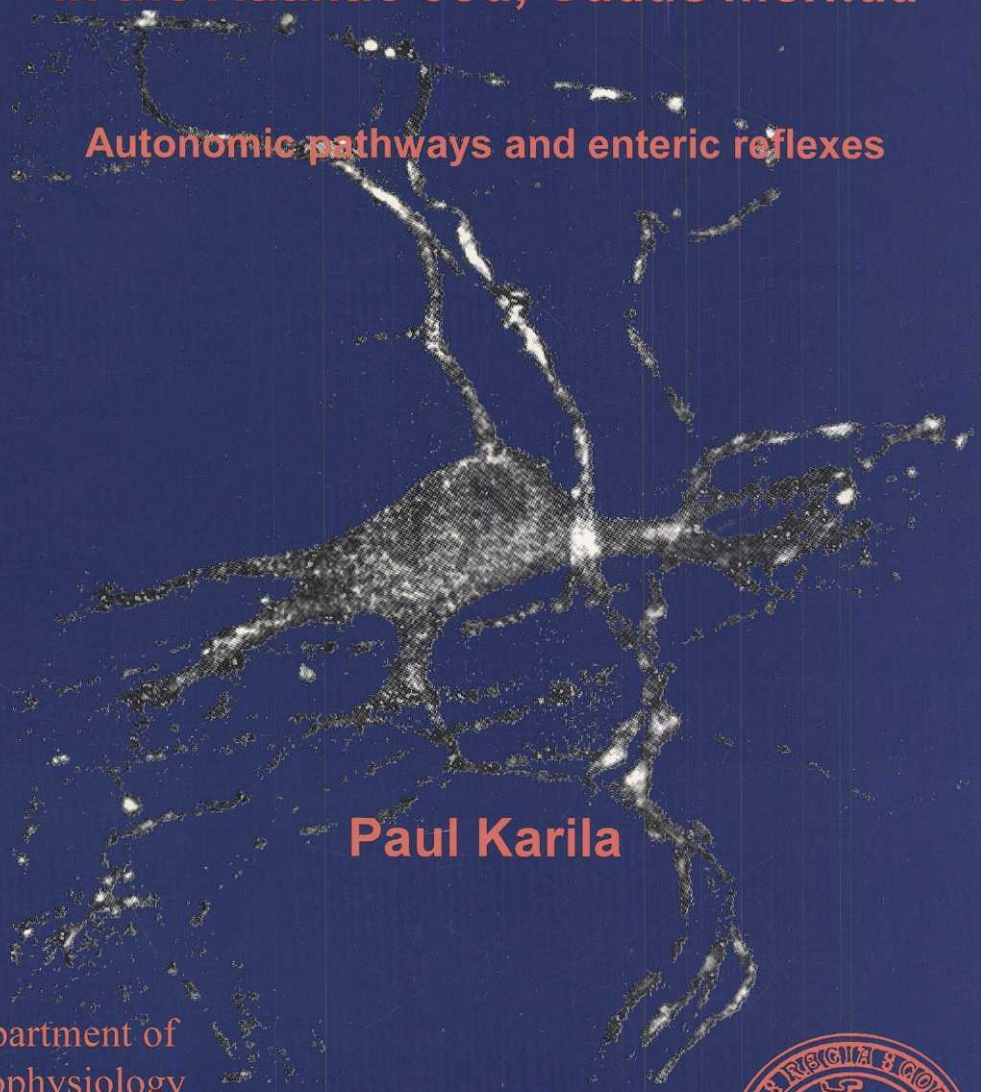
Nervous control of gastrointestinal motility in the Atlantic cod, *Gadus morhua*

Autonomic pathways and enteric reflexes



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1997

Nervous control of gastrointestinal motility in the Atlantic cod, *Gadus morhua*

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Akademisk avhandling

för filosofie doktorsexamen i zoofysiologi
som enligt biologiska sektionens beslut kommer att
offentligen försvaras onsdagen den 7 maj 1997, kl 13.00
i föreläsningssalen, Zoologiska institutionen,
Medicinaregatan 18, Göteborg

Göteborg 1997

ISBN 91-628-2503-8

Cover photo: *A confocal laser scanning microscopy image of a multipolar tachykinin immunoreactive neurone in the myenteric plexus of the cod stomach.*

Back cover: *A speculative arrangement for the neuronal circuitry underlying the peristalsis in the intestine of the cod. See also fig. 4.*

Till Anna och Klimpen

Abstract:
Karila, Paul: **Nervous control of gastrointestinal motility in the Atlantic cod, *Gadus morhua;* autonomic pathways and enteric reflexes.**
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The aim of the study was to elucidate the neuronal pathways within and to the gastrointestinal canal of a commercially important teleost fish, the Atlantic cod (*Gadus morhua*), and to relate the results to findings from other vertebrate groups.

A number of enteric neuronal populations were identified, as were sympathetic, parasympathetic and extrinsic sensory neuronal populations. The different projections of the neuronal populations were revealed by combining the results from myotomy operations and from physiological experiments on isolated intestine. A model has been proposed where populations containing acetylcholine, serotonin and tachykinins are involved in the ascending (orally directed) excitatory part of the peristaltic reflex of the intestine. In the descending (anally directed) inhibitory part of the reflex, neurones containing nitric oxide, vasoactive intestinal peptide and galanin participate. The arrangement of the different neuronal types in the polarised motility reflexes appears to have been highly conserved during evolution since the same pattern is present in teleost fish and in mammals which have been separated for over 400 million years.

The sympathetic innervation to the gastrointestinal canal is chemically coded with adrenergic neurones innervating the smooth muscle and myenteric plexus of the stomach, and adrenergic neurones also containing neuropeptide Y innervating the submucosa and, to a lesser extent, blood vessels. In addition, adrenergic neurones containing nitric oxide synthase are found in sympathetic ganglia.

In is concluded that many features previously believed to be present only in "higher vertebrates", as the mammals, are found in the autonomic nervous system of a teleost fish. It argues against the common opinion that the "lower vertebrates" have less developed organ systems; many similarities exist in the distribution and projection of neurones in and to the gut. I suggest that the simplistic view is almost entirely due to the previous lack of research in these animals in contrast to the extensive efforts that have been put down on research on the mammalian autonomic nervous system.

Keywords:
Nervous system, enteric, autonomic - Myenteric plexus - Immunohistochemistry - Myotomy - Retrograde tracing - Neuronal projections - Coexistence - Neurotransmitters - *Gadus morhua* (Teleostei)

The thesis is based mainly on the following papers, which will be referred to by their Roman numerals:

I. P Karila, AC Jönsson, J Jensen and S Holmgren (1993) Galanin-like immunoreactivity in extrinsic and intrinsic nerves to the gut of the Atlantic cod, *Gadus morhua*, and the effect of galanin on the smooth muscle of the gut. Cell Tissue Res 271:537-544

II. J Jensen, P Karila, AC Jönsson, G Aldman and S Holmgren (1993) Effects of substance P and distribution of substance P-like immunoreactivity in nerves supplying the stomach of the cod, *Gadus morhua.* Fish Physiol Biochem 12:237-247

III. C Olsson and P Karila (1995) Coexistence of NADPH-diaphorase and vasoactive intestinal polypeptide in the enteric nervous system of the Atlantic cod (*Gadus morhua*) and the spiny dogfish (*Squalus acanthias*). Cell Tissue Res 280:297-305

IV. P Karila, J Messenger and S Holmgren, Nitric oxide synthase- and neuropeptide Y-containing subpopulations of sympathetic neurones in the coeliac ganglion of the Atlantic cod, *Gadus morhua*, revealed by immunohistochemistry and retrograde tracing from the stomach. J Auton Nerv Syst, resubmitted

V. P Karila and S Holmgren (1995) Enteric reflexes and nitric oxide in the fish intestine. J Exp Biol 198:2405-2411

VI. P Karila and S Holmgren (1997) Anally projecting neurons exhibiting immunoreactivity to galanin, nitric oxide synthase and vasoactive intestinal peptide, detected by confocal laser scanning microscopy, in the intestine of the Atlantic cod, *Gadus morhua.* Cell Tissue Res 287:525-533

VII. P Karila, F Shahbazi, J Jensen and S Holmgren, Projections and actions of tachykinin containing, cholinergic, and serotonergic neurones in the intestine of the Atlantic cod. Cell Tissue Res, submitted

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List of abbreviations

Ach	acetylcholine
BM	bombesin
Calb	calbindin
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
ChAT	choline acetyltransferase
CLSM	confocal laser scanning microscopy
CM	circular muscle layer
EC	endocrine cells
GAL	galanin
GRP	gastrin-releasing peptide
5-HT	5-hydroxytryptamine
IR	immunoreactive
LM	longitudinal muscle layer
M	mucosa
MEP	myenteric plexus
NA	noradrenaline
NADH-d	nicotinamide dinucleotide diaphorase
NADPH-d	nicotinamide adenine dinucleotide phosphate diaphorase
NKA	neurokinin A
NOS	nitric oxide synthase
NPY	neuropeptide Y
NT	neurotensin
PACAP	pituitary adenylate cyclase-activating polypeptide
RIA	radioimmunoassay
SM	submucosa
SOM	somatostatin
SP	substance P
Tach	tachykinin(s)
TH	tyrosine hydroxylase
TTX	tetrodotoxin
VAchT	vesicular acetylcholine transporter
VIP	vasoactive intestinal peptide

Introduction

General

In this thesis, I have concentrated on a single teleost fish species, the Atlantic cod, *Gadus morhua*, and the control of the gastrointestinal canal motility by the autonomic nervous system in this species. As a background, I will describe what is known on the autonomic nervous regulation of the gut motility in fish and I will then relate my findings to this knowledge. However, since the information on the non-mammalian autonomic nervous system is limited, the mammalian literature will have to serve as a frame of reference in many cases and I will refer the reader to recent reviews on the mammalian literature.

There are more than 20.000 teleost fish species which have evolved over a period of 200 million years and they occupy a variety of habitats (see Romer 1977). Consequently, the alimentary canal of these fish shows considerable anatomical variety. In most species, an oesophagus, a stomach and an intestine can be distinguished, but some fish are stomachless and the first part of the intestine serves a storage function. This variation is reflected by different innervation patterns between the various species (see Nilsson 1983). Although teleost fish are a heterogenous group, I will sometimes draw conclusions from results obtained in different species.

The autonomic nervous system

The autonomic nervous system is the part of the nervous system that controls the viscera. Amongst other things it affects blood flow and gastrointestinal functions such as gastrointestinal motility and secretion (see Guyton 1991). The autonomic nervous system is either activated by centres in hypothalamus, brainstem and spinal cord, or by reflexes originating from the viscera. Anatomically, the autonomic nervous system can be divided into three more or less separate parts: the sympathetic nervous system, the parasympathetic nervous system, and the enteric nervous system (see Langley 1921). Nilsson (1983) suggested a slightly modified terminology where the sympathetic and sacral parasympathetic subdivisions are collectively named "spinal autonomic" and the cranial parasympathetic system is called "cranial autonomic". This because the anatomical distinction between the lumbar sympathetic and sacral parasympathetic subdivisions is hard or impossible to make in non-mammalian vertebrates.

The thesis discusses the enteric nervous system, *i.e.* the part of the autonomic nervous system that has its neuronal cell bodies entirely within the gut wall, and the parts of the sympathetic (spinal autonomic) and parasympathetic (cranial autonomic) systems that are affecting the gastrointestinal canal. In teleost fish like the cod, the cranial autonomic nerve fibres reach the gastrointestinal canal via the vagi, and the spinal autonomic fibres mainly

via the anterior and posterior splanchnic nerves and the vagi (see Nilsson 1983). The anterior splanchnic nerves in teleost fish carry postganglionic fibres from the single coeliac ganglion (which is paravertebral in cod as opposed to prevertebral in mammals; Nilsson 1983). In teleosts, spinal autonomic fibres join the vagus nerves and the vagi are therefore considered to be vago-sympathetic trunks. The vagal (cranial autonomic) influence on the gastrointestinal canal is, as in mammals (see Gershon *et al.* 1994), most important in the anterior parts and does not extend beyond the stomach or proximal intestine in most teleosts (see Nilsson 1983).

The enteric nervous system

The enteric nervous system originates, as the other autonomic divisions, from the neural tube. However, the cells are derived from a region discrete from the sympathetic and parasympathetic divisions of the autonomic nervous system (see Le Douarin 1982). Other major distinctions are that the neurones mainly lack preganglionic inputs from the central nervous system and that the enteric nervous system can work independently of extrinsic innervation. This is possible because the enteric nervous system consists of integrated circuits with sensory, inter- and motor neurones present within the gut wall (see Furness and Costa 1987; Bornstein 1994; Gershon *et al.* 1994; Wood 1994).

The enteric nervous system consists of two major ganglionated plexuses present throughout the length of the gastrointestinal canal: the myenteric (Auerbach's) plexus in-between the muscle layers, and the submucous (Meissner's) plexus. Neurones in the plexuses project to other neurones and to muscle layers, submucosa and mucosa (see Gershon *et al.* 1994). In the most developed plexus, the myenteric plexus, the structure can be divided into different levels of organisation: primary (the ganglia and their thick interconnectives), secondary (the next order of branching) and tertiary (fine nerve fibres ramifying on the surface of smooth muscle fibres) plexuses (see Gershon *et al.* 1994). The neurones in the myenteric plexus project mainly to the smooth muscle layers and other ganglia, whereas the major effectors (target organs) of the submucous neurones are glandular and vascular tissue besides other neurones. Although teleost fish possess both a myenteric plexus and a submucous plexus, it has been reported that the submucous plexus contains no or only few neuronal cell bodies, and that the myenteric plexus is not as well organised compared to mammals. This indicates a simpler arrangement of the enteric nervous system in fish compared to mammals (*e.g.* Kirtisinghe 1940; see also Nilsson 1983; Nilsson and Holmgren 1989; Gibbins 1994).

The role of the enteric nervous system in a wider sense is to control the food uptake by the gastrointestinal canal. More specifically, the enteric nervous system controls a number of functions such as food transport (peristalsis), mechanical division of food particles (mixing movements), acid and enzyme secretion, absorption and cleaning (see Furness and Costa 1987; Gershon *et al.* 1994).

Many of the neurotransmitters of the gastrointestinal canal also function as hormones released from paracrine or endocrine cells. The endocrine system of the gastrointestinal canal co-operates with the enteric nervous system in many reflexes. However, the hormones produced from the endocrine cells are primarily affecting secretion of acid, enzymes, bicarbonate or mucus and not gut motility (see Furness and Costa 1987).

Gastrointestinal motility

A part of this thesis focuses on the intestinal peristaltic reflex. The peristaltic movements, together with mixing- and restrictive movements and accommodation constitute the gastrointestinal motility (see Furness and Costa 1987). A century ago Bayliss and Starling (1899) described "the law of the intestine". The "law" (later called the peristaltic reflex; Trendelenburg 1917; Furness and Costa 1987) described that application of pressure to the intestinal lumen gives rise to a stereotyped wave of descending (or anally directed) propulsive activity. The peristaltic reflex thus co-ordinates the transport of food from the oral end of the gastrointestinal canal to the anal end. The reflex to the circular muscle of the intestine consists of two parts: an ascending (or orally directed) excitatory reflex and a descending inhibitory reflex (see Furness and Costa 1987; Bornstein 1994). Both these reflexes are triggered by distension of the gut by the passing of foodstuff. The anal reflex relaxes the circular smooth muscle anally to the food and the oral reflex contracts the circular muscle orally to the food. Although there is also evidence that distension elicits reflexes to the longitudinal muscle layer, little detail is known about this part of the reflex compared to the ascending excitatory and descending inhibitory reflexes to the circular muscle layer (see Costa *et al.* 1992a; Bornstein 1994). The peristaltic reflex has been considered completely enteric since it functions without extrinsic innervation (Trendelenburg 1917; see also Furness and Costa 1987). In fact, it functions well *in vitro* in an isolated segment of the gut (*e.g.* Holzer 1989; Tonini and Costa 1990). However, Grider and Jin (1994) have shown, at least in the rat colon, that the cell bodies of the sensory neurones responding to stretch of the muscle layers are located in the dorsal root ganglia. That the stretch triggered peristaltic reflex still can be elicited in intestinal preparations is explained by the viability of collaterals from afferent sensory neurones in the acute *in vitro* experiments commonly used.

There are also other means than distension to elicit the enteric reflexes, like deformation of the mucosa by brushing the epithelium (mechanical stimulation of the mucosa) or by changing the composition of the chyme (chemical stimulation of the mucosa; see Furness and Costa 1987; Gershon *et al.* 1994). The cell bodies of the sensory neurones responding to mechanical stimulation of the mucosa are located in the submucous plexus (Grider and Jin 1994; see also Gershon *et al.* 1994). The mechanoreceptors responding to mucosal stimulation could be part of the sensory neurones initiating the peristaltic reflex. However, an alternative (Bülbring's hypothesis) is that the nerve cells are secondarily excited by hormones released from cells in the gastrointestinal mucosa. Bülbring has suggested that enterochromaffin cells act as sensory receptors (Bülbring and Crema 1959; see also Gershon *et al.* 1994). The enterochromaffin cells secrete 5-hydroxytryptamine (5-HT;

serotonin) in response to a variety of mucosal stimuli (*e.g.* pressure, mechanical stimulation, acidification and cholera toxin; Bülbring and Crema 1959; Cassuto *et al.* 1982; see also Gershon *et al.* 1994) and the 5-HT is then suggested to act as a mediator that excites the sensory nerve fibres in the lamina propria (Grider *et al.* 1996; see also Gershon *et al.* 1994).

The extrinsic innervation to the intestine is believed to have a modulatory role on the peristaltic reflex only. This influence is mainly inhibitory, acting presynaptically to inhibit release of acetylcholine from enteric motor neurones (see Furness and Costa 1987). The extrinsic (especially vagal) input to the stomach is more direct and can be very powerful. The vagal innervation is involved in reflexes to the stomach elicited by mechanical stimulation in the pharynx and oesophagus (gastric receptive relaxation) or the proximal stomach (accommodation reflex; see Furness and Costa 1987; Lefebvre 1993). The fundus and corpus of the stomach relaxes in response to these stimuli and the relaxation allows the stomach to store large volumes of food without major pressure changes. The antrum disintegrates the food and propels it towards the duodenum. This part of the stomach also exhibits peristaltic waves. However, they are not generated in the same way as the intestinal peristaltic reflexes (see Furness and Costa 1987).

Although the fish gut has been studied extensively both *in vivo* and *in vitro*, and the effects of several neurotransmitters on gut smooth muscle have been established (elasmobranchs, Andrews and Young 1988; teleosts, Jensen and Holmgren 1985; Kitazawa *et al.* 1990; for a recent review see Jensen and Holmgren 1994), little knowledge has been gathered about the participation of these neurotransmitters in reflex pathways (elasmobranchs, Andrews and Young 1993; teleosts, Grove and Holmgren 1992a, b). In the teleost fish stomach, the gastric receptive relaxation and accommodation reflexes seem to be independent of the vagus (Grove and Holmgren 1992a, b) whereas these reflexes depend on extrinsic pathways in mammals (Martinson 1965; Abrahamsson 1973). This indicates that in teleost fish, enteric neurones perform the tasks of at least some of the extrinsic gut reflexes seen in mammals.

Neurotransmitters of the autonomic nervous system

Until recently, it was believed that adrenaline and/or noradrenaline (NA) is the only transmitter in sympathetic postganglionic neurones, and that the only transmitter of the parasympathetic nervous system is acetylcholine. I will refer to these as "the classical neurotransmitters". Later, other substances like amines (dopamine and 5-HT), purines (ATP), amino acids (GABA, glutamate) and peptides were discovered to have transmitter roles (see Burnstock 1972; Lundberg and Hökfelt 1986; Guyton 1991). The neuropeptides, in contrast to the small-molecule transmitters, consists of chains of up to 40 amino acids. The peptides are usually slow-acting and thus complement the faster acting small-molecule transmitters (see Guyton 1991). About the same time as my study began, the free radical nitric oxide was added to the list of putative transmitters; nitric oxide was discovered to play a role as a neurotransmitter in both the central and autonomic nervous

systems (Bredt *et al.* 1990; Bult *et al.* 1990; Li and Rand 1990; see also Moncada 1994; Zhang and Snyder 1995).

In the enteric nervous system, some neurotransmitters are restricted to neurones with consistent projections, whereas others are located in nerves with various projections in different animals. The projections of neurones containing a certain neurotransmitter may also vary between different gut regions within the same animal. It has been speculated that the transmitters found in conserved pathways are more important for the function of these pathways than those transmitters that are also found in neurones with other projections (Messenger and Furness 1990; see also Furness *et al.* 1992). For example, vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS; the enzyme responsible for nitric oxide formation from L-arginine; Bredt and Snyder 1990) are found in descending (anally projecting) neurones in mammalian intestine (see Furness *et al.* 1992). Both VIP and nitric oxide have relaxing actions on the smooth muscle of the gut in mammals (see Lefebvre 1995) and are considered to be general transmitters of the descending inhibitory reflex of the gut (see Furness *et al.* 1992; 1995).

In mammals, the ascending motor neurones use acetylcholine and the neuropeptide substance P (SP; or other peptides of the tachykinin family) as primary transmitters (see Barthó and Holzer 1985; Grider 1989; Tonini and Costa 1990; Holzer-Petsche 1995). The results of histological studies have confirmed the existence of acetylcholine and SP in motor neurones with anal to oral projections (especially the guinea-pig; *e.g.* Brookes *et al.* 1991; Steele *et al.* 1991; see also McConalogue and Furness 1994; Costa *et al.* 1996), although the projections of SP containing neurones vary between animals and also between different gut regions (see Messenger and Furness 1990). For example in the rat small intestine, SP containing neurones project anally (Ekblad *et al.* 1987).

Nothing was known about the projections of fish enteric neurones prior to this study.

Target-specific chemical coding

For two decades it has been known that a single neurone can contain multiple transmitters (Burnstock 1976; see also Lundberg and Hökfelt 1986). The neuropeptides were found to coexist - both with the classical transmitters and with each other (see Lundberg and Hökfelt 1983). Subsequently, in the eighties the idea that the combination of different transmitters can be utilised to give neurones a "chemical code" arose (see Costa *et al.* 1986; Lundberg and Hökfelt 1986; Furness *et al.* 1989). In the sympathetic division of the autonomic nervous system of the guinea-pig, for instance, NA coexists with neuropeptide Y (NA/NPY) in a subset of neurones, whereas other neurones contain coexisting NA/somatostatin and a third set of neurones contain NA alone (NA/-; *i.e.* lacks any other known transmitter). By establishing the chemical code of the sympathetic neurones, it was found that the three classes provided different targets with sympathetic innervation (see Costa *et al.* 1986).

The functional significance of coexistence in the enteric nervous system is poorly understood and has received little attention. In the other divisions of the autonomic nervous system, and especially the sympathetic innervation of blood vessels, the significance of cotransmission has been more thoroughly investigated in mammals and amphibians. NPY often potentiates the contractile effect of adrenaline/NA on the circulatory system (Thorne and Horn 1997; see also Håkanson *et al.* 1986; Morris and Gibbins 1992). Nothing is known about the effects on cod vessels, but synergistic actions of NPY and adrenaline have been reported on vessel and heart preparations from other teleosts and elasmobranchs (Bjenning *et al.* 1993; Xiang *et al.* 1994; Uesaka 1996).

A comparative approach to the autonomic nervous system

Although the autonomic nervous system is important in everyday activities, *e.g.* by its control of blood flow and gastrointestinal functions, the knowledge of the autonomic nervous system is limited compared to the immense information on the central nervous system. If we look to non-mammalian vertebrates, the information is even more scanty, when it logically should be the other way around; in the earlier developed vertebrates, the nervous system is simpler (yet diverse) than in mammals and this is fundamental for obtaining basic knowledge on the nervous system since the unknown variables are fewer. Thus, as with other biological questions, the comparative approach provides a powerful tool for investigating general principles of autonomic physiology. It is for these reasons that I have devoted myself to studies on a non-mammalian vertebrate.

The presence and function of many of the classical as well as peptide transmitters have been demonstrated in the teleost fish gastrointestinal canal (see Jensen and Holmgren 1994). Adrenaline and acetylcholine were early proven to be transmitters in the fish gut. In the cod, adrenaline has a contractile effect on the stomach and an inhibitory effect on the intestinal smooth muscle (*e.g.* Nicholls 1934; Young 1936; von Euler and Östlund 1957; Nilsson and Fänge 1969; see also Nilsson 1983). Using fluorescence histochemistry, adrenergic nerve fibres are demonstrated in the gastrointestinal canal of the cod (Jensen and Holmgren 1985). However, lack of selective methods to demonstrate cholinergic neurones has hampered the progress in the study of the neuroanatomy of cholinergic neurones in the autonomic nervous system of fish, as well as in mammals (see Nilsson 1983; Schemann *et al.* 1993). Pharmacological methods have, on the other hand, successfully been used to demonstrate the action and sites of action of acetylcholine in the fish gastrointestinal canal (see Nilsson 1983; Jensen and Holmgren 1994). In the cod as in several other teleosts, acetylcholine stimulates muscarinic receptors on the smooth muscle (Jensen and Holmgren 1985; see also Nilsson 1983).

Both fluorescence histochemistry and immunohistochemistry have been employed to demonstrate enteric 5-HT neurones in the fish gastrointestinal canal (Watson 1979; Jensen and Holmgren 1985). 5-HT has an excitatory effect on the fish gastrointestinal canal smooth muscle (von Euler and Östlund 1957; see also Nilsson 1983; Jensen and Holmgren

1994). In the cod intestine, the effect is partly mediated via other enteric neurones (Jensen and Holmgren 1985).

The first neuropeptide to be discovered, a SP-like peptide, was demonstrated in the fish gut (actually in the cod) already in 1956 by von Euler and Östlund. Since then, SP has been established as a primary transmitter in the gastrointestinal canal of many teleosts and the amino acid sequence of native SP has been determined in several fish species (*e.g.* Jensen and Conlon 1992b; see Jensen and Holmgren 1994). VIP has been identified as an inhibitory substance in the gut of a variety of animals (see Dimaline 1989). Although VIP-like peptides are present abundantly in the fish gut and the VIP sequence has been determined in the cod, it has been harder to demonstrate an inhibitory effect here (see Jensen and Holmgren 1994).

A number of other peptides frequently found in mammals have also been demonstrated in the fish gastrointestinal canal: immunoreactivity to bombesin/gastrin-releasing peptide (GRP) is found in the cod stomach and intestine (Bjenning and Holmgren 1988) and bombesin has an excitatory effect in the stomach (Holmgren and Jönsson 1988) whereas the effect on the intestinal smooth muscle is inhibitory (Jensen and Holmgren 1985; Holmgren and Jönsson 1988). The amino acid sequence of a peptide from the bombesin/GRP family has been determined from the rainbow trout (*Oncorhynchus mykiss*). This neuropeptide was structurally more similar to mammalian GRP than to bombesin (Jensen and Conlon 1992a). Another peptide family, gastrin/cholecystokinin (CCK), has also been demonstrated with histochemical methods in the fish gut. In addition to its presence in endocrine cells, gastrin/CCK immunoreactivity is also present in neurones in the fish gastrointestinal canal. The intensities varies between species and regions of the gastrointestinal canal. In the cod, there is a rich nerve net in the stomach whereas gastrin/CCK immunoreactivity is absent from the intestine and rectum (Jönsson *et al.* 1987; Bjenning and Holmgren 1988). Peptides from the gastrin/CCK family have excitatory actions on gastrointestinal preparations from the cod (Jönsson *et al.* 1987). In general, the immunohistochemical demonstration of neuropeptides in the fish gut has been restricted to nerve fibres, whereas the neuronal cell bodies only have been identified in a few cases. This has of course made it difficult to state the origin of the immunoreactive (IR) nerve fibres.

Aims of the study

The aims of the study were to identify and characterise extrinsic and intrinsic nerve pathways in the gut of a teleost fish species, and to correlate these findings with the effects of putative neurotransmitters on gastrointestinal motility. The overall goal of the study has been to propose a model of neuronal transmission in the teleost fish gastrointestinal canal using a comparative approach.

Methods

The thesis is based on immunohistochemical visualisations of a variety of neuronal cell populations within or projecting to the cod gastrointestinal canal. To support the observations, *in vitro* preparations of different kinds have been employed for pharmacological testings. Below follows a discussion on the methods used to elucidate the projections of different neuronal populations immunohistochemically since this has been the most important goal of the thesis study. For a detailed description of the methods used, the reader is referred to the individual papers I-VII.

Immunohistochemistry

Immunohistochemistry employs antibodies raised against substances of interest (transmitter substances and enzymes involved in transmitter substance synthesis in the thesis study) for their visualisation. The method is both specific and sensitive since it can be used to demonstrate the presence of substances present in low concentrations with a high signal-to-noise ratio. The antibodies are usually directed against the active parts of transmitter substance molecules. In this way the specificity becomes great since, *e.g.* antibodies raised against peptide transmitters seldom bind to peptides from other peptide families. However, it cannot be guaranteed that the antibodies do not also bind to peptides with similar sequences (like other peptides from the same family of neuropeptides) or to unrelated structures in the tissue under investigation that happens to have a similar structure (see Polak and Van Noorden 1986). A common way to express this uncertainty about the binding is to use the term "-like immunoreactivity" (*e.g.* galanin-like immunoreactivity). As the sequences of related peptides from a peptide family are determined, allowing for testing of cross-reactivity, one can be reasonably sure what the antibodies bind to and the term "immunoreactivity" (*e.g.* galanin immunoreactivity) is justified. There is also a trend in modern papers to leave out the "-like" as the methodological uncertainty is understood.

A few antibodies directed against native fish neuropeptides have been available [see VII], but the majority of the research is made with antisera directed against mammalian neuropeptides. Since the peptide sequences differ more or less between mammals and non-mammalian vertebrates, these differences may cause low or non-existing binding of antisera directed against mammalian neuropeptides in fish tissue. Therefore, one cannot interpret a negative immunohistochemical result as lack of the investigated peptide since it does not exclude the presence of a native peptide in the fish tissue.

In this study, the indirect fluorescence immunohistochemical method (developed from Coons 1956; see Polak and Van Noorden 1986) was employed.

Nerve lesions, retrograde tracing

An early aim was to elucidate the origin of neurones innervating the gastrointestinal canal of the cod, *i.e.* if the neurones were of an extrinsic (cranial-, spinal autonomic, or sensory) or intrinsic (enteric) origin. The direction along the gastrointestinal canal intrinsic neurones containing identified neurotransmitters project was also of immediate interest since this, together with the known actions of the neurotransmitters, can give clues on the functions of the neuronal populations. By severing the nerve pathways in enteric nerve plexuses or in extrinsic nerves to the gut, nerve pathways in and to/from the mammalian gut have been delineated. This can be done since the transmitter immunoreactivity disappears in the degenerated nerve endings that have been isolated from the cell body (see Costa *et al.* 1986; Furness and Costa 1987). Due to the changes in immunoreactivity, conclusions can be drawn of the extrinsic or intrinsic nature of the nerves and of the direction along the gastrointestinal canal nerves containing a certain transmitter project. However, due to differences in the way neurotransmitters are degraded in mammals and fish [see VI], we have had problems achieving the aims using these methods (see results and discussion). Thus, another method, retrograde tracing, was applied to investigate the origins of nerve fibres in the cod stomach [IV]. Retrograde tracing uses the axonal transport system of neurones since, *e.g.* fluorescent dye molecules can be transported along the axons and accumulate in the neuronal cell bodies (see Kuypers and Huisman 1984). Neuronal tracing has also been successfully performed in fixed tissue, *e.g.* the teleost brain (Holmqvist *et al.* 1992), using lipophilic compounds that diffuse through the myelin sheaths.

Confocal laser scanning microscopy

Since there is little or no degeneration of the portion of the axon isolated from the cell body in fish neurones [see VI and below], we concentrated on the accumulations of IR material on the proximal side of the lesion. The use of confocal laser scanning microscopy (CLSM) in papers VI and VII have aided in the quantification of the results on accumulation after the myotomy operations. The confocal laser scanning microscopy technique reduces the background fluorescence and improves the depth resolution since only light from the focal plane is allowed to pass to the photodetector (see Majlof and Forsgren 1993). This makes it ideal for reproducible acquisition of images from the oral and anal sides of the cuts in myotomy operated fish. The images were subsequently analysed using the built-in image analysis software [see VI].

Results and discussion

In this section, I will discuss a hypothesis that has emerged during the thesis work: the fish autonomic nervous system is not as simple as previously believed. In contrast to the current opinion expressed in older literature (*e.g.* Kirtisinghe 1940; see also Nilsson 1983; Nilsson and Holmgren 1989), I will show that the neuronal density, morphology and neurochemical complexity of fish autonomic neurones resemble that found in other vertebrates.

Extrinsic and intrinsic innervation of the cod stomach

Figures 1 and 2 summarise the immunohistochemical and histological results from papers I-IV on the presence of neuronal cell bodies and nerve fibres within the stomach wall, or the location of neurones projecting to the cod stomach (extrinsic innervation). Where neuronal cell bodies have been found in the myenteric plexus, it is probable that, at least part of, the nerve fibres are of intrinsic (enteric) origin. Some physiological data have also been obtained that can aid in elucidation of the physiological significance of the neuronal populations demonstrated.

Are there any truly enteric neurones in the cod gut?

In its early stages, the study focused on the identification of distinct populations of both extrinsic and intrinsic nerves to the gut of the cod. The immunohistochemical method was engaged and developed in several investigations. A marker for neuronal tissue, anti-neurone-specific enolase, was employed to localise intrinsic and extrinsic neurones of the cod gastrointestinal canal. Using this antiserum in combination with the secondary antisera available at the time did not give specific staining of neuronal structures in the myenteric plexus of the stomach or intestine. Rather, the background was high making it impossible to discern any neuronal cell bodies. However, neuronal cell bodies were found in microganglia along the vagal branches to the gut [I]. Similar neurones have also been found in the toad, *Bufo marinus* (Gibbins *et al.* 1987), but have not been reported from mammalian studies. The neuronal cell bodies along the vagi may represent "enteric" neurones with an external location (or, in other words, parasympathetic postganglionic neurones situated outside the target tissue). One explanation for their location could be that the neuronal cell bodies for some reason become "trapped" in the vagi during the migration from the neural crest to the gastrointestinal canal. In the abnormal gastrointestinal canal of "lethal spotted mice", the distal gastrointestinal canal is aganglionic. The mechanism behind this is believed to be an abnormal extracellular matrix in the gastrointestinal canal; if the concentration of laminin, that lead to cessation

of migration of neural precursors, is abnormally high within the gut, the neurones stop migrating prematurely (see Gershon *et al.* 1993).

Galanin neurones in the vagus nerve

A portion of the neuronal cell bodies in the cod vagus are galanin-IR [I; see fig. 1]. Galanin, a 29 amino-acid peptide (Tatemoto *et al.* 1983), is present in a variety of central and peripheral nervous tissues, including a dense population of neurones in the gastrointestinal canal of many animals (*e.g.* Ekblad *et al.* 1985b; Bishop *et al.* 1986; Morris *et al.* 1992; Holmgren *et al.* 1994). Other neuronal cell bodies in the cod vagus are NOS-IR. There is also a coexistence of NOS/VIP immunoreactivity in some of the neuronal cell bodies (own unpublished results). There are no galanin-IR neuronal cell bodies in the sympathetic coeliac ganglion and in the presumed sensory ganglion of the vagus outflow [the nodose ganglion; I]. On the cod stomach and intestinal smooth muscle, the effects of galanin are direct and weakly contractile. In mammals, galanin has diverse effects and both excitatory and inhibitory responses are reported on gut smooth muscle (Ekblad *et al.* 1985a; Bauer *et al.* 1989; Holzer-Petsche and Moser 1993; see also Rattan 1991). The weak direct effect on smooth muscle in the cod, as in the rat stomach (Holzer-Petsche and Moser 1993), indicates a modulatory role rather than a role as a primary transmitter.

Tachykininergic neurones in the vagus nerve

A population of the vagal neuronal cell bodies are SP-IR [II]. SP is a peptide transmitter belonging to the tachykinin family of regulatory peptides which shares a common C-terminal amino acid sequence (see Jensen and Holmgren 1994). In the thesis, I will refer to SP-IR neurones as "tachykininergic" or "tachykinin-IR" since antibodies raised against the native (and structurally similar; Jensen and Conlon 1992b) tachykinins cod SP and cod neurokinin A (NKA) cross react with cod NKA and cod SP, respectively [VII]. In paper II we also found that tachykinin immunoreactivity is located in presynaptic boutons on postganglionic neurones in the vagus nerve, and that the majority of the axons derive from the cranial direction (see Fig. 1). It was concluded that the axons synapsing on neuronal cell bodies in the vagus are of preganglionic, probably cranial autonomic origin [II]. Indeed, tachykinin immunoreactivity is found in vagal motor nuclei in other fish species (Vecino and Sharma 1992; Weld and Maler 1992).

At this stage, neither galanin nor tachykinin immunoreactivities were found in but a few enteric neuronal cell bodies of the stomach. In the case of tachykinins this was very disappointing, since SP is known to be involved in the ascending excitatory reflex of peristalsis in many animals (see Holzer-Petsche 1995). In the perfused cod stomach, mammalian SP has an excitatory effect not affected by tetrodotoxin (TTX), indicating a direct effect on smooth muscle, and probably a presence in enteric neurones in the systems investigated [II]. Despite the lack of IR neuronal cell bodies, an abundant innervation with varicose fibres in the myenteric plexus of the stomach could be demonstrated using antisera to both peptides. The few enteric neuronal cell bodies found were all large and had a multidendritic appearance [see fig 3 C in II]. It was (in error) concluded that both peptides were confined to neurones mainly extrinsic to the gut. Reasons for the possible lack of enteric neurones in the cod were also discussed [I]. In other fish species, enteric neuronal cell bodies are frequently visualised with antisera against neuropeptides

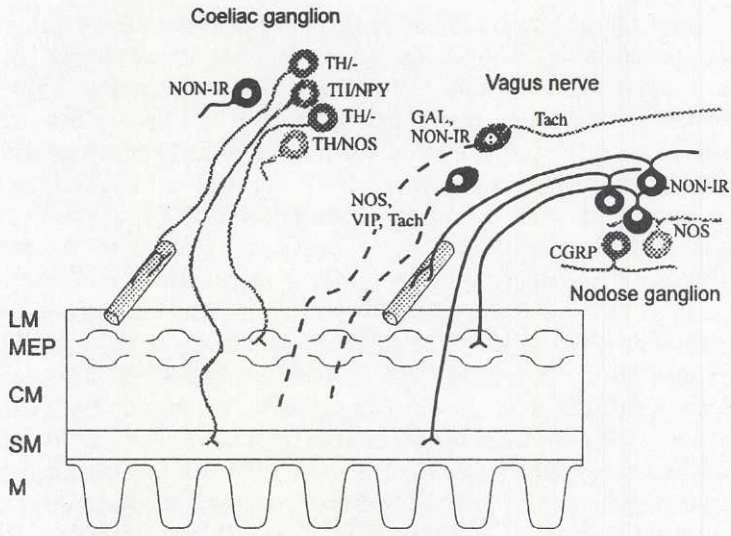

Fig. 1. Extrinsic innervation to the cod stomach.

The immunohistochemical identity of neuronal cell bodies in the coeliac ganglion (spinal autonomic), the nodose ganglion (sensory) and along vagal branches to the stomach (possibly cranial autonomic) is shown. The figure is based on immunohistochemical results in papers I, II, IV, and on own unpublished results. If two immunoreactivities have been shown to coexist, it is indicated by the dash (for example TH/NPY). TH, Tyrosine hydroxylase immunoreactive (IR) neurones; CGRP, calcitonin gene-related peptide; GAL, galanin; NPY, neuropeptide Y; NOS, nitric oxide synthase; Tach, tachykinin(s); VIP, vasoactive intestinal peptide; NON-IR, neurones with unknown identity; LM, longitudinal muscle layer; MEP, myenteric plexus; CM, circular muscle layer; SM, submucosa; mucosa, M

(Bjenning and Holmgren 1988; own unpublished results). However, the myenteric neurones in the fish gastrointestinal canal are not organised in distinct ganglia as in mammals [see Kirtisinghe 1940; fig. 2 in III].

NOS in enteric neurones

In a study on the distribution and projection of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reactive neurones in the gut of the cod [III], a high number of neuronal cell bodies were observed in the myenteric plexus of all regions of the gut. By this finding we were finally convinced that an abundance of enteric neurones *do* exist also in the cod gastrointestinal canal. In mammals, NADPH-d is identical to NOS and is responsible for the production of neuronal nitric oxide. Thus, the NADPH-d method can be used to visualise NOS neurones. Nitric oxide is involved in gut smooth muscle relaxation and is an important transmitter in the peristaltic reflex (Li and Rand 1990; Shuttleworth *et al.* 1991; Calignano *et al.* 1992; Maggi and Giuliani 1993). Also in the fish gastrointestinal canal, NADPH-d reactivity and NOS immunoreactivity coexist in neuronal cell bodies (Li and Furness 1993; C. Olsson personal communication).

The total density of myenteric neuronal cell bodies in the cod gastrointestinal canal [approximately 7000 neuronal cell bodies/cm^2; as measured using NADH (nicotinamide dinucleotide) diaphorase staining; III] is comparable to that in small mammals (Gabella

1987). Since occasional neuronal cell bodies IR to VIP and the related peptide pituitary adenylate cyclase-activating polypeptide (PACAP) had been found in the cod gastrointestinal canal (Olsson and Holmgren 1994), and since VIP and NOS immunoreactivities coexist in the enteric nervous system of other species, we tested for coexistence also in the cod. With the improved immunohistochemistry protocol, adapted from Smart *et al.* (1992), numerous VIP/PACAP-IR neuronal cell bodies were found. The main improvements in this study compared to previous work [I, II] are: 1. A considerably shorter fixation period and switching from Zamboni's fixative to paraformaldehyde fixation. 2. The use of secondary antibodies with high specificity resulting in a higher signal to noise ratio. We found that about 40% of the NADPH-d positive neuronal cell bodies also contain VIP/PACAP [III]. The degree of coexistence of NOS and VIP varies in species investigated but the results are also inconclusive in-between investigations from different laboratories (Costa *et al.* 1992b; Aimi *et al.* 1993; Forster and Southam 1993; Ekblad *et al.* 1994). The differences are believed to be due mainly to the use of colchicine in some studies and not in others (see Ekblad *et al.* 1994) since colchicine enhances the peptide immunoreactivity in neuronal cell bodies. In the toad, the same three populations are found as in the cod study but the proportion of cells showing coexisting NOS/VIP is smaller (Li *et al.* 1993).

Other enteric populations in the stomach

Using the improved immunohistochemistry protocol, populations of for example galanin-, NOS-, NPY- and tachykinin-IR neuronal cell bodies have been found in the cod stomach [IV; own unpublished results] in addition to nerve fibres in all the layers of the stomach. Fig. 2 summarises the occurrence of neuronal cell bodies and fibres using antisera against some putative transmitters and transmitter enzymes. In some cases, this has been done with the same antisera that has given negative results previously, *e.g.* anti-galanin and anti-NPY [I; Bjenning and Holmgren 1988], illustrating the improvements of the modified method. There appears to be colocalisation of galanin-IR and NOS (as seen using NADPH-d) in some of the neurones of the cod gastrointestinal canal whereas galanin and VIP are found in separate neuronal populations [own unpublished results; VI]. For the tachykinins, antisera raised against native cod tachykinin sequences were used and were found to give much higher immunoreactivity than antisera raised against mammalian SP [VII].

Summary

In this section, I have shown that there is a significant population of postsynaptic neurones projecting to the stomach in the vagus nerve. Some of these neurones contain galanin and are contacted by tachykininergic preganglionic neurones. I have also shown that there is a similar coexistence of NOS/VIP in enteric neurones of the cod as in other vertebrates, but more important: the density of neuronal cell bodies in the cod gut is similar to that in small laboratory animals. In mammals, the number of neurones in the enteric nervous system is comparable to that of the spinal cord (see Costa *et al.* 1986). The fish enteric nervous system has been considered to contain comparably fewer neurones (Kirtisinghe 1940; see also Nilsson 1983; Nilsson and Holmgren 1989). It is more difficult to dissect the gut and to make thin, flat preparations out of it in fish and other non-mammalian vertebrates than in mammals (and especially guinea-pig). This has resulted in two things:

1. In the early work using silver impregnation and methylene blue (Kirtisinghe 1940), a large population of enteric neuronal cell bodies was undetected. 2. Slower progress in the non-mammalian enteric nervous system research. This is probably why the fish enteric nervous system is considered to be simpler than the mammalian enteric nervous system.

Projections of extrinsic neurones, retrograde tracing

To determine the origin and transmitter content of the sympathetic innervation to the stomach, retrograde neuronal tracing with fluorescent dye was combined with immunohistochemistry in the spinal autonomic coeliac ganglion of the cod [IV]. Sympathetic postganglionic neurones innervating blood vessels often contain NPY and/or galanin in addition to adrenaline/NA (*e.g.* Morris *et al.* 1986; Ahrén *et al.* 1990; Karila *et al.* 1995a). In the sympathetic coeliac ganglion of the cod, the previous findings of catecholamines, using the Falck-Hillarp fluorescence histochemical method (Nilsson 1976), were confirmed by immunohistochemistry to the catecholamine synthesising enzyme tyrosine hydroxylase (TH), and in double labelling experiments, NPY was found to coexist with TH in a subset of these neuronal cell bodies [IV; see fig. 1]. However, the TH/NPY innervation to the blood vessels of the fish gut is sparse and the majority of the sympathetic fibres to the gut vessels are TH/- [IV]. In a separate population of neuronal cell bodies in the coeliac ganglion, NOS-IR was surprisingly found to coexist with TH-IR. Indeed, NOS is found in a subpopulation of postganglionic neuronal cell bodies in sympathetic ganglia also in other vertebrates, but these neurones are non-noradrenergic (*e.g.* Kummer 1992; Anderson *et al.* 1995; Sann *et al.* 1995) in contrast to the adrenergic NOS-containing neurones in the cod.

Sympathetic innervation to the myenteric plexus

Stronger and more direct anatomical evidence for neuronal projections than chemical coding comes from experiments where a small amount of a dye is injected in a putative target organ and retrogradely transported to the neuronal cell bodies of interest (see Kuypers and Huisman 1984). In this way, for instance neural pathways from sympathetic ganglia to the airways (Kummer *et al.* 1992), blood vessels, skin (Horn *et al.* 1988; Gibbins 1991, 1992) and different gut regions (Zhang *et al.* 1991; Trudrung *et al.* 1994; Uddman *et al.* 1995) have been revealed in frogs (*Rana catesbeiana*) and mammals. Similarly, application of the retrogradely transported dye Fast Blue in the muscle wall and myenteric plexus of the cod stomach resulted in the accumulation of dye in neuronal cell bodies of the coeliac ganglion [IV]. The fast blue labelled neurones in the coeliac ganglion were nearly all TH/-. This confirms an adrenergic sympathetic innervation of the cod gut wall, as previously indicated from nerve stimulation experiments (Nilsson and Fänge 1969) and by the presence of catecholamine fluorescent nerve fibres in the gut wall (Jensen and Holmgren 1985).

Sympathetic innervation to the submucosa

If the Fast Blue injections were made close to vessels at the stomach surface some of the TH/NPY neurones were also labelled. However, more of the TH/NPY-IR neurones were fast blue positive after dye application in the submucosa. Thus, based on the relatively low number of neuronal cell bodies in the coeliac ganglion that were Fast Blue positive after injection close to blood vessels and the sparse TH/NPY innervation of the blood vessels, the TH/NPY neurones are suggested to project mainly to non-vascular sites of the submucosa, whereas the TH/- neurones project to the myenteric plexus of the stomach [IV; see Fig. 1]. This is in stark contrast to the situation in other animals, where catecholamine containing sympathetic neurones with coexisting NPY project to blood vessels. In the guinea-pig, the subpopulation projecting to the submucosa contains somatostatin [which we were not able to demonstrate immunohistochemically in the cod; IV] in addition to NA (see Costa *et al.* 1986). Since very few Fast Blue labelled neuronal cell bodies in the cod coeliac ganglion contained NOS-IR, the TH/NOS neurones of the coeliac ganglion are suggested to have targets other than the stomach. Although the chemical coding appears different in the cod, this is the first evidence for chemically specified neurones to have different projections in the autonomic innervation of fish.

Summary

A study on goldfish sympathetic neurones also implicates that the teleost postganglionic sympathetic neurones are complex. Using intracellular dye injections in fixed neurones, we found a wide range of dendritic morphologies in the coeliac ganglion (Karila *et al.* 1995b). This was surprising since a phylogenetic trend has been observed with the simplest neurones thus far found in amphibians and more complex neurones in birds and mammals (see Gibbins 1994). Together with the target-specific neurochemical diversity in the cod ganglia, this implies that the neurones in sympathetic ganglia of fish are capable of complex integration and not only function as simple relay stations as previously believed.

Sensory neurones

Neuronal cell bodies in sensory ganglia and a population of nerve fibres in peripheral organs contain coexisting calcitonin gene-related peptide (CGRP)-IR and tachykinin-IR in a variety of vertebrate species including mammals, crocodiles, snakes and amphibians (*e.g.* Wiesenfeld-Hallin *et al.* 1984; Gibbins *et al.* 1985; Morris *et al.* 1986; Davies and Donald 1992; Karila *et al.* 1995a). Coexistence of CGRP/tachykinin immunoreactivity has also been used as a marker for sensory neurones in the Australian lungfish gut and airways (Holmgren *et al.* 1994) and in the heart of several other fish species (Davies *et al.* 1994). We early tried to demonstrate a sensory tachykininergic innervation of the cod gut and gut arteries using capsaicin [II]. Capsaicin treatment depletes the transmitter content of primary afferent (sensory) neurones in many vertebrates (see Lundberg and Hökfelt 1986), but was fruitless in the cod. A possible explanation for the lack of visible effect may be

that the sensory tachykinin-IR nerve fibres are outnumbered by enteric tachykinin-IR nerve fibres in the examined regions.

CGRP/tachykinin coexistence in the myenteric plexus

A better way to investigate the sensory innervation would therefore be to use double labelling immunohistochemistry for CGRP and tachykinins in combination with capsaicin treatment. Enteric neurones with coexisting CGRP/tachykinin-IR are found in the eel (*Anguilla anguilla*) and in another fish species, the five bearded rockling (*Ciliata mustela*), a low number of CGRP/tachykinin-IR nerve fibres were discovered, exclusively surrounding gut vessels (own unpublished results). Using a CGRP-antibody which gives immunoreactivity also in nerve fibres in the cod stomach, we have recently, in double labellings with a SP antibody raised in guinea-pig, found that there is nearly 100% coexistence between CGRP and tachykinins in the myenteric plexus and muscle layers of the stomach (P. Karila and S. Holmgren unpublished). However, the SP antibody raised in guinea-pig gives only a fraction of the immunoreactivity compared to antisera raised against cod tachykinin sequences [VII] and it is thus unlikely that all tachykininergic nerve fibres contain CGRP immunoreactivity. Rather, it is probable that there is a large fraction of intrinsic tachykinin/- neurones that are not detected with the antibody raised in guinea-pig.

CGRP in sensory neurones?

In the cod and the tadpole fish (*Raniceps ranius*), CGRP-IR neuronal cell bodies were found in the nodose ganglion, but these did not contain tachykinin immunoreactivity (P. Karila, J. Messenger and S. Holmgren unpublished; see Fig. 1). In the nodose ganglion of the cod, 15% of the neuronal cell bodies contain NOS-IR and 11% contain CGRP-IR. Which transmitter(s) the other neurones contain and whereas the CGRP and NOS immunoreactivities coexist remains to be elucidated.

Generally, the low reactivity to peptides in neuronal cell bodies may be due to a too low peptide concentration, or alternative processing with only pro-peptides with poor immunoreactivity present in the neuronal cell bodies. Attempts to stop the vesicular transport in the neurones with axonal transport antagonists, and thus to elevate the transmitter concentrations in the neuronal cell bodies, have all been fruitless (II; own unpublished results). After fast blue injections in the stomach [see IV], accumulation of dye also occurred in neuronal cell bodies of the nodose ganglion (P. Karila, J. Messenger and S. Holmgren unpublished). Presuming that the nodose ganglion in the cod is a sensory ganglion like in other vertebrates, this shows that there is a significant extrinsic sensory innervation of the gut wall also in teleosts. Their transmitter identity remains unidentified since the neurones projecting to the stomach mainly lack detectable CGRP and NOS immunoreactivities (P. Karila, J. Messenger and S. Holmgren unpublished).

Summary

Although the origin of the CGRP/tachykinin-IR nerve fibres of the cod stomach thus remains to be elucidated, circumstantial evidence suggests that the wide-spread combination of CGRP/tachykinin immunoreactivity in primary sensory neurones may occur also in teleost fish.

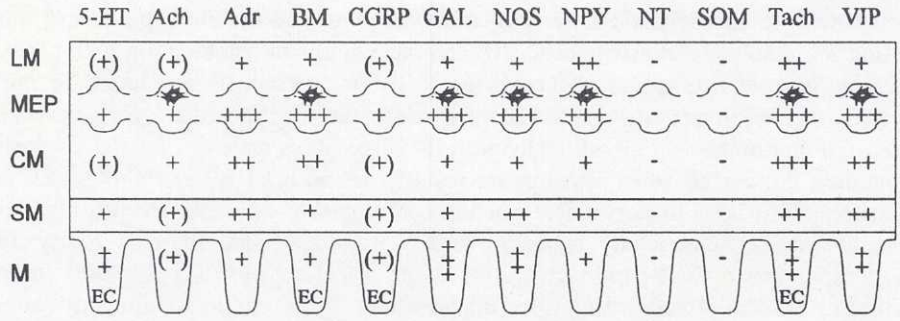

Fig. 2. Innervation of the cod stomach.

The distribution of immunoreactive neuronal cell bodies in the myenteric plexus and nerve fibres in the different layers of the cod stomach. The appearance of the immunoreactive (IR) fibres was evaluated as follows: +++, densely packed IR fibres; ++, moderate density of IR fibres; +, sparse distribution of IR fibres; (+), occasional IR fibres; -, lack of IR fibres. The figure is based on immunohistochemical results in papers III, IV, Olsson and Holmgren 1994, and on own unpublished results. Where immunoreactive enteric neuronal cell bodies have been found, the immunoreactive nerve fibres are probably of intrinsic origin. 5-HT, 5-hydroxytryptamine; Ach, acetylcholine; BM, bombesin/GRP; NT, neurotensin; SOM, somatostatin; EC, endocrine cells; for other abbreviations see fig. 1

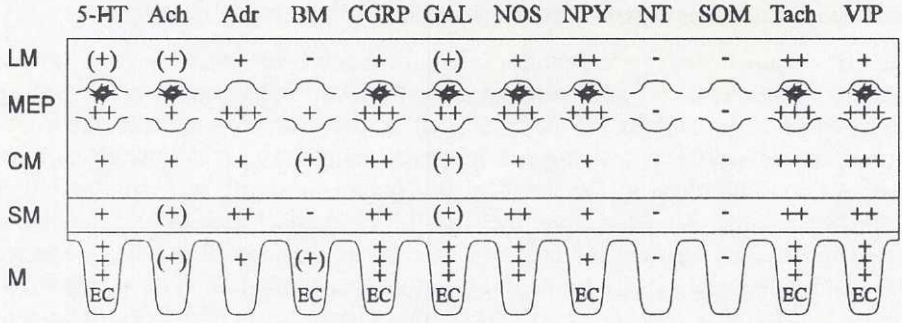

Fig. 3. Innervation of the cod intestine.

The distribution of immunoreactive neuronal cell bodies in the myenteric plexus and nerve fibres in the different layers of the cod intestine. The figure is based on immunohistochemical results in papers VI, VII, and on own unpublished results. Where immunoreactive neuronal cell bodies have been found, the nerve fibres are probably of intrinsic origin. For abbreviations see Fig. 1 and 2

Distribution of neurones intrinsic to the cod intestine

Previous studies have demonstrated the presence of dense nets nerve of fibres, for example showing NPY, tachykinin and VIP immunoreactivities, in the cod intestine (Jensen and Holmgren 1985; Bjenning and Holmgren 1988; Olsson and Holmgren 1994). However, neuronal cell bodies have been seen only occasionally with antisera against tachykinins, VIP or other peptides. With the immunohistochemical protocol used in [III] as discussed above, I have re-evaluated the distribution of neurones intrinsic to the cod intestine. Fig. 3 summarises the results on distribution of neuronal cell bodies and fibres in the different intestinal layers. I will discuss the functional implications of some of the findings in relation to projections of enteric neurones in the next section.

NOS neurones

Neuronal cell bodies containing the enzyme producing nitric oxide, NOS, have been demonstrated both histochemically and immunohistochemically in the cod intestine as well as in the stomach [see above; III; VI]. As in the stomach, a large proportion of the total neuronal population [approximately 40% of the NADH-d positive neurones; III] in the intestine is NOS positive. This proportion varies amongst the investigated species: in the rainbow trout and mammalian intestinal myenteric plexus, 10% to 20% of the total cell population is NOS-IR (Nichols *et al.* 1992; Li and Furness 1993; Costa *et al.* 1996). In the toad, the NOS positive cells represent an even larger part of the NADH-d positive population (approximately 70%; Li *et al.* 1992) than in the cod intestine. Using a neuronal cell body antiserum, it has been shown that NADH-d histochemistry can visualise about 80% of the total cell population in the guinea-pig small intestine (Young *et al.* 1993). The number of NADH-d positive neurones per cm^2 in the cod intestine is comparable to that of small laboratory mammals (Gabella 1987), and although this may represent only 80% of the total neuronal population, the nitrergic neurones of the cod intestine are likely to constitute a large, and thus important, population in the cod gastrointestinal canal.

Galanin and VIP neurones

Galanin immunopositive neuronal cell bodies and fibres were found in the intestinal myenteric plexus [VI] and we could thus deduce that, at least part of the galanin-IR nerve fibres detected in [I] are intrinsic to the intestine. VIP immunoreactivity was also seen in neuronal cell bodies in the myenteric plexus of the intestine, in addition to the presence in nerve fibres [VI]. Coexistence of galanin/VIP seems to be common in enteric neurones of many fish species as in mammals (own unpublished results; see Furness *et al.* 1992) but in the cod, no coexistence of galanin and VIP immunoreactivity was detected in any layer of the intestine. In contrast, galanin and VIP coexist in perivascular nerve fibres of the cod (M. Johnsson pers. comm.). As reported with the NADPH-d method [III] and with a NOS antiserum [VI], a proportion of the neuronal cell bodies and fibres exhibited coexisting NOS/VIP immunoreactivities in the intestine as in the stomach (see above). From the relative percentages of the three NOS and VIP populations [namely NOS/-, NOS/VIP and VIP/-; see III], it can be deduced that VIP neurones are approximately as frequent in the cod gastrointestinal canal as the NOS neurones.

Tachykininergic, cholinergic and serotonergic neurones

Tachykinin-IR neuronal cell bodies and fibres were abundant in the cod intestine [VII] and we can thus, as with galanin, deduce that the previously reported populations of fibres IR to tachykinins (Jensen and Holmgren 1985), at least in part, are intrinsic to the intestine. Further, immunoreactivities to two specific markers for cholinergic neurones, choline acetyltransferase (ChAT; the enzyme synthesising acetylcholine in the neuronal cytoplasm) and vesicular acetylcholine transporter (VAchT) were found in neuronal cell bodies. VAchT is located specifically in small synaptic vesicles of cholinergic neurones and is responsible for packaging of acetylcholine into the synaptic vesicles (Gilmor *et al.* 1996; Weihe *et al.* 1996). Thus, for the first time, the distribution of cholinergic neuronal cell bodies has been described in the non-mammalian autonomic nervous system.

Although the relatively weak staining with the cholinergic markers did not allow quantifications, a portion of the cholinergic neurones showed coexisting 5-HT or tachykinin immunoreactivities [VII]. In the guinea-pig intestine, approximately 85% of the neuronal cell bodies in the myenteric plexus are cholinergic; 90% of these in addition contain SP (Costa *et al.* 1996). This, together with the abundant presence of tachykininergic neurones in the cod intestine makes it likely that we have identified a major enteric neuronal population in the cod intestine.

In paper VII, the possible coexistence of tachykinins and NOS/VIP was examined and it was found that, as in the toad and mammalian gastrointestinal canal, tachykinin immunoreactivity is found in enteric neurones separate from the NOS- or VIP-IR neurones (Wattchow *et al.* 1988; Costa *et al.* 1992b; Li *et al.* 1993; Schemann *et al.* 1995; Costa *et al.* 1996). This indicates that the tachykinin and NOS/VIP immunoreactivities can be used as markers for separate neuronal populations in the intestine of a wide range of vertebrates.

CGRP in enteric neurones

CGRP, a peptide often found in extrinsic sensory neurones (see above) and in intestinal enteric neurones of other vertebrate species, has also been found in the cod intestine. Frequently occurring neuronal cell bodies and densely packed fibres IR to CGRP are present in the myenteric plexus (F. Shahbazi, P. Karila, J. Jensen and S. Holmgren unpublished). This is in contrast to the moderate (and probably extrinsic) nerve fibre distribution in the cod stomach (see above, Fig. 2). The difference in neuronal density between the gut regions agrees with a radioimmunoassay (RIA) study of the concentrations of CGRP-IR material in the cod gastrointestinal canal (J. Jensen pers. comm.). Also in other animals there are concentration differences between stomach and intestine. In the rainbow trout, for example, concentrations of CGRP immunoreactivity measured by radioimmunoassay are higher in the intestine than in the stomach (Fouchereau-Peron *et al.* 1990). CGRP/tachykinin colocalisation (which is a common marker for primary sensory neurones in mammals; *e.g.* Wiesenfeld-Hallin *et al.* 1984; Gibbins *et al.* 1985) was not detected in the cod intestine, except for in a few nerve fibres in the circular muscle layer. This, in addition to the distinct distributions of the immunoreactivities to tachykinins and CGRP, indicates that CGRP and tachykinins are present in separate enteric populations.

The distribution of NPY immunoreactivity in the stomach and intestine is unique since the immunoreactivity is more abundant in nerve fibres of the longitudinal muscle layer than in the circular muscle layer. Immunoreactivity to the catecholamine synthesising enzyme TH and to peptides in the bombesin/GRP family did not reveal any neuronal cell bodies in the cod intestine, although TH-IR nerve fibres frequently occur (own unpublished results). These fibres are probably, as the adrenergic fibres in the stomach [IV], of spinal autonomic origin. The distribution of bombesin immunoreactivity in the intestine differs from the stomach where both neuronal cell bodies and fibres are present (own unpublished results). The few fibres in the intestine are of unknown origin.

Lack of immunoreactivity

The negative results with antisera against neurotensin (NT) and somatostatin in the cod gastrointestinal canal may indicate too large species differences between the native cod peptides and the mammalian forms (that the antibodies are directed against) to allow recognition of the fish peptides with the antisera used. However, since immunoreactivities to the two peptides (using the same antisera) are found in the autonomic nervous system of other non-mammalian vertebrates (Holmgren *et al.* 1994; Karila *et al.* 1995a), a "true" absence of the putative transmitters from the cod gastrointestinal canal is also possible. In an early study on the peptidergic innervation of the gastrointestinal canal in different teleost fish species, half of the species investigated had neurotensin-IR gastrointestinal nerve fibres whereas the other half lacked these fibres (Langer *et al.* 1979). This also indicates that there might be true species differences in the innervation pattern among the teleosts.

Summary

The overall distribution pattern of neuronal cell bodies and fibres in the intestine is comparable to the pattern in the cod stomach (see figs. 2, 3). There are only slight variations except for 5-HT-IR and CGRP-IR nerve fibres which are more abundant in the intestine and bombesin/GRP-IR nerve fibres which are more abundant in the stomach. Correspondingly, 5-HT and CGRP containing neuronal cell bodies have not been found in the stomach and bombesin/GRP containing neuronal cell bodies have not been found in the intestine. The origin of these nerve fibre populations remains to be elucidated.

Projections of neurones intrinsic to the cod intestine

The presence of polarised enteric reflexes has not been previously established in fish. The aim of this part of the thesis study was therefore dual: first to investigate the presence of descending inhibitory and ascending excitatory reflexes in the fish intestine, and second, to see if the anatomical arrangement of nerves in the cod gastrointestinal canal could support such a reflex.

Methods to assess enteric projections

√ A method frequently used in mammals to investigate the mechanisms underlying the peristaltic reflex is an *in vitro* preparation of the intestine where the stimulus sites (for eliciting the reflexes) and recording sites (for circular muscle responses) are separated and drugs can be administered independently to the different compartments (*e.g.* Costa and Furness 1976; Tonini and Costa 1990; Grider and Jin 1994). Local distension of the gut by a balloon (as by the passing of foodstuff) triggers a polarised enteric reflex. The reflex to the circular muscle of the intestine consists of an ascending excitatory reflex and a descending inhibitory reflex (see Furness and Costa 1987). In the cod intestine, however, we were not able to elicit enteric reflexes in either direction by distending the muscle wall with a balloon [V]. The inability to elicit peristaltic reflexes by distending the cod intestine may suggest that distension is not the physiological stimulus triggering peristalsis, and that other types of receptors than stretch receptors form the sensory link of the reflex. Instead, the stimulus may be mechanical irritation of the villi or it may be chemical in nature, since the chyme does not form pellets in the cod intestine, and as a result, no local distension of the intestinal wall is likely to occur in such a way that stretch receptors are triggered.

Intramural electrical stimulation, on the other hand, caused a contraction oral to the stimulation site (in the proximal section of the bath) and a more pronounced relaxation anal to the stimulation site [in the distal section of the bath; V]. TTX added to the intermediate compartment (the site for the electrical stimulation) blocked or reduced the response to electrical stimulation in both directions indicating selective local stimulation of nerves with the method used. Although the reflex could not be triggered by distension in the cod preparation *in vivo*, the results points to the presence of a basic, polarised peristaltic reflex also in the teleost intestine. Indeed, an ascending excitatory reflex could be elicited by distending the intestine in a recent experiment on another teleost fish, the rainbow trout (D. Petersson, P. Karila and S. Holmgren unpublished). The differing results may perhaps be correlated to differences in the consistency of the chyme in the two species. These in turn may be due to different habitats (saltwater - freshwater) or reflect differences in the preferred food between the two species.

√ The second goal was to histochemically analyse which direction along the gastrointestinal canal neurones containing certain neurotransmitters project. This can be done by severing the axons in the myenteric nerve plexus with microsurgical nerve lesions (myotomy operations). Using this method, many nerve pathways in the mammalian gut have been delineated due to degeneration and hence the disappearance of IR material in the cut nerve endings (*e.g.* Furness and Costa 1979, 1982; Ekblad *et al.* 1987, 1988; see also Furness *et al.* 1992). The disappearance occurs preferentially on the distal side of the myotomy operation (where the axons are no longer in contact with the neuronal soma) and thus conclusions can be drawn on the direction along the gastrointestinal canal nerves containing a certain transmitter project. Together with the known actions of the substance, this gives clues to the functions of the nerve type containing the substance.

In the cod, however, we found that the peptide-IR material on the distal side of the myotomy (where the axons are isolated from their cell bodies) was reduced for a very short distance only [II, VI, VII]. In mammals the reduction in immunoreactivity extends from several millimetres up to centimetres (*e.g.* Ekblad *et al.* 1985b, 1987; Messenger and Furness 1990; see also Furness and Costa 1987) and we concluded that the short distance of reduced immunoreactivity in the cod gastrointestinal canal cannot correspond to the length of the neurones. Instead, accumulations of IR material were readily observed on the proximal side of the myotomy (*i.e.* the side where the axons are still connected to the cell bodies).

Several studies have shown that the degradation of the cut axons after axotomy has an entirely different time course in invertebrates and poikilotherm vertebrates compared to mammals. In the central nervous system of the goldfish, cut Mauthner axons in the spinal cord are reported to stay intact for several months *in vivo* as opposed to mammalian axons which typically degenerate after a few days (see Furness and Costa 1987; Moehlenbruck *et al.* 1994). The long survival time was accompanied by an intact composition of neurofilaments (Moehlenbruck *et al.* 1994) and is proposed to be due to low activity of the calcium-dependent proteolytic enzyme calpain (Raabe *et al.* 1995). Also in the amphibian enteric nervous system, degeneration of cut axons has a similar time course as in our studies on the cod gut (Murphy *et al.* 1993; Torihashi and Kobayashi 1993). Indeed, both fish and amphibian brains have lower calpain activity than other vertebrates (see Raabe *et al.* 1995) which could be a factor that enables the long-term survival in fish neurones.

√ In paper III we traced the NADPH-d positive axons manually, finding that the largest population of the intestinal nitrergic neurones projected anally although there were also populations of neurones projecting orally or along the circumference of the intestine. However, the method has a limitation since it is only possible to trace axons when the density of nerve fibres is low, as it is in the cod gastrointestinal canal using the NADPH-d method.

√ The myotomies applied in papers II, VI and VII give limited information since they only indicate the dominating oro-anal polarity of the neurones, without any information on length of the projections or location of the cell bodies. Neurones projecting along the gut circumference are also overlooked. In mammals, retrograde tracing and intracellular dye injection are methods that have been successfully used to elucidate projections of enteric neurones (*e.g.* Song *et al.* 1991; Schemann and Schaaf 1995). These methods have the advantage that individual neurones can be exactly traced from terminal fields to neuronal cell bodies or vice versa. In the cod, injection of retrograde tracers does not give any repeatable results in the gastrointestinal canal (*in vitro* or *in vivo*; own unpublished results), although extrinsic pathways to the stomach have been successfully revealed using similar methods [IV].

In the following sections, I have attempted to use the pharmacological results in combination with the immunohistochemical results from myotomy operated animals to delineate the neuronal populations responsible for the polarised enteric reflex to the circular muscle layer in the cod intestine.

Descending projections in the cod intestine

In two studies using myotomy operations in the cod intestine [VI, VII], we concentrated on the accumulations on the proximal side of a myotomy and could thus outline the projections of some immunohistochemically distinct neuronal populations in the intestine. In order to quantify the dominating direction of projection along the intestine for various neuronal populations, a method using the confocal laser scanning microscope was developed: equal regions anal and oral to a myotomy operation were analysed and the area that was covered with IR material was measured.

Galanin and VIP in anally projecting neurones

It was found that galanin and VIP immunoreactivities accumulated on the oral side of the myotomy, indicating an oral to anal projection of neurones containing these peptides [VI]. A reduction in immunoreactivity in the myenteric plexus and muscle layers was also observed anal to the cut, but only for a very short distance [see fig. 2 D in VI; *c.f.* II]. VIP is found in descending neurones in all species so far investigated (Furness and Costa 1979; Ekblad *et al.* 1987; Messenger and Furness 1990; Murphy *et al.* 1993; Barbiers *et al.* 1995). VIP has a relaxing effect on the smooth muscle of the gut in many mammals (Li and Rand 1990; Bojö *et al.* 1993; Maggi and Giuliani 1993). Due to the inhibitory effects and the consistent anal projections of VIP neurones, VIP is regarded a general transmitter of the descending inhibitory reflex of the gut (see Furness *et al.* 1992; 1995; Costa *et al.* 1996; see fig. 4).

In the cod intestine, VIP has only a weak depressant effect on the spontaneous activity in *in vitro* preparations of the circular muscle, whereas the related peptide PACAP abolishes the spontaneous activity (C. Olsson pers. comm.). Since VIP does not seem to mediate relaxation of smooth muscle in the fish gastrointestinal canal its role is perhaps modulatory. Varicose VIP-IR nerve fibres also surround many enteric neurones in the cod gut, indicating a presence of VIP in sensory neurones or interneurones of the myenteric plexus [III; see fig. 4].

Galanin has also been found in descending nerve pathways in mammalian and toad intestine (Ekblad *et al.* 1985b, 1988; Messenger and Furness 1990; Murphy *et al.* 1993; see also Furness *et al.* 1992). It often coexists with VIP and NOS, although its physiological role in the descending inhibitory reflex is not obvious; galanin is therefore considered to be a secondary transmitter in this system (see Furness *et al.* 1992). Rather than having direct effects on the smooth muscle, galanin acts presynaptically to suppress synaptic transmission as well as postsynaptically to inhibit excitability of neurones (see Rattan 1991; Wood 1994).

In the cod intestine, as in the stomach, the excitatory action of galanin is a weak, direct effect on the smooth muscle [I]. In the rainbow trout, native trout galanin has no detectable effect on intestinal smooth muscle (J. Jensen pers. comm.). The weak effects in the fish gut, together with the sparse distribution of galanin-IR nerve fibres in the muscle layers [VI], suggests a secondary role for galanin in the control of fish gut motility. The distribution of galanin immunoreactivity in the cod gastrointestinal canal, with nerve fibres surrounding mucosal glands [see I], perhaps indicates a more important role for

galanin in mucosal events than in gut motility. Indeed, in the teleost fish tilapia (*Oreochromis mossambicus*), galanin is involved in absorption over the intestinal epithelium (Kiliaan *et al.* 1993).

Nitric oxide in anally projecting motor neurones

In contrast to the lack of a notable degeneration of neuropeptide-IR neurones in fish, there was a reduction in the area covered with NOS immunoreactivity in the cod intestine on the anal side of a myotomy [VI]. This may be due to different time courses for breakdown of peptides (galanin, VIP) and for the enzyme (NOS). The degeneration on the anal side indicates an oral to anal projection of neurones expressing NOS immunoreactivity. NOS is, as VIP, found in descending neurones in all species investigated (Costa *et al.* 1992b; Murphy *et al.* 1993; McConalogue and Furness 1993; Timmermans *et al.* 1994; Barbiers *et al.* 1995; Schemann and Schaaf 1995).

The anal projections of nitrergic neurones in the cod intestine agrees with the results using the partitioned bath to record peristaltic reflexes in the cod intestine [V]: the nitric oxide synthesis inhibitor L-N^G-nitro-arginine methyl ester (L-NAME) abolished the descending inhibitory reflex and the nitric oxide donor sodium nitroprusside relaxed the intestinal circular muscle [V; see fig. 4]. Nitric oxide also relaxes gut smooth muscle in mammals (Li and Rand 1990; Shuttleworth *et al.* 1991; Calignano *et al.* 1992; see also Lefebvre 1995) and is considered to be a general transmitter of the descending inhibitory reflex of the gut (see Furness *et al.* 1992; 1995; see fig. 4).

In the cod intestine, L-NAME also potentiated the ascending contractions elicited by electrical stimulation and increased the frequency and/or amplitude of the spontaneously occurring contractions. This indicates the presence of an inhibitory nitrergic tone on the circular muscle of the intestine. In mammals, an inhibitory neural tone is always present to the circular muscle layer during resting conditions (see Makhlouf 1994; Wood 1994). This may be the case also in the cod intestine, where this tone is suggested to be nitrergic. However, the spontaneously occurring contractions in the cod intestine are often abolished or reduced by TTX [Jensen and Holmgren 1985; V], indicating a predominating excitatory neural tone in the cod intestine.

Summary

Although the functional roles for galanin and VIP in fish intestinal motility are not as clear as for nitric oxide, the consistent anal projections of galanin-, VIP- and NOS-IR neurones in all vertebrates investigated implicates that the oral to anal projections of neurones containing VIP, NOS and galanin are evolutionary highly conserved features and thus probably important for the descending inhibitory reflex. In fig. 4, I have indicated the most probable locations of the nitrergic and galanin- and VIP-containing neurones in the descending inhibitory reflex.

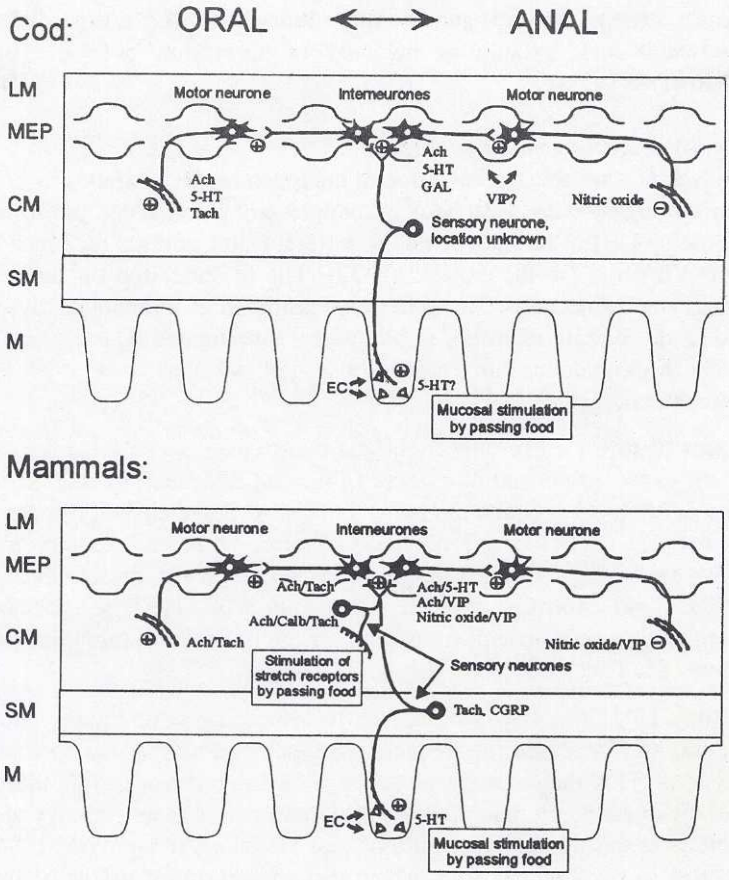

Fig. 4. (see also back cover) The basic circuit for the peristaltic reflex.

A comparison between the neuronal circuitry in the mammalian and cod intestine. The cod model is based on papers [V-VII] where either pharmacological studies on peristaltic reflexes or immunohistochemical methods were used, and on pharmacological studies on peristaltic reflexes in the rainbow trout (D. Petersson, P. Karila and S. Holmgren unpublished). The mammalian model is based on several different studies, especially in the guinea-pig intestine (see Bornstein 1994; Gershon *et al.* 1994; Wood 1994; Goyal and Hirano 1996; Costa *et al.* 1996; Grider *et al.* 1996).

In mammals, the enteric reflexes are triggered by either mucosal stimulation caused by passing food (mechanical or chemical irritation of the villi) or by distension of the muscle layers caused by passing food. The mucosal stimulation causes release of 5-HT from endocrine cells. 5-HT acts on nerve endings of sensory neurones (with neuronal cell bodies located in the submucous plexus). If the reflex is elicited by distension, sensory neurones (with neuronal cell bodies located in either the myenteric plexus or in the dorsal root ganglia) with stretch receptors are excited. The sensory neurones release their transmitter(s) and stimulate interneurones of both the ascending excitatory reflex and the descending inhibitory reflex. The interneurones in turn stimulate the motor neurones.

In the cod, the enteric reflexes are possibly triggered by mucosal stimulation caused by passing food (mechanical or chemical irritation of the villi). The sensory neurones and the interneurones have not been identified. The anally projecting motor neurones use nitric oxide, whereas the orally projecting motor neurones use acetylcholine and 5-HT as transmitters. The most likely locations in the reflexes of the anally projecting cholinergic, serotonergic, VIP- and galanin-containing neurones and the orally projecting tachykininergic neurones are indicated in the figure.

Together, the neurones represent a basic circuit that gives rise to the polarised reflexes with an oral contraction and an anal relaxation of the circular muscle layer. The basic circuit for peristalsis depicted is repeated serially along the intestine. Calb, Calbindin containing neurones; for other abbreviations see Fig. 1 and 2

Ascending projections in the cod intestine

Acetylcholine and the tachykinins SP and NKA are well established as neurotransmitters in excitatory motor neurones in the mammalian gut (*e.g.* Barthó and Holzer 1985; Grider 1989; Tonini and Costa 1990; Allescher *et al.* 1992; Holzer and Maggi 1994; see also Holzer-Petsche 1995) and are often colocalised in enteric neurones (*e.g.* Brookes *et al.* 1991; Schemann *et al.* 1995; see also McConalogue and Furness 1994; Costa *et al.* 1996). The combination of tachykinins and acetylcholine is also found in orally projecting interneurones in the guinea-pig intestine (see Costa *et al.* 1996; see fig. 4).

Orally projecting cholinergic and serotonergic motor neurones

The aim of this part of the study was to elucidate the mechanisms underlying the ascending excitatory reflex of peristalsis demonstrated in the teleost fish intestine [V; see above]. In the *in vitro* preparation, electrical stimulation was used to elicit polarised enteric reflexes. The effects of the muscarinic acetylcholine antagonist atropine and the serotonergic antagonist methysergide on these reflexes were investigated. The cholinergic and serotonergic antagonists were chosen since the effects of both acetylcholine and 5-HT are excitatory in the cod intestine (Jensen and Holmgren 1985). In the experiments on the vascularly perfused cod intestine, where the combined activity of longitudinal and circular muscle was measured, the serotonergic excitation could partly be antagonised by TTX. This indicates a participation of 5-HT in interneurones to excitatory motor neurones as well as in excitatory motor neurones (Jensen and Holmgren 1985). In paper V, we could not show an effect on the ascending excitatory reflex when the antagonists were added to the section of the bath where the electrical stimulation took place. On the other hand, both antagonists had inhibitory effects on the ascending excitatory reflex when added to the oral section of the bath (at the site of registration of the circular muscle contraction). This indicates a participation of cholinergic and serotonergic neurones in the ascending excitatory reflex of the cod intestine. The site of action of the antagonists suggests that the muscarinic and serotonergic receptors are located on the smooth muscle rather than on neurones in the ascending excitatory reflex (see fig. 4).

Anally projecting cholinergic and serotonergic interneurones?

To confirm the involvement of acetylcholine and 5-HT in the ascending excitatory reflex of the intestine histologically, we used antibodies against 5-HT and the cholinergic marker ChAT on sections from myotomy operated cod [VII]. Individual sections showed accumulation of 5-HT- and ChAT-IR material on either sides of the myotomy or only at the oral or anal side. Consequently, there was no difference in the average areas covered with 5-HT-IR or ChAT-IR material between the two sides [VII]. Although the pharmacological experiments showed that both 5-HT and acetylcholine are involved in the ascending excitatory reflex and presumably present in orally projecting neurones [V], the inconsistent accumulations of 5-HT and ChAT immunoreactivities on the two sides of the myotomy suggest that 5-HT and acetylcholine are present both in orally and anally projecting neurones. Since acetylcholine and 5-HT have excitatory effects on intestinal circular muscle [VII] but the effect on the smooth muscle in the descending reflex is inhibitory, the anally projecting cholinergic and serotonergic neurones are probably interneurones rather than effector neurones to the smooth muscle. There is also a possibility that these neurones are anally projecting motor neurones to the longitudinal

muscle layer. In the guinea-pig myenteric plexus, cholinergic neurones project mainly orally, but acetylcholine is also commonly present in other types of enteric neurones, including descending interneurones (Brookes *et al.* 1991; Steele *et al.* 1991; see also McConalogue and Furness 1994; Costa *et al.* 1996; see fig. 4), which might also be the case in the cod intestine. 5-HT is colocalised with SP or VIP in the guinea-pig stomach (Schemann *et al.* 1995), and thus probably present both in anally and orally directed interneurones, although 5-HT neurones of the mammalian intestine in general project anally. The anally projecting neurones are suggested to be interneurones of the descending inhibitory reflex (Furness and Costa 1982; Wardell *et al.* 1994; Barbiers *et al.* 1995; see also Costa *et al.* 1992a; Costa *et al.* 1996). In fig. 4, the most likely locations of the serotonergic and cholinergic neurones in the ascending and descending reflexes are indicated.

It would be very interesting to apply neuropeptide antagonists to the partitioned bath preparation and thereby find out if for instance the tachykinins have a role in the ascending excitatory reflex of the fish intestine. The neuropeptide antagonists available so far have, however, no effects (or unspecific actions) in non-mammalian systems; recently, it was shown that the non-peptide NK_1 receptor antagonist CP-96,345 was without effect in the rainbow trout circulation (Kågström *et al.* 1996). The lack of effect of neuropeptide receptor antagonists in non-mammalian species may either be due to lack of this particular receptor type or changes in the binding site for the antagonist, whereas the binding site for the peptide is conserved (see Kågström *et al.* 1996).

Tachykinins in orally projecting neurones

To verify the presumed excitatory effects of native tachykinins (cod NKA and cod SP; see Jensen and Conlon 1992b) in the cod intestine, cod NKA and cod SP were applied to *in vitro* ring preparations of the cod intestine. The native cod tachykinins had potent excitatory effects [VII]. This indicates a role as primary transmitter for the tachykinins in the control of gut motility in the cod intestine. In accordance, massive accumulations of tachykinin-IR material were found on the anal side of the myotomy operation indicating an oral projection of neurones containing these substances [VII; see fig. 4]. The results from the physiological and immunohistochemical experiments agree with the situation in mammals where tachykinins clearly are involved in the ascending excitatory reflex (*e.g.* Barthó and Holzer 1985; Grider 1989; see also Holzer-Petsche 1995; Costa *et al.* 1996). The main projections, as revealed by nerve lesions, of SP-IR neurones in the mammalian gut are not exclusively oral (*e.g.* Ekblad *et al.* 1987; Messenger and Furness 1990). However, SP seems to be present in all orally projecting interneurones in addition to its presence in nearly all excitatory motor neurones to circular and longitudinal muscle in the guinea-pig small intestine (Brookes *et al.* 1991; Costa *et al.* 1996).

Summary

On the basis of the presence of a polarised reflex in the cod intestine, seemingly employing neurones with different transmitters, I conclude that there is a synaptic circuitry in the enteric nervous system of teleost fish that resembles the basic mammalian circuitry (see fig. 4). Thus, the circuit for peristalsis appears to have arisen early in the evolutionary process and to have been conserved as a reflex over the range of vertebrates from teleost fish to mammals. Based on the above sections on the distribution and projections of enteric neurones, Fig. 4 summarises the possible arrangement of the cod peristaltic reflex.

General conclusions

√ The density of neuronal cell bodies in the cod gastrointestinal canal is comparable to that found in small mammals.

√ Galanin, tachykinins, nitric oxide and VIP are present along the vagus nerves in postganglionic, presumably cranial autonomic, neurones to the gut. The galanin containing neurones are innervated by preganglionic (presumably also cranial autonomic) tachykininergic neurones.

√ Different populations of chemically coded sympathetic neurones (adrenergic neurones and adrenergic neurones that in addition contain NPY) innervate different target tissues in the gut. This arrangement shows some similarities to the situation in other investigated vertebrates although differences are present; NOS in adrenergic, postganglionic sympathetic neurones has not been reported from other vertebrates.

√ In the sensory nodose ganglion, neurones projecting to the stomach are numerous. The main transmitter in this population does not seem to be CGRP, since few neurones retrogradely labelled from the stomach contain this neuropeptide.

√ Nitric oxide is an important transmitter in the fish intestine, produced in neurones with anal projections and probably released from motor neurones in the descending inhibitory reflex. Myotomy studies indicate that also galanin and VIP are involved in descending pathways in the intestine. This is consistent with result from other vertebrates studied.

√ Acetylcholine, 5-HT and tachykinins are present in motor neurones of the ascending excitatory reflex. Acetylcholine and tachykinins seem to be present in the ascending excitatory reflex in all animals studied so far.

Taken together, these results show that an advanced teleost, the Atlantic cod, shares many of the complex features of the autonomic nervous system of "higher vertebrates". It argues against the common opinion that the "lower vertebrates" have less developed organ systems; many similarities exist in the distribution and projection of neurones in and to the gut. Of course, there are also obvious differences which may be attributed to adaptations to different habitats or remnants from an ancient lineage of animals. I suggest that the simplistic view is almost entirely due to the previous lack of research in these animals in contrast to the extensive efforts that have been put down on research on the mammalian autonomic nervous system.

Acknowledgements

I wish to thank the following persons:

Anna, my woman, for making this worthwhile,
Susanne Holmgren, my supervisor, for her time, criticism and support,
Stefan Nilsson, head of the department, for the regular discussions on biology,

Michael Axelsson for invaluable advise on statistics and computerised data sampling,
Barbro Egnér for expert help with radioimmunoassay,
Christina Hagström and
Inger Holmquist for skilful technical assistance in histochemistry and photography,
Colin Johnston for initiating me to the confocal technique,
Gunilla Rydgren for expert technical assistance with perfusion experiments,
Bengt Svensson for the skilful assembly of the partitioned bath,
Birgitta Vallander for expert assistance in photography,
Ann Wikström for expert technical assistance with muscle strip experiments.

I would also like to acknowledge:
The co-authors in the thesis papers and elsewhere for their inspiration and
Ulrika Aronsson,
Peter Bomgren,
Susanne Holmgren,
Jörgen Jensen,
Marianne Johnsson,
Jens Kågström,
Catharina Olsson and
Fatemeh Shahbazi for reading and commenting on this manuscript,
the rest of the comparative neuroscience groups at the 4th floor,
the staff/students at the department of Zoophysiology and
the staff at the Zoological Institute for making it all possible.

The study was funded by grants from:
The Adlerbertska Research Foundation and The Adlerbertska Foundation,
The Ahlstrand Foundation,
The Anna Ahrenberg Foundation,
The Helge Ax:son Johnson Foundation,
The Paul and Marie Berghaus Foundation,
The Lars Hierta Foundation,
The Hierta-Retzius Foundation,
The Carl Jönsson Foundation II,
The Kungliga and Hvitfeldtska Foundation,
The Wilhelm and Martina Lundgren Research Foundation,
The Viktor Rydberg Foundation,
The Swedish Natural Science Research Council and
The Hjalmar Théel Foundation.

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