALD, H.B. 1994. Neuropeptides in the myenteric ganglia and nerve fibres of the forestomach and abomasum of grey, white and black Karakul lambs. *Onderstepoort Journal of Veterinary Research*, 61:207–213

Previous studies indicated large, thin-walled, milk-filled rumens in lethal grey and white Karakul lambs. There was also a significant decrease in the number and size of the myenteric plexuses and the number of ganglion cells in these lambs. The purpose of this study was to determine whether the myenteric ganglia of the affected lambs are functional, by testing for the presence of vaso-active intestinal peptide, somatostatin, neurotensin, neuropeptide Y, met-enkephalin, calcitonin gene-related peptide and substance P in the myenteric ganglia and nerve fibres in the forestomach and abomasum of grey, white and black Karakul lambs.

Four 1-cm^2 samples were taken from analogous areas of the wall of the rumen, reticulum, omasum and abomasum of five grey, five white and five black newborn Karakul lambs. They were pinned to wax squares, fixed for 18 h in Zamboni's fixative, dehydrated and rehydrated through graded alcohols and stored in phosphate-buffered saline. The outer longitudinal muscle layer of each sample of the rumen, reticulum, omasum and abomasum was separated from the rest of the tissue layers, stained for each of the seven neuropeptides by employment of the immunofluorescence technique, and studied with a Leitz Orthoplan fluorescent microscope.

All the material studied tested positive for all the neuropeptides.

It is concluded that all the peptides tested for were present in all the lambs and that the myenteric ganglia are therefore functional in the lethal lambs.

INTRODUCTION

Macroscopical studies on 24-h-old lethal grey and white lambs (Groenewald & Booth 1992a) demonstrated enlarged, thin-walled, milk-filled rumens. Lethal grey and white Karakul lambs can be identified at birth by the lack of pigmentation of the tongue, palate and ears (Nel & Louw 1953). The oesophageal groove is responsible for the channelling of milk to the abomasum and it has been shown that its normal functioning depends on an intact nerve supply (Newhook & Titchen 1974). Since the vagus nerve and its myenteric ganglia and neurones are responsible for movement of the stomachs (Duncan

Received 7 July 1994—Editor

& Phillipson 1951), the inability of the stomachs of affected lambs to contract, and the presence of milk in the rumen, strongly suggested a deficiency in the nerve supply to the stomachs.

Subsequent histological studies indicated a decrease in the thickness of the *tunica muscularis* (Groenewald & Booth 1992b), a decrease in the number and size of the myenteric ganglia, and a decrease in the number of myenteric neurones in the affected lambs (Groenewald & Booth 1992a).

The question that had to be answered was whether the limited number of neurones in the affected lambs was functionally normal. The functioning of neurones depends on the presence of neurotransmitters (Cooke 1986; Llewellyn-Smith 1987; Mawe, Schemann, Wood & Gehrson 1989). Neuropeptides act as putative neurotransmitters in the enteric neurones (Cooke 1986; Mawe *et al.* 1989; Scharrer 1990). Various authors have detected several neuropeptides immunocytochemically in the myenteric and submucosal neurones in the gastro-intestinal tract of a number of animals (Costa, Buffa, Furness & Solcia 1980; Cooke 1986; Llewellyn-Smith 1987; Anglade, Michel, Ozaki, Tsuji, Vignon & Yanaihara 1988; Llewellyn-Smith, Furness & Costa 1989; Mawe *et al.* 1989; Lehman 1990; Timmermans, Scheuermann, Stach, Adriaensen & De Groot-Lasseel 1990; Vergara-Esteras, Harrison & Brown, 1990; Yamamoto, Kitamura, Yamada & Yamashita 1994). Neuropeptides detected include calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), enkephalin, galanin, 5-hydroxytryptamine (5-HT), neuropeptide Y (NPY), neurotensin (NT), somatostatin (SOM), substance P (SP) and vaso-active intestinal peptide (VIP).

In this study the functioning of the myenteric neurones in the lethal grey and white lambs was determined by testing immunocytochemically for the presence of the neuropeptides most commonly described in the enteric neurones, viz. CGRP, met-enkephalin (ENK), NPY, NT, SOM, SP and VIP. The results were compared with the data obtained in black control lambs.

MATERIALS AND METHODS

Five grey and five white lambs with unpigmented tongues, palates and ears, and five black control lambs were injected with 1 mg colchicine (which is known to promote the retention of neuropeptides) intraperitoneally immediately after birth (Lee, Takami, Kawai, Girgis, Hillyard, Macintyre, Emson & Tohyama 1985; Llewellyn-Smith 1987). They were slaughtered 18 h later and four 1-cm^2 samples were collected from analogous areas of the wall of the rumen, reticulum, omasum and abomasum, respectively.

For wax sections, the samples were rinsed in phosphate-buffered saline (PBS) and fixed with Bouin's fixative for 12 h. The specimens were then washed in 80 % alcohol until clear of picric acid and stored in 70 % alcohol. In the laboratory, the samples were processed in a Shandon Elliot Duplex Processor (Shandon). They were dehydrated through graded alcohols (60 %, 70 %, 80 %, 96 %, 3,5 h in each, 100 %, 2 × 2,5 h and cleared in xylol, 2 × 2 h). They were then transferred to two changes of wax at 56 °C for 4 h and 8 h, respectively, and embedded in a Thermoline Histocenter 2 (Thermoline). Five-micron-thick sections were cut on an Anglia Scientific microcrotome (Anglia) and fixed to slides with egg albumin. The sections were then stained both with the peroxidase-antiperoxidase and the Biotin-Streptavidin techniques for each of the seven neuropeptides.

The samples for whole mounts were rinsed in phosphate-buffered saline (PBS), pH 7,4, pinned to wax squares with insect pins and fixed in Zamboni's fixative (Stefanini, De Martino & Zamboni 1967) for 18 h at 4 °C. The fixed specimens were then removed from the wax squares and washed in 80 % ethanol until clear of picric acid. Then they were dehydrated through graded ethanols, cleared in 100 % xylol, rehydrated through graded ethanols and stored in PBS, pH 7,4 at 4 °C.

In the laboratory, the outer longitudinal muscle layer of each sample was stripped off under a dissection microscope by means of a pair of watchmaker's forceps. The separated layers of each of the stomachs of the three groups of lambs were incubated overnight at room temperature in diluted primary antisera, against seven selected peptides according to the method of Costa & Furness (1983).

The details of the primary antisera employed are listed in Table 1. A positive and a negative control were included for each peptide. The positive control was a sample of the outer longitudinal muscle layer of the rumen of an adult black Karakul sheep injected with 10 mg colchicine intraperitoneally, and in the negative control the primary antiserum was replaced with PBS. The incubation took place in a humid chamber. After the primary incubation, the tissue samples were washed in PBS (3 × 5 min). They were then incubated with biotinylated anti-rabbit immunoglobulin (Serotec) at a dilution of 1:200 for 1 h, washed with PBS, incubated with R.-Phycoerythrin-labelled Streptavidin (Serotec) at a dilution of 1:300 for another hour and washed again with PBS. The laminae were mounted in PBS:Glycerol (1:1) and studied with a Leitz Orthoplan fluorescent microscope.

The immunoreactivity in each specimen was judged subjectively by the amount of fluorescence within the 1-cm^2 field, on a scale of 0–3+.

RESULTS

The peroxidase-antiperoxidase (PAP), Biotin-Streptavidin and immunofluorescence techniques were employed on sections and on whole mounts to determine the presence of neuropeptides in these organs. The immunofluorescence technique on whole mounts described by Costa & Furness (1983) gave the best results and was subsequently used in this study. The results obtained from sections of wax-embedded samples were unsatisfactory, owing to the paucity of myenteric ganglia and neurones in the grey and white lambs (Groenewald & Booth 1992a). In a number of sections, either very few or no myenteric ganglia and neurones could be detected.

TABLE 1 Details of the primary antisera employed against neuropeptides (source: Amersham)

Primary antisera	Code	Dilution
Calcitonin gene-related peptide (CGRP) (synthetic rat CGRP)	RPN.1842	1:300
Enkephalin (5-L-Metionin) (ENK) (synthetic met-enkephalin)	RPN.1562	1:200
Neuropeptide Y (NPY) (synthetic porcine)	RPN.1702	1:400
Neurotensin (synthetic bovine) (NT)	RPN.1632	1:400
Somatostatin (synthetic-14) (SOM)	RPN.1612	1:200
Substance P (SP) (synthetic)	RPN.1572	1:200
Vaso-active intestinal peptide (VIP) (natural porcine)	RPN.1582	1:500

A summary of the immunoreactivity of the seven neuropeptides tested in the whole mounts is presented in Table 2.

The immunoreactivity for CGRP in the myenteric neurones of the rumen, reticulum and omasum of black lambs was low to moderate (Fig. 1) and no immunoreactivity could be detected in the myenteric neurones of the abomasum. In the grey and white lambs, the immunoreactivity for this peptide was low in the myenteric neurones of the rumen, reticulum and omasum (Fig. 2), and no immunoreactivity could be detected in the neurones of the abomasum.

In the neurones of the rumen, reticulum and omasum of the grey, white and black lambs, the immunoreactivity for ENK was moderate to high (Fig. 3) and the immunoreactivity in the neurones of the abomasum of all three groups was low. The immunoreactivity for NPY in the myenteric neurones of the rumen, reticulum, omasum and abomasum in the grey, white and black Karakul lambs was low to moderate (Fig. 4).

TABLE 2 Neuropeptides present in the forestomachs and abomasums of grey, white and black Karakul lambs

Area of sampling	Lamb group	Neuropeptides						
		CGRP	ENK	NPY	NT	SOM	SP	VIP
Rumen	Grey	+	++	+	+	++	++	++
	White	+	++	+	++	+++	+++	++
	Black	++	+++	++	+++	+++	+++	+++
Reticulum	Grey	+	++	+	++	++	++	++
	White	+	++	+	++	++	++	++
	Black	++	++	+	++	+++	+++	+++
Omasum	Grey	+	++	+	+++	++	++	+
	White	+	++	+	+++	++	++	+
	Black	+	+++	+	+++	++	+++	+
Abomasum	Grey	0	+	+	+	++	++	+
	White	0	+	+	+++	++	++	+
	Black	0	+	+	+++	+++	+++	++
Control	Positive	++	+++	+++	+++	+++	+++	+++
	Negative	0	0	0	0	0	0	0

0 = no immunoreactivity; + = low immunoreactivity ; ++ = moderate immunoreactivity; +++ = high immunoreactivity

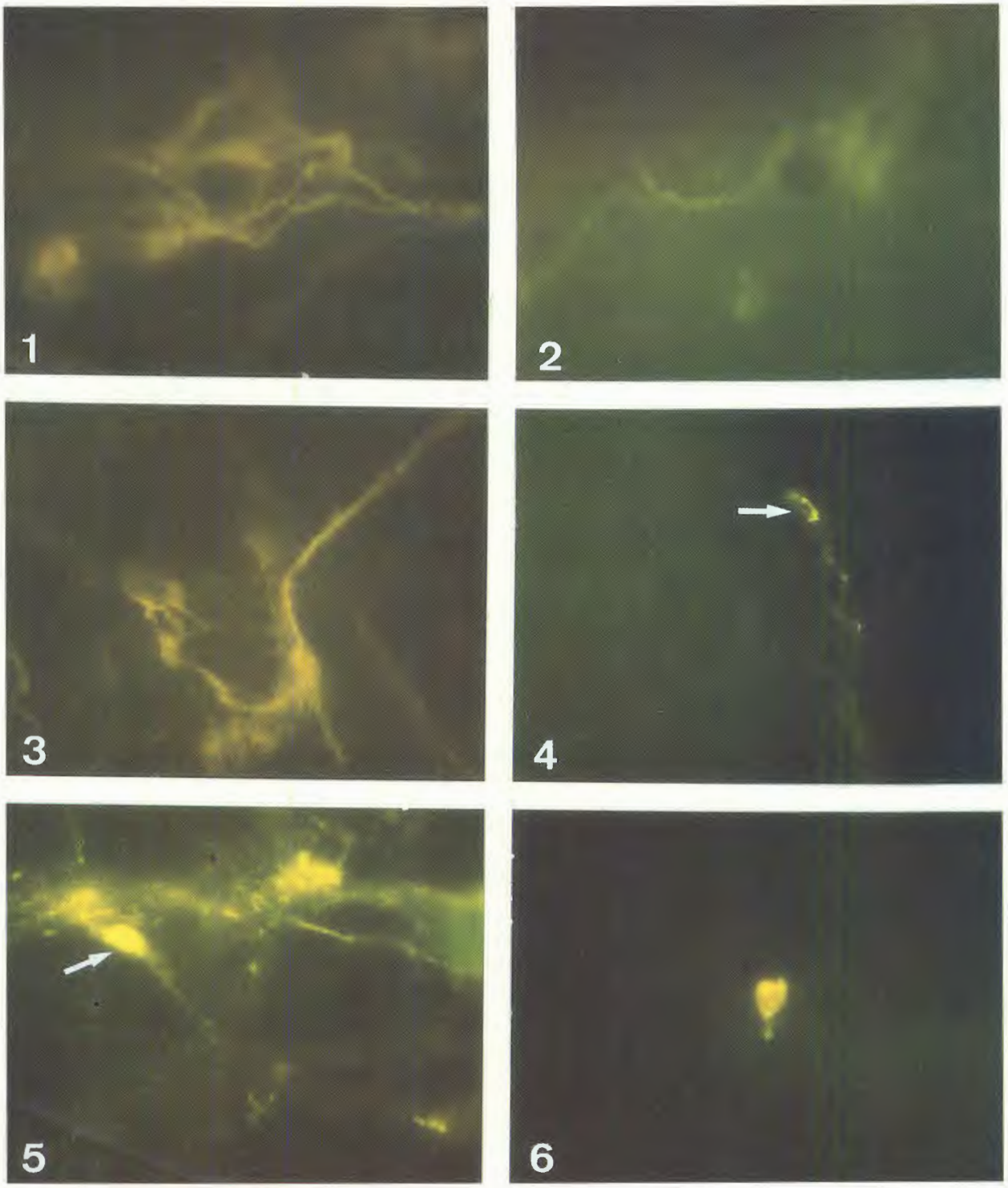

FIG. 1 Moderate immunoreactivity against CGRP is indicated in nerve fibres of the rumen of a black lamb (400 x)
FIG. 2 Low immunoreactivity against CGRP is indicated in nerve fibres of the rumen of a white lamb (400 x)
FIG. 3 High immunoreactivity against ENK is indicated in the nerve fibres of the rumen in a black lamb (400 x)
FIG. 4 Low immunoreactivity against NPY in a neuron (arrow) and axon in the reticulum of a white lamb (400 x)
FIG. 5 High immunoreactivity against NT is demonstrated in a myenteric ganglion of the reticulum of a black lamb. The arrow indicates a neuron (400 x)
FIG. 6 Low immunoreactivity against NT is displayed in a neuron of the abomasum of a grey lamb (400 x)

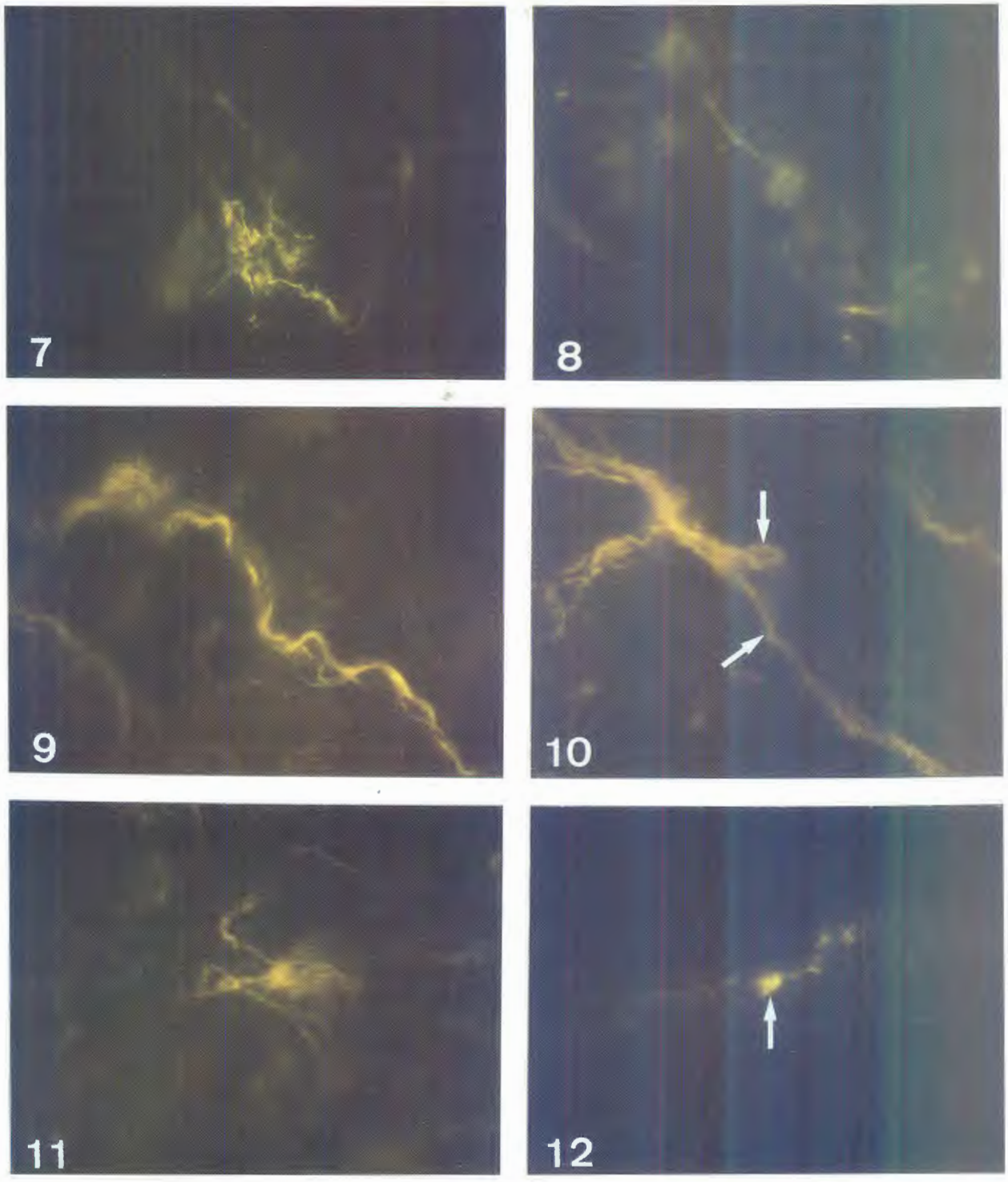

FIG. 7 Moderate immunoreactivity against NT is displayed in the nerve fibres of the reticulum of a grey lamb (400 x)
FIG. 8 Moderate immunoreactivity against NT is displayed in a myenteric ganglion of the omasum of a white lamb (400 x)
FIG. 9 High immunoreactivity against SOM is indicated in the nerve fibres of the rumen of a grey lamb (400 x)
FIG. 10 High immunoreactivity against SP is displayed in a neuron (top arrow) and nerve fibres (bottom arrow) of the rumen of a white lamb (400 x)
FIG. 11 High VIP immunoreactivity indicated in the myenteric ganglion of the rumen of a black lamb (400 x)
FIG. 12 Low VIP immunoreactivity displayed in a neuron (arrow) with its axon in the abomasum of a white lamb (400 x)

In the black group, the immunoreactivity for NT was high in the neurones of all the stomachs (Fig. 5). In the grey group, it was low in the neurones of the rumen and abomasum (Fig. 6), and moderate to high in the neurones of the reticulum and omasum (Fig. 7), and in the white group, it was moderate to high in the neurones of all the stomachs (Fig. 8). The immunoreactivity for SOM was moderate to high in the neurones of the stomachs of all three groups (Fig. 9).

SP immunoreactivity was moderate to high in the myenteric neurones of the rumen, reticulum, omasum and abomasum of all the lambs (Fig. 10). In the grey, white and black lambs, VIP immunoreactivity in the neurones of the rumen and reticulum was moderate to high (Fig. 11), and in the neurones of the omasum and abomasum, it was low to moderate (Fig. 12).

DISCUSSION

The normal functioning of the enteric nervous system is dependent on the presence of mediators for synaptic events (Cooke 1986; Llewellyn-Smith 1987; Mawe *et al.* 1989). Putative neurotransmitters in the form of neuropeptides have been shown to act as synaptic mediators (Cooke 1986; Llewellyn-Smith 1987; Mawe *et al.* 1989; Scharrer 1990). The finding that enteric neurones contain neuropeptides was a major breakthrough for the study of the enteric nervous system (Llewellyn-Smith 1987). Pearce & Polack (1975) and Nilsson, Larsson, Hakanson, Brodin, Pernow & Sundler (1975) were the first workers to find neuropeptides in enteric neurones and since then many neuropeptides have been discovered within nerve- cell bodies and nerve fibres (Furness, Llewellen-Smith, Bornstein & Costa 1988). Of these peptides CGRP, ENK, NPY, NT, SOM, SP and VIP are the most frequently described.

In this study, all the neuropeptides tested for were present in the rumen, reticulum and omasum of the grey, white and black Karakul lambs. The presence of these neuropeptides has also been described in the stomach of the guinea pig (Costa *et al.* 1980; Llewellyn-Smith 1987; Llewellyn *et al.* 1989; Mawe *et al.* 1989) and pig (Timmermans *et al.* 1990). SOM immunoreactivity in the rumen, reticulum, omasum and abomasum in sheep (Vergara-Esteras *et al.* 1990) and SP, ENK, VIP and NPY immunoreactivity in the omasum of the sheep (Yamamoto *et al.* 1994) was confirmed by this study. This work also revealed that the neuropeptides in the myenteric ganglia and neurones of the stomach and small intestine, as described by various authors (Costa *et al.* 1980; Cooke 1986; Llewellyn-Smith 1987; Anglade *et al.* 1988; Llewellyn-Smith *et al.* 1989; Mawe *et al.* 1989; Lehman 1990; Timmermans *et al.* 1990; Vergara-Esteras *et al.* 1990), are present in the rumen, reticulum and omasum of sheep.

Gastric myenteric ganglia of the guinea pig appear to lack intrinsic CGRP immunoreactive neurones (Mawe *et al.* 1989). Lee *et al.* (1985) found no immunoreactivity against CGRP in the myenteric ganglia of the rat stomach, even after pretreatment with colchicine. Mawe *et al.* (1989) proposed that CGRP immunoreactive fibres in the guinea-pig stomach are of extrinsic origin and that they are mostly gastric branches of visceral sensory neurones. The absence of intrinsic CGRP immunoreactive fibres in the muscle layers of the stomach suggests that this neuropeptide plays no role as a transmitter at neuromuscular junctions (Mawe *et al.* 1989). In this study no immunoreactivity against CGRP could be detected in the abomasa of the grey, white and black lambs, which also suggests that this neuropeptide does not function as a transmitter in the abomasum.

All the other neuropeptides tested for were present in the grey, white and black lambs. However, in most cases there seemed to be less fluorescence in the grey and white lambs as compared with the black lambs. The apparent decrease in immunoreactivity in the grey and white lambs is probably due to the paucity of myenteric ganglia and neurones and not to diminished production of the neuropeptides. When individual neurones and fibres were compared there seemed to be little difference in the intensity of the fluorescence between the three groups.

Although Colchicine was injected into the lambs 18 h before they were slaughtered to promote retention of the neuropeptides in the nerve cells and fibres (Lee *et al.* 1985; Llewellyn-Smith 1987), the neuropeptides were difficult to demonstrate in the 24-h-old lambs, probably owing to the small amount of neuropeptides present at this early stage. The situation was further complicated by the paucity of myenteric ganglia and neurones in the grey and white lambs.

It is concluded that the myenteric neurones present in the affected lambs, although limited in number, are functional, and that the decreased immunoreactivity is due to the paucity of the myenteric ganglia and neurones.

ACKNOWLEDGEMENTS

I would like to thank Messrs D. Kotze and B. Oberholzer, and Dr J.T. Soley for their invaluable assistance and advice in collecting the research material, and Prof. A.J. Bezuidenhout and Dr J.T. Soley for their critical reading and contributions.

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