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Selectivity in sympathetic innervation during development and regeneration in the rat

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Much attention has been directed to the description and investigation of the specificity of neurones of the central nervous system. During regeneration, retinal neurones form synapses with tectal neurones in an organized fashion under a variety of experimental conditions presumably via some form of chemospecificity[18]. Similarly, early specification of motoneurones is suggested from experiments in which these cells can find their appropriate muscle targets even after manipulations of the spinal cord or of the limbs themselves[28].

By comparison, much less in known about specificity within the autonomic nervous system. As early as the 1890's, Langley described an apparent anterior-posterior matching of preganglionic spinal efferent fibers with the position of postganglionic sympathetic targets in the head [29-31]. Stimulation of efferent fibers in anterior spinal roots elicited responses in rostrally situated sympathetic target tissues. The cellular basis for these observations has been investigated by Nja and Purves[38] using more sophisticated physiological techniques. The preganglionic input for any one sympathetic neurone in the superior cervical ganglion of the guinea pig comprises at least 10 fibers which emerge from the spinal cord in a contiguous set of spinal roots, one of which represents the dominant input. The anterior-posterior position of this set of preganglionic fibers is then correlated with the rostrocaudal position of the postganglionic sympathetic target tissue[38]. Thus, sympathetic neurones appear to be or to become specified by either their preganglionic input to contact only particular target tissues, or alternatively by connection with their target tissues to receive only particular preganglionic inputs. Sympathetic neurones of the superior cervical ganglion lose their preganglionic synapses after axotomy[36,44] and do not regain them if the postganglionic axons are prevented from reinnervating their targets in the periphery[44], suggesting that contact with peripheral targets is essential for the maintenance of appropriate preganglionic connections.

Following section of the preganglionic nerve trunk to the superior cervical ganglion, reinnervation of ganglion cells is selective although the actual number of preganglionic synapses may vary from normal[39,40]. From the earliest stages of reinnervation of the ganglion, sympathetic neurones innervating rostrally placed target tissues are contacted by preganglionic fibers emerging in anteriorly situated spinal roots[39,40]. Furthermore, thoracic and lumbar ganglia transplanted to the neck are reinnervated more frequently by axons arising from more caudal thoracic segments than are neurones of transplanted superior cervical ganglia[47]. These results suggest that the postganglionic neurones have become specified by their peripheral connection to receive only a particular subset of inputs. Surprisingly, however, reinnervation of peripheral targets following crushing of the major postganglionic branches of the superior cervical ganglion did not result in restablishment of selective end-organ effects[31,46]. When the preganglionic nerve trunk was cut at the same time as the postganglionic trunks, the synapses formed within the ganglion were selective in that the fibers to any one ganglion cell were derived from contiguous spinal roots with the contribution from one spinal root being dominant[46]. Thus, while the reinnervation of the ganglion by the preganglionic fibers appeared to be specific, that of the peripheral target organs appeared to be nonspecific. The authors suggest that this difference may reflect differences in the ease with which the respective fibers can reach their targets. They stress, however, that their parameters for selectivity of ganglion cell innervation by preganglionic fibers may be an intrinsic factor relating to the neurone itself and have nothing to do with specifications transmitted from the periphery[46].

With the advent of the fluorescence histochemical technique for the demonstration of catecholamines[14], much energy was devoted to the description of the patterns of sympathetic innervation within peripheral target tissues in a variety of animals. From these studies it emerged that there was an enormous variation in the density of the terminal plexus from tissue to tissue. Moreover, even within any one type of tissue there was variation. For example, some blood vessels, such as the rabbit thoracic aorta, had a relatively sparse network of adrenergic fibers while others, such as the rabbit ear artery, had a dense plexus of fibers[1]. Cardiac muscle, too, could be divided into the densely innervated atria and the sparsely innervated ventricles. But what distinguished one area from the other as far as the sympathetic nerve fibers were concerned?

The direction for the pattern of innervation seemed to reside within the target tissue rather than as a property of the sympathetic neurones. Transplantation studies in which various sympathetic target tissues were placed in the anterior chamber of the eyes of adult rats showed that these tissues were reinnervated functionally by the sympathetic fibers of the host iris in a pattern typical of the transplanted tissue[35,43]. Furthermore, neurones from a variety of ganglia were equally competent at reinnervating the host iris when it was denervated[43]. Specificity dictated by target organs has also been described in tissue culture. The dilator and constrictor muscles of the rat iris are penetrated by adrenergic and cholinergic sympathetic fibers as they are in vivo, regardless of the source of the ganglion cells[23].

The most obvious mechanism for these effects was that target organs released trophic agents which 'attracted' nerve fibers and hence densely innervated tissues attracted more fibers through release of more factor. Indeed, when sympathetic ganglia were placed in culture equidistant from different target tissues, normally

densely innervated tissues elicited a greater nerve fiber outgrowth than did sparsely innervated tissues[3,4,10]. These effects were only seen over short distances under conditions which would permit establishment of stable concentration gradients between the tissues. Initially, variations in the content of the protein nerve growth factor (NGF) were implicated in these phenomena. NGF could be shown to increase the ramification of sympathetic nerve fibers within iris explants in culture[53], and to orient the tips of growing sensory fibers in vitro[5,19,32], and apparently of sympathetic fibers in vivo following NGF injections[37]. In addition, NGF contents of irides and hearts of rats were increased after denervation[11,12,56].

Recently however, an astonishing range of other 'factors' which permit survival of and neurite extension from sympathetic neurones have been described both in extracts of normally innervated target tissues[2,9], denervated tissues[54] and media conditioned by such cells in vitro[7,13,20]. Smooth muscle cells isolated from certain blood vessels and grown in tissue culture have also been shown to release extracellular matrix materials such as collagen, elastin and glycosaminoglycans[42], all of which may assist in providing favorable adhesive pathways into target tissues. Indeed, there appears to be some specificity in the glycoprotein complexes released by smooth and skeletal muscle cell lines in that the former promotes cell-substrate adhesion of the sympathetically derived cell line, PC12, while the latter inhibits adhesion of these cells[52].

The possibility exists, then, for different target tissues to be identified by a unique combination of macromolecules which may be either matched to a similar set of recognizer molecules on the surface of the neurones or may somehow modify the neurones directly.

Many of the studies mentioned so far have attempted to make comment about events which occur during the development of sympathetic innervation by using systems which in fact describe events occurring during regeneration. To what extent is this valid? If initial contact with target tissues can modify neurones, then reinnervation may be much more structured than development and conclusions drawn from these studies may not be true for the processes occurring during de novo innervation. Alternatively, if sympathetic neurones are indeed specified initially before contact with the periphery, then regenerative capacities may also vary from neurone to neurone. The studies involving transplantation to the anterior eye chamber indicate that, at least for the neurones of the superior cervical ganglion, there is a marked capacity for regeneration and an ability to support additional synapses but how universally true is this for sympathetic neurones?

The sympathetic innervation to branches of the superior mesenteric artery of the rat develops over the first 2–3 postnatal weeks[22]. The intrinsic ganglia of the gastrointestinal tract are supplied by sympathetic noradrenergic fibers[6,17,26,41] whose axons lie in bundles beside these same mesenteric arteries[8]. Our studies have employed physiological and anatomical techniques to study the processes of innervation of these two sympathetic target tissues and compare them with reinnervation following denervation effected by a freezing technique[15].

The mesenteric arteries at birth were essentially devoid of catecholamine containing fibers while the adjacent paravascular nerve bundles were visible until they disappeared into the gut wall[22]. By day 4 postnatal, fluorescent fibers had penetrated the intramural ganglia (Hill, unpublished results). The mesenteric arteries at this stage had weakly fluorescent fiber bundles running alongside them and occasionally branching over their surface. The number of fibers ramifying on the arteries increased over the next few days so that, by day 9, there was a moderately dense plexus[22]. Within the gut, varicose fluorescent fibers were found within both the myenteric and submucous plexuses and also within the circular smooth muscle and between the longitudinal and circular muscle layers (Hill, unpublished results). The pattern was thus identical at postnatal day 9 to that seen in the adult rat[6,17]; although the innervation of the submucous blood vessels was not mature. Further increases in density of the plexus on the mesenteric arteries and in the intensity of the fluorescent reaction occurred with time so that by day 16 it was comparable to that of adult rats[22]. During the period of innervation, the nerve network on the most distal vessels in the mesentery was consistently denser than that on the main mesenteric arteries (fig. 1, solid circles versus open circles respectively[22]).

Intracellular recordings from the smooth muscle cells of the distal mesenteric arterioles during the first three weeks of postnatal development revealed three distinct, sequential postjunctional membrane potential changes in response to a single perivascular stimulus[22]. Between postnatal days 1 to 8, a slow depolarizing potential (s.d.p.) was recorded in the majority of preparations (fig. 2A). In those arteries in which a single stimulus failed to initiate a detectable membrane potential change, brief trains of perivascular stimuli (10–20 pulses at 10–50 Hz) initiated a long latency depolarization usually of small amplitude (1–8 mV). In animals aged 9 days and older, a single stimulus evoked a excitatory junction potential (e.j.p.; fig. 2B) and repetitive stimulation at 20 Hz caused summation of e.j.p.s and initiation of a muscle action potential. Between days 9 and 17, e.j.p.s increased in amplitude from 1–5 mV to 8–15 mV[22]. The time of appearance of each of the three, distinct postjunctional responses described above in the distal mesenteric vessels consistently preceded its appearance in the proximal mesenteric arteries (table).

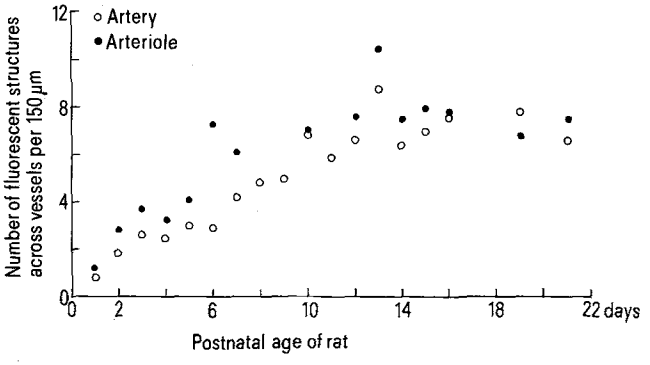

Figure 1. Changes in the density of the adrenergic innervation of the proximal arteries (open circles) and distal arterioles (closed circles) during postnatal development of the rat mesentery. An estimate of the density of the nerve plexus was made by measuring the number of fluorescent fibers crossing the vessel per 150 µm.

During the late prenatal and the early postnatal period, then, sympathetic nerve fibers grew alongside the mesenteric arteries and into the gut wall to innervate the ganglia of the submucous and myenteric plexuses. Subsequent to this initial ingrowth, fibers began to ramify over the surfaces of the mesenteric arteries themselves. These fibers, however, were not closely associated with the arterial smooth muscle cells until day 9[34], when e.j.p.s were first recorded from those cells. At this time, the pattern of sympathetic nerve fibers within the intramural plexuses was similar to that seen in the gut of adult animals although the transverse submucous blood vessels were only sparsely innervated. During the next week, the innervation to the mesenteric arteries and the submucous blood vessels matured. Thus the development of the sympathetic innervation to the neurones of the intramural plexuses preceded that to the mesenteric and submucous blood vessels by several days. This was apparently not due to any physical barrier between the 'gut fibers' and the arteries since the two populations of fibers ran together in the same paravascular nerve bundles.

To study regenerative capacities of these two populations of fibers we chose 3-week-old rats in which the innervation to both the mesenteric arteries and the intrinsic neurones of the ileum appeared to be comparable to those of more mature animals[22] (Hill, unpublished observations). During the first week after the denervation procedure, sympathetic fibers disappeared from the surface of the arteries peripheral to the point of freezing and within the segment of ileum supplied by the denervated artery[21]. At this time, perivascular stimulation proximal to the point of freezing did not evoke any response in the smooth muscle cells distal to the denervation (fig. 3A). A single stimulus near the recording site, however, did evoke an e.j.p. but successive e.j.p.s became reduced in amplitude at stimulation frequencies as low as 0.25 Hz. These results indicated that the denervation technique had been successful but that some nerve terminals persisted for a few days after the operation.

Catecholamine containing nerve fibers reappeared on the artery during the second week but formed only sparse

Developmental changes in post-junctional responses in proximal arteries and distal arterioles of the rat mesentery

Postnatal age of rat (days)	s.d.p. Proximal artery	s.d.p. Distal arteriole	Train response Proximal artery	Train response Distal arteriole	e.j.p. Proximal artery	e.j.p. Distal arteriole
1	0	0	0	0	0	0
2	+	+			0	0
4–5	+	0		+	0	0
	+	0		+	0	0
6–7	+	+		+	0	0
	+	0		+	0	0
	+	0	+	+	0	0
8–9	+	0		+	0	0
	0	0	+	+	0	0
	0	0	+		0	+
10–11	+	0		+	0	0
	0	0	+		0	+
12–13	+	0			0	+
	0	0	+		0	+
	0	0	+		0	+
14–16	+	0			0	+
	0	0			+	+
	0	0			+	+
	0	0			+	+
17–19	0	0			+	+
	0	0			+	+
20–23	0	0			+	+
	0	0			+	+
	0	0			+	+
	0	0			+	+
	0	0			+	+

Each horizontal row represents data from one animal in which intracellular records were made from both a proximal artery and a distal arteriole in response to stimulation of the periarterial nerves. The responses recorded following a single stimulus were either a slow depolarizing potential (s.d.p.) or an excitatory junction potential (e.j.p.) (+ = a response; 0 = no response). If a single stimulus was ineffective in initiating a response, then a train of stimuli was applied. The resulting response was termed a 'train response'.

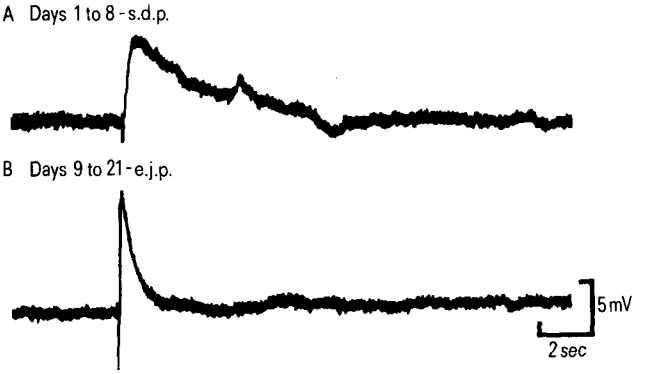

Figure 2. Postjunctional membrane potential changes recorded from distal mesenteric arterioles in response to a single perivascular stimulus in rats of differing postnatal ages. Panel A shows a slow depolarizing potential (s.d.p.) typically recorded in animals aged from 1 to 8 days postnatal. Panel B shows an excitatory junction potential (e.j.p.) recorded in a 20-day-old animal. Such potentials could be recorded from animals aged from 9 to 21 days. Calibration bars apply to both records.

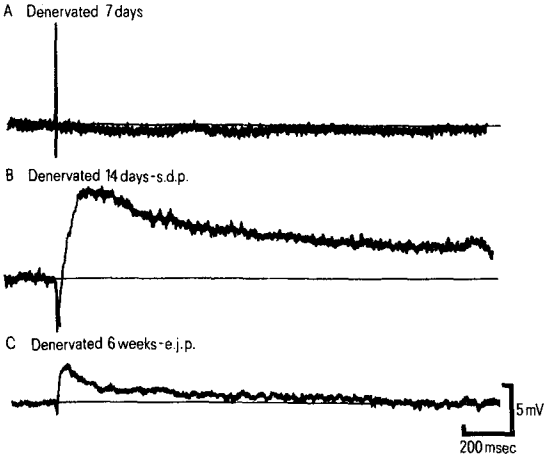

Figure 3. Changes in postjunctional membrane potentials recorded from distal mesenteric arteries at various times after denervation. In A, a single stimulus applied proximal or distal to the point of denervation failed to elicit any response in the smooth muscle cells distal to the denervation indicating that the nerves were no longer continuous through the denervation site. In B, a single stimulus evoked a slow depolarizing potential (s.d.p.) in arteries between 11 and 21 days after the denervation. In C, an excitatory junction potential (e.j.p.) recorded in a rat 6 weeks after the operation. Note that the e.j.p. is markedly smaller than those recorded during development (fig. 2). Calibration bars apply to each record.

plexuses like those seen during development in the first postnatal week. S.d.p.s could be recorded from 2 to 3 weeks post operatively (fig. 3B) while e.j.p.s were recorded in response to a single perivascular stimulus during the third week[21]. These latter responses were of small, variable amplitude (fig. 3C) and fatigued even at low stimulation frequencies of 0.5–1.0 Hz. Unlike e.j.p.s recorded during the first week after the denervation, these responses recovered in amplitude after a few minutes rest. After 6 weeks, such low frequency stimulation produced facilitation of e.j.p.s, while high frequency stimulation (10–20 Hz) caused e.j.p.s to summate without initiating an action potential or a constriction of the artery. Over the next 4 ½ months, there was a slight increase in the range of amplitudes of e.j.p.s, but even 6 months after the operation they were still smaller than those recorded from control vessels[21].

Four months after the denervation, small constrictions were observed with long trains of repetitive stimulation at 10 and 20 Hz. In control vessels, 10 Hz stimulation was sufficient to consistently initiate contractions. Between 2 weeks and 6 months after the operation, there was a progressive increase in the density of the plexus of fluorescent fibers over the surface of the arteries but even by 6 months the network was only about 70% as dense as that over control arteries[21].

Thus there were similarities and differences between the processes of innervation and reinnervation of mesenteric arteries in rats. While a similar sequence of postjunctional membrane potential changes were recorded in the smooth muscle cells in response to periarterial stimulation, the overall time course of the reinnervation was much longer than that of the initial innervation. Even 6 months after the operation, the extent of the reinnervation was not entirely comparable to control either anatomically or functionally. Furthermore, from the earliest time of appearance of e.j.p.s during development, even high frequency stimulation did not produce fatigue of e.j.p.s as it did between 3 and 6 weeks after the denervation. These observations may suggest that the pool of transmitter available for release was less in the regenerating fibers than in the developing fibers.

Stimulation of the paravascular nerves supplying control segments of ileum produced an inhibition of spontaneous gut movements along with a decrease in resting tone of the preparation[25]. During the first few days after the denervation procedure, paravascular nerve stimulation had no effect on gut motility. Between 1 and 3 weeks, however, there was a progressive return of extrinsic sympathetic control so that all preparations tested at 3 weeks responded to paravascular nerve stimulation with the characteristic inhibition (fig. 4)[21]. This response was abolished by guanethidine (10^{-5} g/ml) or a combination of the α and β blockers, phentolamine and propranolol (10^{-5} g/ml), suggesting that it was due to the release of catecholamines as was the case for control segments.

One week after the operation, at a time when the arteries were devoid of fibers, fluorescent nerve trunks were observed to have regenerated from the site of the lesion to the various points at which they entered the gut wall. Within the gut wall these fibers ran beside the submucous blood vessels and ramified within the first ganglia which they encountered[21]. With further time, the number of reinnervated ganglia increased transversely along the submucous vessels towards the antemesenteric border and anteriorly and posteriorly from each major transverse submucous vessel. Even 4 months after the operation, however, the submucous vessels themselves were only sparsely innervated, while the majority of the ganglia in both plexuses had been reinnervated. By 6 months, the ganglia were innervated throughout the gut wall. The submucous blood vessels at this time exhibited nerve plexuses over their surfaces similar to those in control preparations[21].

The observations made both during development and during regeneration point to the existence of two distinct populations of postganglionic fibers. The first of these begin to develop and regenerate earlier than the second. They comprise a group of catecholamine containing fibers which selectively innervate the neurones of the myenteric and submucous plexuses of the wall of the ileum. This occurs in spite of the close proximity of the mesenteric and submucous blood vessels which are at that moment either uninnervated or denervated. Indeed the 'gut fibers' appear to use the mesenteric arteries and their smaller branches as a means of reaching the gut wall.

The second group of fibers innervate the mesenteric and possibly the submucous blood vessels. They grow to their targets within the same paravascular nerve bundles as do the 'gut fibers' and leave them at periodic intervals to contact the smooth muscle cells. While there is a temporal difference in the onset and conclusion of the inner-

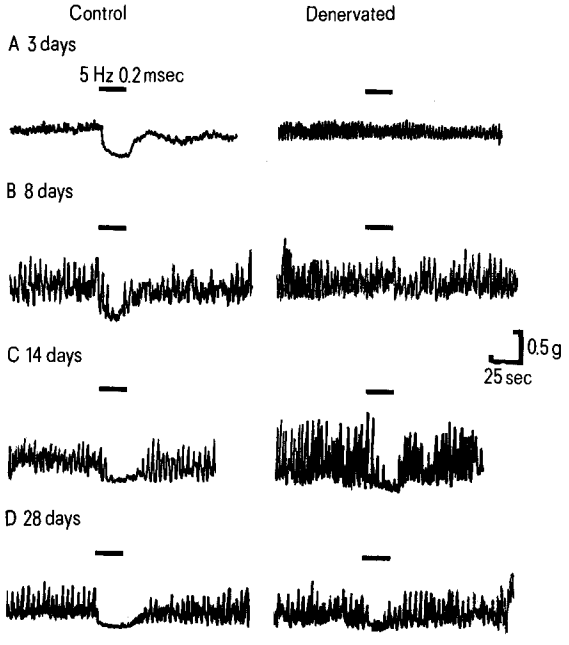

Figure 4. Tension recordings from segments of ileum innervated by paravascular sympathetic nerves (Control) or from segments of ileum which have been extrinsically denervated (Denervated). Note that a 20 s train of stimuli at 5 Hz (pulse width 0.2 ms, pulse amplitude 70 V) produced an inhibition of spontaneous gut motility. There was no response to stimulation of sympathetic nerves to ileal segments 3 days after the operation (A) and in 80% of preparations 8 days after the operation (B). Stimulation at 14 days produced inhibition of gut motility in 75% of preparations (C) and in 100% of preparations after 28 days (D). Calibration bars apply to each record.

vation of the two different target areas of the smooth muscle cells of the blood vessels and the intramural ganglion cells, the major period of innervation or reinnervation is coincident. Selectivity of innervation of the two targets would be maintained during this time if later regenerating fibers were attracted to the pathways of fibers of the same type. Such cell adhesion molecules have been described to be responsible for nerve fiber fasciculation[50,51].

The two populations of sympathetic fibers also differed in the efficacy of the reinnervation of their respective targets. There was a rapid return of extrinsic sympathetic control of gut motility, while stimulation induced constrictions of the mesenteric artery were not recorded for several months after the denervation procedure. In the gut, this functional reinnervation was observed at a time when few ganglia had been penetrated by adrenergic fibers. 6 months after the operation, reinnervation of the gut as assessed anatomically was completed while the nerve fiber networks associated with the artery were still less dense than those on control vessels. Thus, regeneration of sympathetic nerve fibers in general was a slow process. Reinnervation of postganglionic sympathetic neurones by preganglionic fibers is similarly slow and incomplete even 15 months after the operation[45].

It is possible that the selectivity in target organ innervation might reflect some organization of like neurones into groups within the sympathetic ganglia. No such topographical relationship existed in the superior cervical ganglion of guinea pigs[33,48]. Ganglion cells with similar segmental preganglionic innervation were found at different points along the major axes of the ganglion[33] and cells innervated by a common axon were often several hundred μm apart[48]. Within the sympathetic trunk, axons from different spinal segments were intermingled[48]. Finally, neurones retrogradely labeled from horseradish peroxidase injection into the eye or ear were randomly distributed over a large area generally associated with neurones whose axons exit the ganglion through the same postganglionic nerve[33].

The cell bodies of the noradrenergic fibers supplying the intrinsic ganglia of the gastrointestinal tract are reported to lie in the prevertebral autonomic ganglia[16,27], but data for the location of the cell bodies of fibers innervating the mesenteric arteries is not to hand. Retrograde labeling of both arterial and gut fibers with the fluorescent dye, Fast Blue, revealed cells predominantly in the prevertebral, coeliac and superior mesenteric, ganglia but also a smaller contribution from thoracic and, to a lesser extent, abdominal paravertebral sympathetic ganglia[24]. While separation between the two populations is difficult, our preliminary results indicate considerable overlap in their distributions. Thus, specificity of postganglionic innervation conferred by topographical distribution of the cell bodies is highly unlikely.

The selectivity in sympathetic innervation described here is perhaps suggestive of the release of different trophic factors from the two target areas. The two populations of fibers would be attracted by the substance appropriate to their class of neurones and unaffected by the alternative substance. It is interesting that mouse atrium and stomach both stimulated the outgrowth of neurites from superior cervical and coeliac ganglia, but antiserum to NGF totally abolished only that induced by the atrium[49]. The authors concluded that the gut may produce a trophic factor for sympathetic neurones which is immunochemically distinguishable from NGF.

Whatever the mechanism it would appear that at least some sympathetic neurones are specified for particular targets. Furthermore, the specificity does not appear to be conferred on the sympathetic neurones by contact with the periphery since the first fibers during development also grow alongside the uninnervated mesenteric arteries to preferentially penetrate the gut. Within the mesenteric arterial population, the distal vessels were consistently innervated earlier both anatomically and physiologically than the proximal vessels[22]. Perhaps also in the gut, the intrinsic neurones are innervated before the submucous blood vessels. In this case the submucous vessels would be innervated by the population of gut fibers rather than by the population of fibers innervating the mesenteric arteries. Selectivity described in the connections on and by the neurones of the superior cervical ganglion has in general been associated with position of the peripheral targets in the head rather than with a common function of those targets[33,55]. Thus sympathetic neurones may be specified to respond to particular growth factors released by target tissues in particular regions. Within those regions there may be some form of distal to proximal gradient for innervation.

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