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Synaptic specificity in frog sympathetic ganglia during reinnervation and development

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SYNAPTIC SPECIFICITY IN FROG SYMPATHETIC GANGLIA DURING REINNERVATION AND DEVELOPMENT

by

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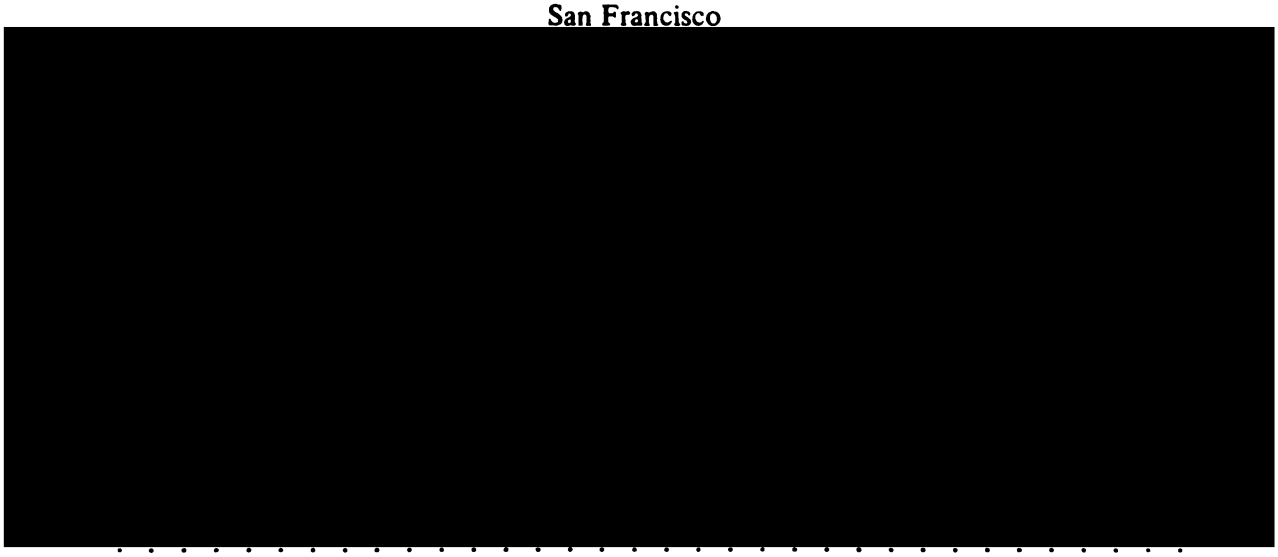

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DISSERTATION ABSTRACT

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In frog lumbar sympathetic ganglia, B cells and C cells are innervated respectively by B fibers and C fibers. The two preganglionic fiber populalations enter the sympathetic trunk at different segmental levels; thus, either population may be selectively stimulated or lesioned by selecting the appropriate preganglionic nerve roots. Using intracellular recording, I have examined the specificity of regenerating synapses in mature ganglia, and the specificity of normal synapses in developing ganglia.

Following complete denervation of ganglia, appropriate (i.e. B fiber→B cell, C fiber→C cell) synapses are eventually restored. Two experimentally separable processes underlie this specific reinnervation. First, there appears to be a "preference" for appropriate connections from the outset of reinnervation; when either preganglionic fiber population is allowed to return in the absence of the other population, the returning fibers form synapses most often with their appropriate targets. However, despite the overall preference for appropriate connections, many inappropriate synapses are formed. Most of these are eliminated within 6 weeks, provided that both preganglionic fiber groups are allowed to return. Thus, a second process underlying specific reinnervation is the selective elimination of inappropriate synapses, which results from a competitive interaction between appropriate and inappropriate synapses.

When only B fibers are cut, the intact C fibers sprout and form

synapses on about half of the B cells. The signal that induces sprouting must therefore not be specific for neuronal type. When the B fibers eventually return, they reinnervate the B cells, and the sprouted C fiber synapses can no longer be detected. Thus, after a transient loss of specificity, specific connections are restored.

Studies of the innervation of neurons in normally developing tadpole ganglia indicate that at least 8% of the neurons receive synaptic input from both B and C fibers; cells receiving both B and C fiber input are virtually never encountered in normal mature ganglia. This suggests that competition between appropriate and inappropriate synapses plays a role in the establishment of specific neuronal connections during normal ontogeny.

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PREFACE

Three related projects are presented in this dissertation. Chapter 1 comprises a general background, including information about the experimental preparation. Chapters 2, 3, and 4 are derived from manuscripts intended for publication. At the time of this writing, the material of Chapter 2 has been submitted to The Journal of Physiology, and the material of Chapters 3 and 4 are in preparation as two separate manuscripts. For the sake of completeness I have chosen to present the entire manuscripts, with minor modifications to conform to the format of the dissertation, despite the redundandies resulting from this manner of presentation.

I wish to thank Michael Dennis for providing guidance at all stages during the execution of this research, and during the writing of this dissertation. His sage advice has been of inestimable value in my scientific development. I am grateful to Roger Nicoll for his advice and encouragement, without which this research would never have gotten off the ground. I thank Zach Hall and David Van Essen for serving on my thesis committee, and for their help with the writing and content of the dissertation. Finally, I wish to express my gratitude to Dale Branton and Barry Gumbiner for their friendship, and for many helpful and stimulating discussions.

BACKGROUND

In the mature nervous system the connections between neurons, and between neurons and peripheral targets, are characterized by a remarkable degree of specificity. It is convenient to consider this specificity in several contexts; there is type specificity, whereby each neuron is innervated by a precise set of afferents, and each neuron in turn innervates a precise set of targets. In addition, there is quantitative specificity regulating the numbers of synapses given and received by each neuron; geometric specificity is evident in the restricted sites upon receptive cells where synapses of each afferent type are made; molecular specificity results in the distribution of the appropriate postsynaptic receptors, neurotransmitter-degrading enzymes, and uptake systems at the sites of release of various chemical transmitters. The integrative capacities which enable higher organisms to fulfill their bodily needs, and to successfully respond to changing environments, depend upon the orderly precision of neuronal connections. The understanding of the mechanisms through which this grand precision arises is a principal goal of neuroscientists.

Our knowledge of the mechanisms by which patterns of connectivity arise is elementary; much of what we do know is phenomenological. One emerging principle is that during development, there appears to be considerably less precision of neuronal connections than that found in the mature ner-

vous system; early patterns of connections are modified in several ways, culminating in the adult pattern. Furthermore, the adult pattern of connections can be modified experimentally. In the following discussion, I will consider some results of both developmental and regeneration experiments that illustrate such modifications, and which provide clues to the understanding of the mechanisms that underly specific synapse formation.

Multiple Innervation and Synapse Elimination

One type of modification of synaptic specificity observed during development is quantitative. Some neuronal targets are first 'hyper-innervated' by axons of the same type, and redundant synapses are lost during maturation. This phenomenon has been described at neuromuscular junctions of mammals (Redfern, 1970; Bagust et al., 1973), chicken (Bennett & Pettigrew, 1974) and frog (Bennett & Pettigrew, 1975). Fast skeletal myofibers in mature vertebrates are usually innervated by a single motor axon, but in neonatal animals several axons innervate each fiber. The average number of innervating axons per fiber decreases until each fiber is singly innervated. The withdrawal of multiple innervation is due not to the death of motoneurons (the major wave of motoneuron death occurs during prenatal development), but rather to a decline in motor unit size (Brown et al., 1976). Furthermore, when a muscle is partially denervated just after birth, the decline proceeds

(although delayed somewhat) at the expense of leaving many myofibers without innervation. It is thus a process that is inherent at least in some motoneurones, and occurs despite the availability of excess 'periphery'. However, a different result was obtained by Betz, et al. (1980) in rat lumbrical muscles in which all but one of the innervating axons was severed; the remaining axon did not reduce the size of its motor unit in the absence of competition from other axons.

Loss of multiple innervation has been described also for neonatal neurons in autonomic ganglia and in the central nervous system (CNS). In rat submandibular ganglia, each neuron is initially innervated by several preganglionic axons, all but one of which is lost within the first several postnatal weeks (Lichtman, 1977). In the rat cerebellum, Purkinje cells may be innervated by two or more climbing fibers at birth. Within several weeks after birth, only one climbing fiber innervates each Purkinje cell (Crepel et al., 1976).

Excessive innervation occurs during reinnervation of muscle in rat (McArdle, 1975; Brown, et al., 1976) and frog (Rothshenker and McMahan, 1976), and of parasympathetic ganglion cells of frog (Dennis & Sargent, 1978; Ko & Roper, 1978). In the case of rat skeletal muscle, single innervation is eventually restored; however, in both reinnervated muscle and parasympathetic neurons of frogs, excessive

innervation persists. It appears that the mechanism by which some redundant synapses are eliminated during development has been lost in adult frogs.

Transient Inappropriate Innervation During Development

The evidence discussed above implies that in some cases, once developing axons have reached the appropriate target region, they form an excess of synapses and subsequently reduce the number of target cells they innervate. There is also evidence for a second kind of imprecision resulting in innervation of inappropriate targets, which is ultimately corrected by selective synapse elimination. Such inappropriate projections have been found in various parts of the visual system at early stages of development. For example, So, et al. (1978) have demonstrated autoradiographically in hamsters that contralateral retinal afferents reach the lateral geniculate nucleus (LGN) before ipsilateral fibers, and in three- to six-day postnatal animals, they occupy the entire nucleus. The ipsilateral fibers arrive by day 5 in their restricted medial region of the nucleus, but the contralateral projection does not recede from this region until days 7-8 to form the segregated adult pattern. If the ipsilateral eye is removed just after birth, the extended contralateral projection persists in the medial region. Similarly, Rakic (1977) found by autoradiography that when the optic nerve fibers first reach the LGN in fetal monkeys, there is no sharp separation into laminae

of the projections from the two eyes. Within several weeks the projections begin to segregate. Rakic reported similar findings for the ocular dominance patterns of the superior colliculus, and of area 17. With such anatomical techniques, there is no indication whether functional synaptic contacts have been made within these inappropriate regions.

LeVay, et al. (1978) studied newborn kittens with transneuronal autoradiography and physiological recording. They show that at 1-2 weeks of age, functional inputs from the two eyes are continuous and overlapping in layer IV, that they begin to segregate by 3 weeks, and reach a more or less adult pattern by 6 weeks of age. These findings suggest that early-formed connections in inappropriate columns are selectively lost, resulting in the establishment of ocular dominance columns. This system is of particular interest because it shows plasticity during a critical period, when input from both eyes is necessary for the establishment of a normal thalamo-cortical projection in cats (Shatz & Stryker, 1978) as well as primates (Hubel, et al., 1977).

Individual skeletal muscles in mature vertebrates are innervated by pools of motoneurons residing in the ventral horn in nuclei with particular positions along the medial-lateral and rostro-caudal axes of the spinal cord (Landmesser, 1978). The axons innervating particular muscles exit the spinal cord via particular segmental nerves. Dur-

ing embryonic development of amphibians (Lamb, 1976, 1977; McGrath & Bennett, 1978) and chickens (Pettigrew et al., 1979; but see Landmesser & Morris, 1975 and Landmesser, 1978), the motoneurons innervating muscles and their axons are not so strictly distributed; apparently developing motoneurons also transiently innervate targets beyond those they innervate in the adult animal.

Another form of modification of innervation during development is the removal of synapses from inappropriate sites of the target neurons. Axosomatic synapses, not present in adults, have been found on embryonic Purkinje cells of chick (Mugnaini & Forstrőnen, 1967) and monkey (Kornguth, et al., 1968) and on motoneurons of kittens (Conradi & Ronnevi, 1977). In the olfactory cortex of neonatal rats, afferents from the olfactory bulb and associational fibers overlap completely, whereas in adults, the two afferent projections are distinctly laminated within the dendrites of the cortical cells (Price, et al., 1976).

Thus, transient innervation of several forms appears widespread in the developing nervous system. Such findings may indicate that developing neurons are over-zealous in their search for willing partners in synapse formation; additional mechanisms must then correct the 'mistakes' committed early on by the elimination of inappropriate or redundant synapses. Alternatively, these transient synapses may have some vital function during development.

Specificity of Synapse Formation During Reinnervation

Specificity has been studied extensively in reinnervated adult peripheral tissues. Under a variety of circumstances, a particular target may be functionally innervated by an inappropriate input. When a foreign nerve is implanted into a botulinum-toxin poisoned (Tonge, 1974) or a denervated muscle (Elsberg, 1917; Tonge, 1974; Jansen, et al., 1973; Dennis & Yip, 1978), effective synapses are formed. Recent experiments by Bixby and Van Essen show that it is not even necessary to destroy the normal innervation to obtain synapse formation by an implanted foreign nerve, provided it is implanted directly over the normal synaptic region of the muscle (Bixby & Van Essen, 1979) When a nerve innervating several muscles is cut or crushed, the muscles are reinnervated more or less randomly by correct or foreign axons (Bernstein & Guth, 1961) -- a fact with serious clinical consequences. Furthermore, mammalian muscle fibers are reinnervated without regard to the type, 'fast' or 'slow', of myofiber and motoneuron (Miledi and Stefani, 1969; but see below). Denervated muscles have also been innervated by preganglionic autonomic nerves (see, for example, Bennett, et al., 1973). Similarly, denervated neurons of the mammalian superior cervical ganglion (SCG) have been successfully innervated by vagus nerve (see, for example, Purves, 1976) and various somatic motor nerves (McLachlan, 1974; Ostberg et al., 1976). In general, it appears that these peripheral targets will form functional synapses with

virtually any cholinergic nerve fibers. Clearly, during reinnervation, specificity is not absolute.

But are there cases in which synapses are specifically re-established by correct nerves? In both the CNS and the peripheral nervous system there are examples which demonstrate that appropriate synaptic connections can be re-established in adult tissues. In general, connections between neurons in the CNS of higher vertebrates do not regenerate. However, in adult fish and amphibians, retinal ganglion cell axons reinnervate the tectum, restoring a nearly normal retinotopic projection (see, for example, Attardi & Sperry, 1963) and restoring visuomotor coordination (Sperry, 1943). Such observations have spawned a field of intense research involving exhaustive manipulation of the retina, the optic nerve, and the tectum, directed toward elucidation of the mechanisms underlying selective synapse formation (for reviews see Jacobson, 1978; Fraser & Hunt, 1980). However, as Jacobson (1978, p403) notes, "...in spite of much effort, studies of optic nerve regeneration have been of limited value in disclosing the mechanisms of nerve growth, pathway selection, target selection, and formation of synaptic connections. The salient result that emerges from all the investigations is that optic axons have a strong tendency to regain their positions in the retinotectal map, and that functional connections are formed after regeneration on the basis of the inherent specificities of the optic axons."

Information more relevant to the research presented here comes from studies of reinnervation of muscle and peripheral neurons. In mammals, when both correct and foreign nerves are available to reinnervate a muscle, the outcome is generally a lack of specific reinnervation. However, in lower vertebrates, and in the chick, there are cases of successful correct innervation under competitive circumstances. A sequence of experiments by Sperry and Arora (1965) and Mark and collaborators (Mark, et al., 1972; Marotte & Mark, 1970a, 1970b; Mark & Marotte, 1972) examined specificity in reinnervation of extra-ocular muscles of fish, and showed by behavioral criteria that reinnervation appeared to be selective. The Mark group cross-innervated one of the oculomotor muscles with the nerve to the antagonist muscle, and removed the antagonist muscle. By behavioral criteria, the inappropriate connections made after cross-innervation were apparently lost upon regeneration of, and synapse formation by, the proper nerve. They took this as evidence for competitive selective reinnervation. Unable to find morphological evidence for degenerating terminals from the inappropriate nerve, the Mark group went on to postulate that inappropriate synapses had been functionally suppressed but were left physically intact (see below). Scott (1977) reexamined this situation using more sensitive physiological techniques, including tension measurements and intracellular recording, and found no evidence for suppression of incorrect innervation at the level of the neuromuscular

junction -- many muscle fibers remained dually innervated by axons from the correct and incorrect nerves. Similarly, Frank and Jansen (1976) found no suppression of incorrect innervation upon return of the correct nerve in fish gill muscle cross-innervated with fin nerve. Thus, the question of selective reinnervation of fish muscle is at best unresolved.

More convincing evidence for selective reinnervation has been found in studies of salamander limb musculature. Grimm (1971) crossed the two main nerves innervating extensor and flexor muscles, respectively. After regeneration, swimming movements were normal, which resulted because the nerves had succeeded in reinnervating their correct muscles, despite considerable anatomical obstacles. Cass, et al. (1973) created competition between correct and incorrect axons by partially denervating the leg, thereby inducing collateral sprouting from the remaining nerves. (Collateral sprouting will be discussed in the next section.) Sprouts from the incorrect nerve progressively innervated the denervated area. When the correct nerve regenerated, it reinnervated only its own normal territory, and the sprouted nerve no longer caused contraction in the reinnervated muscle. After cutting the correct nerve a second time, the incorrect nerve reinnervated the area within 3 days, compared with 3 weeks after the first cut. These results were interpreted to mean that upon reinnervation by the correct nerve, the sprouted synapses were functionally suppressed while

remaining physically intact.

The methods used in the above experiment were indirect and ambiguous. Yip and Dennis (1976) re-examined competition between correct and incorrect innervation in the salamander more directly. They implanted a flexor nerve into an extensor muscle and cut or crushed the extensor nerve. The foreign nerve innervated the muscle, but when the correct nerve returned, transmission by the foreign nerve declined, with a decrease in quantal content. Also, the number of muscle fibers receiving foreign input dropped from 97% to a mean of 35%, and did not increase rapidly after a second lesion of the correct nerve. Muscles in which foreign transmission had been completely suppressed were stimulated by way of the correct nerve while bathed in horseradish peroxidase (HRP) to histochemically label vesicles in active terminals. Virtually all terminals were so labelled, suggesting that in these muscles, where no foreign synapses were detected with intracellular recording, there were no physically intact, but 'silent' terminals (Dennis & Yip, 1978).

A somewhat different type of selective reinnervation has been found in amphibians and chick. This is reinnervation of muscle fibers according to their type, fast or slow, by axons of the appropriate class. In amphibians, muscle fibers are of two distinct types, normally innervated by separate classes of axons which differ in conduction velo-

city and excitation threshold. Hoh (1971) allowed axons of both types to compete for reinnervation of two different muscles containing either all fast fibers or fibers of both types. Using tension measurements, he was able to deduce that the fast axons innervated mainly fast muscle fibers, and the slow axons only slow muscle fibers. Similar experiments were done in chicks by Feng, et al. (1965) showing selective reinnervation of the 'fast' posterior and the 'slow' anterior latissimus dorsi muscles. While inappropriate innervation was not completely excluded, these experiments suggested that mismatched connections were made only with difficulty. The techniques used by Hoh and Feng, et al. would not have revealed mistakes if they were few in number, or if synaptic inputs were sub-threshold for the muscle fibers. Furthermore, the early stages of reinnervation were not studied systematically, so these results do not address the possibility that incorrect connections may have been made at first, to be eliminated in competition with correct inputs.

Schmidt and Stefani (1976) used intracellular recording to determine if muscle fibers in the frog were reinnervated by axons appropriate to the type of muscle fiber. Myofibers were identified as fast-twitch or slow-graded by their electrophysiological properties. Early after denervation both types of fibers were reinnervated by fast axons. Slow axons began to appear somewhat later, exclusively in slow-graded myofibers. Ultimately, fast axons were found innervating

only fast-twitch myofibers. This suggested that fast axons had regenerated more rapidly, and formed synapses non-selectively on either myofiber type in the absence of competition from slow axons. Once the slow axons returned, they reinnervated the slow myofibers, and 'mistakes' made early by the fast axons were apparently eliminated. Nothing was reported about the fate of the displaced synapses, and there is no information about the processes going on during the period of competition between correct and incorrect inputs. In experiments designed to allow more simultaneous reinnervation of the muscle by both axon classes Elizalde and Stefani (1978) reported selective reinnervation of both myofiber types from the outset.

The specificity of reinnervation of autonomic neurons has been examined also. Landmesser and Pilar (1970) studied the reinnervation of the pigeon ciliary ganglion. There are two populations of neurons in this parasympathetic ganglion, innervated respectively by two populations of preganglionic axons which differ in mean conduction velocity. The postganglionic axons from the two cell populations exit the ganglion via separate nerve trunks. Responses to preganglionic stimulation can be recorded from the postganglionic nerves. After cutting all the preganglionic axons (which run together in a common trunk), these authors examined the latency and form of these extracellularly-recorded postganglionic responses to assess the reinnervation of ganglion cells. The eventual return of response latencies to near

control values was interpreted to mean that pre-synaptic fibers of each class had selectively innervated almost exclusively its own class of ganglion cells. The extracellular recording method used here would reveal only connections which bring the post-synaptic cells to threshold. Furthermore, interpretation of the results is difficult because the latencies measured result from both pre- and post-synaptic conduction times; the two presynaptic populations do not differ greatly (in fact, they overlap somewhat) in conduction velocity. The difference in latency of the responses in the two postganglionic nerves could thus be largely attributable to differences in postganglionic conduction velocity, and this could make crossed-innervation difficult to rule out. Preparations were examined at various times after denervation and the authors state that their results "help to exclude the possibility of initial random re-growth followed by retraction or degeneration of inappropriate synaptic connections;" I find the results completely ambiguous with regard to this matter.

A more thoroughly studied preparation is the mammalian superior cervical ganglion (SCG). The SCG receives input from the first seven thoracic spinal segments. Stimulation of one of the upper rami (T_1-T_3) causes a particular combination of sympathetic responses, mainly in the eye, such as pupillary dilation; stimulation of one of the lower segments (T_4-T_7) causes some combination of effects in other parts of the head, such as vasoconstriction in the pinna. This

suggests that each ramus contributes preganglionic axons which innervate a particular subset of neurons in the SCG, in turn innervating the various peripheral end organs. Langley (1897) observed in cats that after sectioning the superior cervical trunk containing all preganglionic axons, stimulation of various rami caused a substantially normal pattern of responses when the axons had regenerated. This suggested that axons from a given ramus had by and large specifically reinnervated that subset of SCG neurons they had originally innervated. Guth and Bernstein (1961) confirmed Langley's results using somewhat more quantitative behavioral methods. Of course, the behavioral measurements used in these experiments suffer the usual criticisms with regard to possible sub-threshold errors, and again no attempt was made to study the initial stages of reinnervation.

Nja and Purves (1977a, 1977b) used intracellular recording to extend understanding of the cellular basis for specific reinnervation. Recording from cells in the guinea pig SCG, they found that normally each neuron receives input from a contiguous subset of the spinal segments which innervate the ganglion, and is dominated by synaptic input from one segment, with adjacent segments contributing a progressively decreasing synaptic input. After reinnervation, Nja and Purves found essentially the same pattern of synaptic input to the SCG neurons. Relatively early during reinnervation, when substantial numbers of cells first appeared to

be reinnervated, the pattern was essentially the same as at long times (Nja & Purves, 1978). From these findings, along with the restoration of appropriate behavioral responses to mono-segmental stimulation, it was inferred that regenerating axons from a given segment reinnervated roughly the same subset of neurons they originally innervated. Although these results do argue for specific reinnervation, this preparation is not ideal for detecting errors because one can never be certain if a particular input is an error since a given cell is innervated by several segments. With regard to the early reinnervation pattern, it is possible that at even earlier times there may have been more numerous errors, but that they were eliminated rapidly during the first days of reinnervation. This possibility seems an important point to raise because it has important implications concerning the mechanisms involved in specific reinnervation, and because of the relative nature of specificity in the ganglion shown by Nja and Purves' work, and by other experiments to be described below.

Murray and Thompson (1957) partially denervated the SCG in cats by resecting preganglionic rami T_1, T_2, and T_3, containing approximately 90% of the fibers innervating the ganglion. Four to eight weeks later, stimulating the sympathetic trunk caudal to the T_3 level (containing axons from T_4-T_7) elicited pronounced contraction of the nictitating membrane and dilation of the pupil. The remaining presynaptic axons had thus apparently sprouted and formed

'effective', i.e. super-threshold, synapses on the denervated cells -- cells which they normally do not innervate, at least not 'effectively'. Guth and Bernstein (1961) carried this one step further by allowing reinnervation by T_1-T_3 axons after T_4-T_7 axons had sprouted. Using behavioral measurements, they showed that not only would effective inappropriate innervation appear when the preferred innervation was removed, but that when the correct fibers returned, they reformed connections selectively with their own neurons, while inappropriate input was suppressed. It is reasonable to ask if selective reinnervation following complete denervation, or selective innervation during embryonic development of the SCG proceeds with initial non-selective synapse formation, followed by selective enhancement of appropriate synapses and suppression of inappropriate ones. The result of such a competitive process would be the pattern of innervation dominance elucidated by the work of Nja and Purves.

Proctor, et al. (1979) denervated frog cardiac (parasympathetic) ganglia by cutting the vagus nerves and implanted hypoglossal nerves. The hypoglossal fibers formed functional synapses with the ganglion cells. The regenerating vagal axons eventually restored their connections with the ganglion cells, and there was a concomitant loss of the foreign synapses. A second type of foreign synapse elimination has been described by Sargent and Dennis (1981); this is described below under the topic of 'collateral

sprouting'.

Reinnervation specificity has been studied in invertebrates, where well-characterized connections of identified neurons and their targets make it possible to ask whether selectivity occurs precisely at the level of single identified functional connections. Jansen and Nicholls (1972) have reported specific reinnervation between identified cells in adjacent segmental ganglia of the leech, after cutting an intersegmental connective containing thousands of axons. Van Essen and Jansen (1977) examined reinnervation following lesions of peripheral nerves in the leech, and found considerable specificity for both motor and sensory neurons.

Collateral Sprouting

An interesting form of plasticity of synaptic connections in mature animals is so-called 'collateral sprouting', which has been observed in sensory and motor peripheral nerves (Edds, 1953; Weddell, et al., 1946), in the peripheral autonomic nervous system (Murray & Thompson, 1957; Courtney and Roper, 1976; Sargent & Dennis, 1977), and in various parts of the CNS (Liu & Chambers, 1958; Raisman and Field, 1973; see Cotman & Lynch, 1976, for review). The phenomenon results after partial denervation of a target field. This somehow induces remaining nearby axons or terminals to sprout and reinnervate the denervated target field.

In several regions of the CNS, electron microscopic evidence has been found for new synapse formation by remaining afferents after partial deafferentation (Raisman & Field, 1973; Nakamura, et al., 1974; Bernstein & Bernstein, 1973). Electrophysiological evidence, although somewhat indirect, suggests that the new synapses are functional in the red nucleus and (Tsukahara, et al., 1975) and dentate gyrus of the hippocampus (West, et al., 1975). The process shows some selectivity in that only particular afferents react after particular lesions, and the available evidence suggests that new synapses are formed only by afferents which already provide input to the partially deafferented target (Cotman & Lynch, 1976).

At the mouse neuromuscular junction, axons can sprout and functionally innervate about five times their normal complement of myofibers (Brown & Ironton, 1978). When the cut axons reinnervate the muscle, the size of sprouted motor units decreases, though some proportion of sprouted terminals remain fully functional. At least 10% of the myofibers become innervated by both sprouted terminals and the returning interrupted axons, as shown by tension recording, and supported by intracellular recording (Brown & Ironton, 1978). Regression of sprouted terminals upon reinnervation by the normal nerve has been reported in salamander limb muscle (Bennett & Raftos, 1977; Cass, et al., 1973 -- see above) and Xenopus extraocular muscle (Fangboner & Vanable, 1974). Such regression may depend upon the fact that

sprouted axons are supporting an unusually large number of synapses, and may not necessarily represent true selective reinnervation on the basis of correct versus incorrect (Purves, 1976). This idea is supported by the results of Wigston (1980) in salamander muscle, showing that sprouted synapses will regress when cut foreign nerves are allowed to reinnervate the muscle, just as when the native nerve regenerates. Sprouted synapses will persist, however, if the interrupted nerve fails to reinnervate the muscle (Brown & Ironton, 1978; Fangboner & Vanable, 1974). Furthermore, in the rat at least, sprouts acquire stability with time; they are not displaced if the native nerve regenerates only after sprouting has reached its maximal extent (Thompson, 1978).

Sprouting in the mammalian SCG was demonstrated by Murray and Thompson (1957) and Guth and Bernstein (1961), as discussed above. In light of the work of Nja and Purves (1977a), showing the multi-segmental innervation of individual ganglion cells, a somewhat different interpretation should be considered: perhaps inputs already present, but of low synaptic efficacy, became stronger when the dominant innervation was removed, rather than collateral sprouts innervating new and 'inappropriate' neurons, in analogy to the form of sprouting generally observed in the CNS. Morphological evidence does suggest actual sprouting (Murray & Thompson, 1957; Williams, et al., 1973), but the question of whether new cells are innervated remains uncertain. The regression of expanded innervation observed in the SCG could

again be more directly related to over-extension than to competition between correct and incorrect fibers, despite the solid evidence for specific reinnervation after complete denervation.

Sprouting has been demonstrated physiologically in the frog parasympathetic cardiac ganglion after sectioning of one of the vagus nerves. The contralateral vagus quickly innervates the denervated neurons and these sprouts recede somewhat when the (interrupted) ipsilateral vagus fibers return (Courtney & Roper, 1976; Roper, 1976). There is no reason to think of this competition as representing correct versus foreign; more likely it represents a competitive disadvantage of the over-extended sprouted fibers.

The frog cardiac ganglion manifests a second form of sprouting; when the preganglionic axons are all severed and prevented from growing back for two months or more, synapses between ganglion cells, ordinarily never detected, develop (Sargent & Dennis, 1977). A similar phenomenon has been described in the mammalian SCG (Purves, 1976). These 'intrinsic' synapses in the frog persist in the absence of regeneration of the preganglionic axons, but decline sharply if regeneration is allowed after 100 days (Sargent & Dennis, 1981). This is perhaps the only clear demonstration that denervated neurons will induce (although we don't know the real source of the induction) functional sprouts from a source which normally does not innervate them at all.

The Frog Sympathetic Ganglion

Here I describe studies of the specificity of newly formed synaptic connections in frog lumbar sympathetic ganglia. The primary focus is on regenerating synapses in partially or completely denervated ganglia of mature frogs. Additionally, I present a study of normally developing synapses in tadpole ganglia. The adult ganglia lend themselves to examination of the cellular mechanisms underlying synaptic specificity because the neurons are accessible to intracellular recording, the normal physiology and morphology of the synapses have been described (Ginsborg, 1977; Taxi, 1977; Kuba & Koketsu, 1978), and because of their simple pattern of specific synaptic connections: there are two types of principal neurons, B cells and C cells, that receive input from distinct populations of preganglionic fibers, B fibers and C fibers, respectively. (Nishi, Soeda, & Koketsu, 1965). The two preganglionic fiber types enter the sympathetic trunk at different segmental levels (Libet, Chichibu, & Tosaka, 1968; Skok, 1965; Francini & Urbani, 1973), and thus they may be manipulated and stimulated independently. The two cell populations are intermingled within the ganglia, yet in mature frogs each is exclusively innervated by its complementary preganglionic fiber type. This situation is comparable to that which exists in many areas of the central nervous system (CNS) in that the specific targets of distinct afferent fiber populations are intermingled. How such systems develop appropriate synaptic

connections is the broad focus of this study.

The system used for numbering the ganglia is that of Ecker and Weidersheim (Gaupp, 1899), in which the sciatic plexus is composed of spinal nerves 8, 9, and 10. The ninth and tenth nerves are usually the largest. A small eleventh nerve is occasionally present. Ganglia are numbered according to the spinal nerves with which they connect via rami communicantes. I refer to preganglionic axons as 'B fibers' or 'C fibers' and to post-ganglionic axons as 'B cell axons' or 'C cell axons'.

Two principal neuronal types have been distinguished in the ninth and tenth sympathetic ganglia of anuran amphibia. In the toad, B cells have myelinated axons with conduction velocities of 1.2-8 m/sec, whereas C cells have non-myelinated axons, conducting in the range 0.2-0.8 m/sec. B cells are innervated by myelinated preganglionic B fibers conducting at 2-13 m/sec, whereas C cells are innervated by non-myelinated preganglionic C fibers conducting at 0.2-0.4 m/sec (Nishi et al., 1965). In frogs (but not in the toad) the two groups of preganglionic fibers enter the sympathetic trunk at different segmental levels: B fibers enter anterior to the 7th segmental ganglion whereas C fibers enter at the 7th and 8th segmental levels (Libet et al., 1968; Skok, 1965; Francini & Urbani, 1973). Thus, as illustrated in Figure 1, the two preganglionic fiber groups may be stimulated or interrupted independently. The basic design of

Figure 1. Schematic diagram of the lumbar sympathetic chain with associated spinal nerves. The myelinated B fiber and B cell axon populations are represented by segmented lines, and the non-myelinated C fiber and C cell axon populations are represented by unsegmented lines. The innervation pattern for B and C cells in the 9th ganglion is identical to that shown for the 10th. Preganglionic B fibers are stimulated in the sympathetic trunk anterior to the 7th ganglion, and C fibers are stimulated in the 7th and 8th nerves. The postganglionic axons of B and C cells are antidromically stimulated in the 10th (or 9th) nerve. The sites of lesions for various experiments are illustrated here, and are specified in the text and in Table 1.

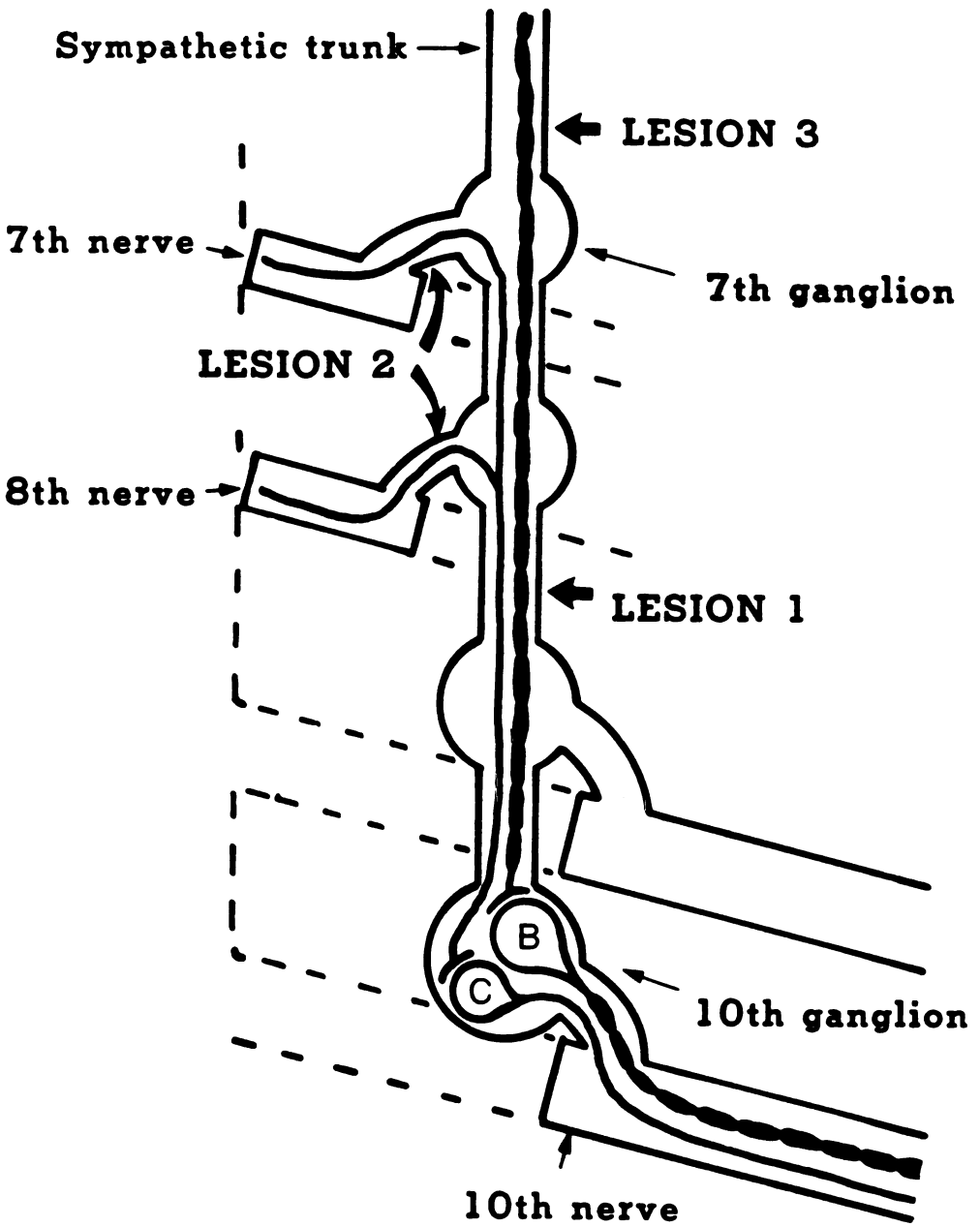

Fig. 1. Schematic diagram of lumbar sympathetic chain.

these experiments is to determine the specificity of mature, regenerating, and developing synaptic connections by recording intracellularly the responses elicited by stimulating the different preganglionic fiber populations.

COMPETITIVE AND NON-COMPETITIVE MECHANISMS UNDERLY SPECIFIC REINNERVATION OF FROG SYMPATHETIC GANGLIA

The developmental mechanisms that underly the orderly formation of synaptic connections in the nervous system are not understood. This lack of understanding derives in part from the difficulty of performing physiological studies on embryonic tissue. One means of avoiding this difficulty has been to study the regeneration of synaptic connections in mature animals. Using this approach, various investigators have explored the specificity of regenerated connections in a variety of systems, including visual projections (for reviews see Fraser & Hunt, 1980; Jacobson, 1978), neuromuscular pathways (see for example Dennis & Yip, 1978; Schmidt & Stefani, 1976), and autonomic ganglia (see for example Nja & Purves, 1977b).

Here I describe a study of the specificity of regeneration of synaptic connections in frog lumbar sympathetic ganglia. These ganglia lend themselves to examination of the cellular mechanisms that underly synaptic specificity because the neurons are accessible to intracellular recording, the normal physiology and morphology of the synapses have been described (Ginsborg, 1977; Taxi, 1977; Kuba & Koketsu, 1978), and because there are two types of principal neurons (B cells and C cells) that receive input from distinct populations (B fibers and C fibers, respectively) of preganglionic fibers (Nishi, Soeda, & Koketsu, 1965). The

two preganglionic fiber types enter the sympathetic trunk at different segmental levels (Libet, Chichibu, & Tosaka, 1968; Skok, 1965; Francini & Urbani, 1973), and thus they may be manipulated and stimulated independently. The two cell populations are intermingled within the ganglia, yet in mature frogs each is exclusively innervated by its complementary preganglionic fiber type. This situation is comparable to that which exists in many areas of the central nervous system (CNS) in that the specific targets of distinct afferent fiber populations are intermingled. How such systems develop appropriate synaptic connections is the broad focus of this study.

In the experiments I describe here, ganglia were denervated, and at various times after denervation the regenerated synapses were examined physiologically. The ganglion cells were found to be selectively reinnervated. At least two separate mechanisms appear to be responsible for this selectivity. Some of these results have been reported in preliminary form (Feldman, 1979, 1980).

Methods

Physiological Techniques

The caudal portion of the sympathetic chain, including ganglia 7 through 10, the proximal (preganglionic) segments of spinal nerves 7 and 8, and the distal (post-ganglionic) segments of spinal nerves 9 and 10, were dissected out and

pinned in a chamber with a Sylgard (Dow Corning) bottom. Suction electrodes were applied to the sympathetic trunk anterior to the 7th ganglion, and to nerves 7 and 8 for orthodromic stimulation of the B and C preganglionic fiber populations. A suction electrode was also applied to the 10th (or 9th) nerve for antidromic stimulation of post-ganglionic axons in the 10th (or 9th) ganglion. The stimulation points are diagrammed in Figure 1. (In some preparations the 9th and 10th ganglia fuse to form a single compound ganglion; in these, antidromic stimulation was applied to the sciatic nerve just caudal to the point of anastomosis of the 9th and 10th nerves.) The post-ganglionic suction electrode was also used for extracellular recording of the synaptically evoked post-ganglionic compound action potentials. This provided a convenient means of monitoring the overall health of a preparation during an experiment and of making a crude assessment of the extent of reinnervation of a ganglion.

Preganglionic and post-ganglionic axons were stimulated with 0.5 msec pulses at $\leq$ 1 pulse/sec. Stimulus intensities were generally in the range of 0.3-2.0 V for B fibers and B cell axons, and 1-5 V for C fibers and C cell axons. These low intensities did not stimulate axons other than those intended. The frequency of stimulation was kept low to reduce fatigue in the regenerated synaptic terminals.

Intracellular recording was carried out under direct

observation at a magnification of 500X using Zeiss-Nomarski interference contrast optics. The resolution provided by this optical system facilitated successful ganglion cell penetrations, particularly in the case of C cells which, due to their size, are difficult to record from. Recordings were carried out at room temperature (20-22^{o}C) in flowing Ringer solution of the following composition (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 3.6; glucose, 10; HEPES buffer, 4 (pH 7.3). The calcium concentration used was twice that of normal frog Ringer, serving to increase the size of synaptic potentials and to increase the stability of intracellular recordings. High resistance micropipettes were used for intracellular recording. Most satisfactory were those pulled from thin-walled quick filling capillary tubing (Federick Haer and Co., Brunswick, ME) on a Brown-Flaming design puller (Brown & Flaming, 1977). Electrodes were filled with 4M potassium acetate and had resistances of 30-50 megohms. A high impedance preamplifier with negative capacitance compensation was used. A bridge circuit in the preamplifier allowed injection of current through the recording electrode.

The short post-ganglionic nerve trunks (usually 4-6 mm) in these preparations made determination of axonal conduction velocities inaccurate; crude estimates were made from the latency of responses and the approximate conduction distances. Ganglionic neurons were classified by type from the latency to arrival in the soma of antidromic action

potentials. This was a reliable means of distinguishing B and C cells in both normal and reinnervated ganglia (see Results). At the end of an experiment, the ganglion from which intracellular recordings had been made was drawn into a suction electrode and the antidromically propagated compound action potentials were recorded extracellularly. Usually, two or three well-defined populations (see Results section regarding intermediate population) of axons were resolved in this way. A large, slow, high threshold wave was considered to represent the C cell axons, and its latency defined as the cutoff value for distinguishing C cells by intracellular determination of antidromic latency. In this way, individual variability due to nerve length, temperature, etc. was normalized. Identifications by this criterion generally agreed with those made while recording from within individual cells, on the basis of their antidromic latency and cell diameter (measured in the living preparation with a micrometer in the eyepiece of the compound microscope).

Only the nicotinic fast excitatory post-synaptic potentials (Kuba & Koketsu, 1978) were examined in this study. Synaptic input was categorized by anatomical source; thus, when stimulation of the sympathetic trunk anterior to the seventh ganglion caused a synaptic response, the cell was considered to receive B fiber input, and a response due to stimulation of the 7th or 8th nerves was taken to indicate C fiber input. The stimulating current was gradually

increased between pulses to search for steps in amplitude of the synaptic response resulting from excitation of additional innervating fibers. Such steps were often better resolved by passing hyperpolarizing current into the cell body while stimulating its preganglionic inputs. When a low-threshold synaptic response gave rise to an action potential, it often obscured additional synaptic steps elicited at higher intensities of preganglionic stimulation. Such masking was avoided by timing the synaptic response so that it fell during the refractory period following a spike elicited directly by a depolarizing current pulse (Purves, 1975). When a cell appeared to receive both a B fiber and a C fiber input, with synaptic potentials of each having similar size and waveform, the possibility of collaterals of one axon being present in both preganglionic roots was checked by means of a collision test; the two inputs almost always proved to be distinct.

Denervation

Frogs were anesthetized by immersion in a solution of tricaine methanesulfonate, 1.3 g/ml (Sigma). The skin and muscle wall were cut 3-4 mm ventral to the pelvic protuberance and the peritoneum was pulled ventrally to expose the sympathetic trunk and spinal nerves. The principal landmark used to determine the proper site for lesion was the sciatic plexus, composed of nerves 8-10. The lesion sites for various experiments are detailed in the appropriate sections of

'Results'. For crush lesions, the nerve trunk was pinched with a pair of fine forceps several times, until there remained only a transparent zone about 1 mm long of epi- and perineural sheaths holding the trunk together. In some operations (see Results) the nerve trunk was intentionally transected.

After denervation, the muscle wall and skin were independently sutured. The animals were revived, and were maintained with a 12 hr. light cycle in tanks where they had access to both a dry platform (air temp 20-22^{o}C) and a reservoir of running tap water (14-18^{o}C). They were fed weekly with homogenized beef liver or pet food.

After intervals of 1-22 weeks, animals were pithed and the ganglionic innervation studied. Twenty to 35 cells were sampled in a given preparation, usually at least 10 each of B and C cells. (The ability to anticipate a cell's type by its diameter made it possible to select for C cells.) The results from ganglia examined at specific post-operative intervals were pooled to obtain generalized information about the time course of innervation.

Results

Normal Ganglia

Neurons in normal ganglia could usually be classified as B or C cells by means of their antidromic propagation rate and their synaptic input, as is so in the toad Bufo

Figure 2. Compound action potentials recorded from the 10th ganglia of two different preparations, following antidromic stimulation of their 10th nerves. In A, three major populations of post-ganglionic axons were present, corresponding to B, I, and C groups. In B, mainly B and C cell axons were present. Calibration bars: horizontal, 10 msec; vertical, 20 V. Photographs retouched.

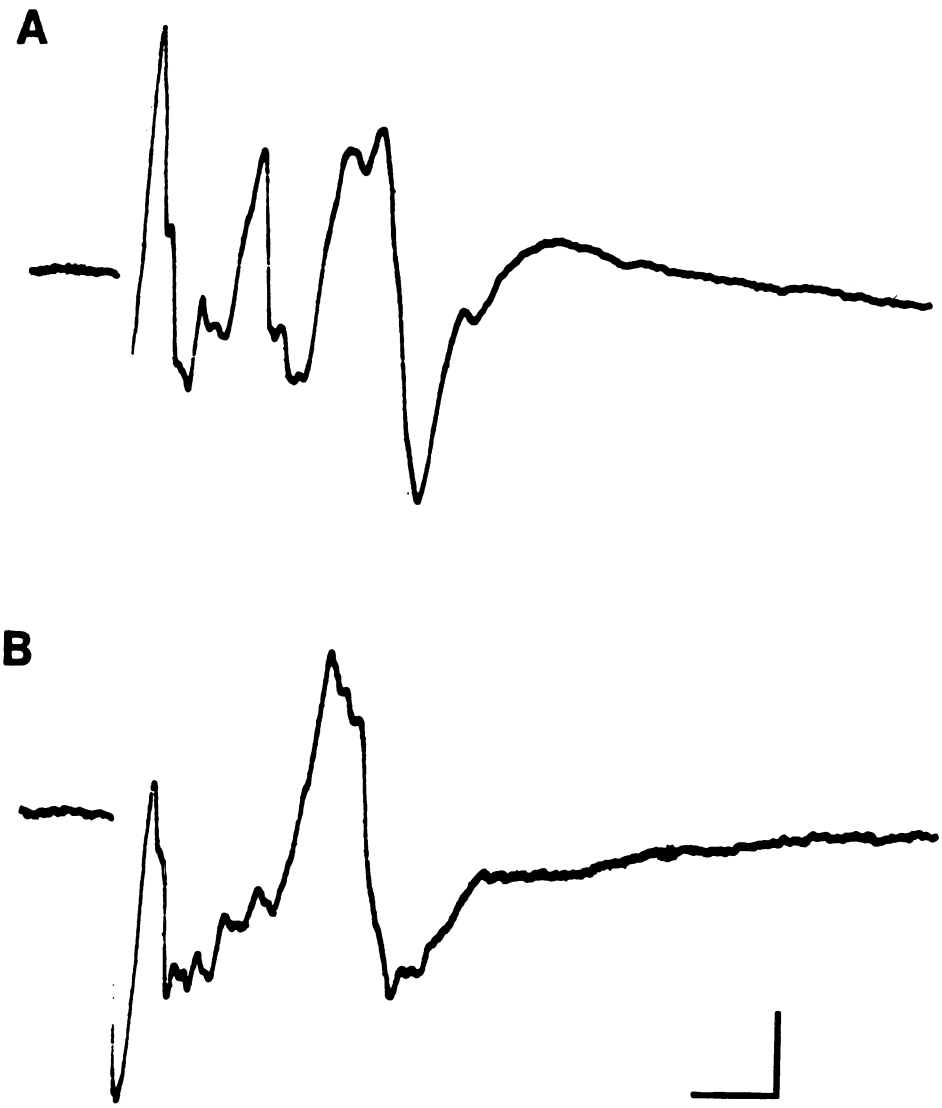

Fig. 2. Compound antidromic action potentials in frog ganglia.

vulgaris japonica (Nishi et al., 1965) and in other species of frog, Rana catesbiana (Libet et al., 1968) and R. esculenta (Francini & Urbani, 1973; Skok, 1965). In some ganglia a class of neurons not recognized in the other anuran species could be distinguished, which will be referred to as intermediate (I) cells. These I cells were characterized by axonal conduction velocities, excitation thresholds, and soma diameters intermediate between those of B and C cell populations (see Figures 3a,b). The synaptic inputs to I cells were B fibers, as judged by their entrance into the sympathetic trunk anterior to the 7th ganglion, and the latencies of orthodromic activation, which were similar to those of B cells. Responses from these three classes of ganglion cell can be seen in Figure 2a which is a recording made from the surface of the 10th ganglion of one preparation, following antidromic stimulation of the nerve approximately 6 mm away. Three well-defined potential peaks are visible, which are correlated with approximate conduction velocities of 1.5, 0.4, and 0.2 m/sec. The first peak results from activity in the axons of B cells. The conduction velocities of the second and third peaks would classify them as C fibers; however, I distinguish them because of their differing conduction velocities, and their differing sources of innervation.

The results of intracellular recording from cells in normal ganglia are shown in Figure 3a. The number of cells (ordinate) of given latencies of antidromic spike invasion

Figure 3. Properties of neurones in normal ganglia. A. Histogram of antidromic action potential invasion latencies for neurones in normal ganglia, tabulated according to source of preganglionic input. Neurones in which synaptic potentials could be elicited by stimulation of the anterior sympathetic trunk (i.e. innervated by B fibers) are indicated by open bars; those in which synaptic potentials were elicited by stimulation of the 7th or 8th nerves (i.e. innervated by C fibers) are indicated by shaded bars. When the data for all normal ganglia are thus pooled, these two populations of neurons overlap slightly in antidromic latency. Within individual ganglia, however, such overlap was never observed; cells innervated by C fibers always had longer latencies than those innervated by B fibers. B. Scatter plot of cell body diameters plotted as a function of antidromic latency. Neurones with B fiber input are plotted with open circles (O); neurones with C fiber input are plotted with filled circles (●). Measurements of the living neurons were made with an eyepiece micrometer when antidromic latency was determined. For oblong shaped neurones, the measurements of long and short axes were averaged.

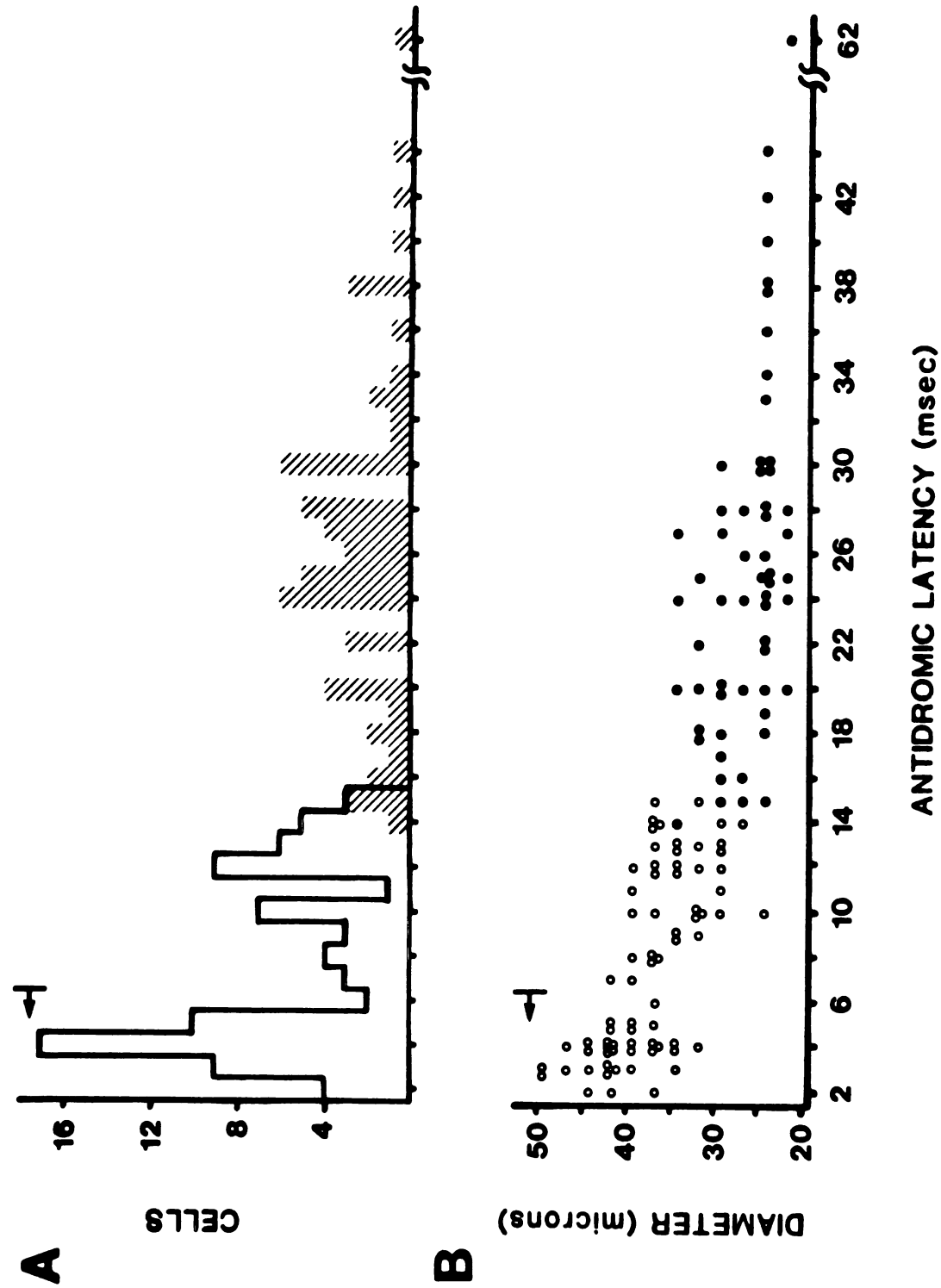

Fig. 3. Properties of neurons in normal ganglia.

(abscissa) are indicated in that figure, along with the type of innervation they receive. The histogram includes data from all normal preparations studied, without correcting for nerve length or other sources of variability of antidromic latency. Cells appear to fall into three populations. The fastest conducting group and the intermediate group are innervated by B fibers, stimulated by shocking the sympathetic trunk anterior to the 7th ganglion, whereas cells in the slowest conducting group are innervated by C fibers, entering via the 7th or 8th spinal nerves. Of the neurons innervated by B-fibers, I have arbitrarily taken those with latencies of 6 msec or less as B cells, and those with latencies longer than 6 msec as I cells. All the cells innervated by C fibers are considered to be C cells. Assuming an average conduction distance of 5 mm, approximate mean conduction velocities for these populations are: B cells, 1.4 $\pm$0.4 m/sec (mean $\pm$ standard deviation); I cells, 0.46 $\pm$0.11 m/sec; C cells, 0.2 $\pm$0.06 m/sec.

The cells of the three populations varied in size, in accordance with their conduction velocities, as shown in Figure 3b. The mean soma diameters of the cells identified as members of these classes were as follows: B cells, 39.7 µm $\pm$3.8 µm; I cells, 32.6 µm $\pm$3.6 µm; C cells, 26.2 µm $\pm$3.4 µm. These differences in size between the three cell types facilitated selection of cells of any desired class for intracellular recording.

The relative numbers of cells in the three populations, judged by the amplitudes of antidromic potentials recorded extracellularly and the antidromic latencies recorded intracellularly, varied among different preparations. The intermediate population was the most variable. In some ganglia no intermediate cells were found by intracellular recording, and few or none were detected by extracellular recording of the antidromic compound action potentials. Such ganglia had only well-separated B and C volleys. Figure 2b is an example taken from one such ganglion.

Experimental Ganglia

In normal ganglia, B and C cells are readily distinguishable by their conduction velocities and their preganglionic innervation. Soma diameters are also useful for distinguishing cell types. However, in denervated and reinnervated ganglia the nature of the innervation to the neuronal types was itself the subject of investigation, and could not be used as an indication of post-ganglionic cell type. Cells were therefore defined by their conduction rates and diameters. These remained reliable criteria, as demonstrated by the fact that the experimental ganglia, regardless of the type of lesion or the post-operative survival period, were similar to normal ganglia in their neuronal populations. This was true also of several ganglia in which preganglionic input was kept away for several weeks by repeated resection of all inputs. Extracellular recordings

of compound action potentials antidromically propagated into the experimental ganglia resembled those from normal ganglia in every respect; two or three major peaks were discernable, with conduction rates resembling those for the B, C, and I populations found in normal ganglia. Antidromic conduction times, measured by intracellular recording, also fell into the normal ranges. The relative numbers of each cell type populating the ganglia were comparable (though variable) in both experimental and normal animals. The diameters of cells classified according to antidromic latency agreed well with the respective diameter ranges found in normal neurons (data not shown).

In 46 animals, both B and C fiber inputs were interrupted by crushing the sympathetic trunk between the 8th and 9th ganglia ('Lesion 1' in Figure 1). The ganglia were examined at various intervals (2 to 22 weeks) after the denervation. During this period, greater than 95% of the several hundred cells identified as B cells were appropriately reinnervated by B fibers, often by two or three different ones. Innervation of B cells by C fibers was rare (~2%), and most of the B cells with C fiber input simultaneously received B fiber input.

The reinnervation of intermediate cells was not systematically studied, although the results were essentially the same as for B cells: I cells were reinnervated primarily by B fibers.

Figure 4. A. Time course of reinnervation of C cells by B fibers in denervated ganglia. Each bar represents the data pooled from several preparations examined during the numbered postoperative week(s). The results from ganglia denervated by a single crush lesion ('Lesion 1' in Fig. 1) are shown with solid bars. The open bar shows the results from ganglia in which the normal C fiber input to C cells was kept away by cutting the rami communicantes connecting nerves 7 and 8 to the sympathetic trunk ('Lesion 2' + 'Lesion 1'). B. Time course of reinnervation of C cells by C fibers in denervated ganglia. The same populations of neurons represented by the solid bars in (A) are represented here. In most ganglia studied from 6 to 22 weeks after denervation, the contribution of C fibers from the 7th nerve was neglected; thus, values for these time bins represent a lower limit to the percent of C cells reinnervated by C fibers. The 7th nerve typically innervates 20-30% of the C cells in a normal ganglion.

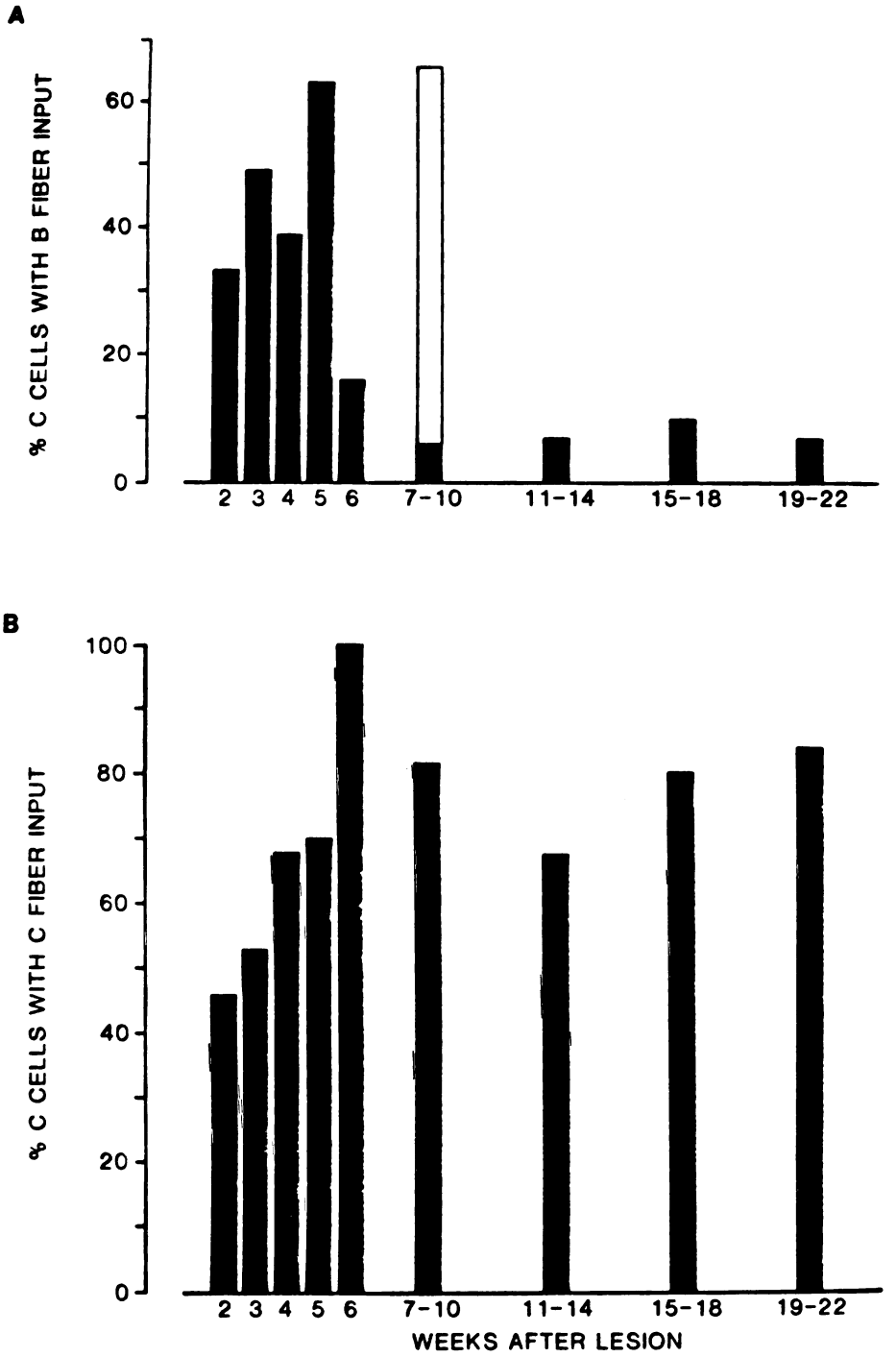

Fig. 4. Time course of reinnervation of C cells.

The nature of the synaptic input to C cells varied with the survival period. As shown in Figure 4a, a large fraction of C cells examined during the first 5 weeks of reinnervation were reinnervated by B fibers, often by two or more. The frequency of such inappropriate innervation dropped off sharply during the 6th week, declining to less than 10% subsequently.

C fiber input to C cells returned during the first several weeks of reinnervation; as shown in Figure 4b, appropriate input is restored to 70% of C cells by the 4th week, prior to the time when inappropriate input begins to disappear. C cells frequently received both B and C fiber input during weeks 2 to 5. Figure 5 shows responses from a cell which received both appropriate and inappropriate inputs.

The inappropriate synapses often gave rise to synaptic potentials of sufficient amplitude to fire the C cells. Though not rigorously studied, the rise times and durations of potentials elicited by inappropriate synapses were comparable to those of regenerated appropriate synapses. For cells simultaneously innervated with both fiber types, there was no consistent trend as to which type of input gave the larger synaptic potential.

The loss of B fiber -> C cell synapses illustrated in Figure 4a could represent a <u>selective</u> loss of inappropriate synapses, or it could represent a <u>random</u> withdrawl of B

Figure 5. Intracellular recordings from a C cell which received both appropriate and inappropriate synaptic input. A. Supra-threshold synaptic potential resulting from stimulation of the B fibers in the sympathetic trunk anterior to the 7th ganglion. B. Supra-threshold synaptic potential resulting from stimulation of the C fibers in the 8th nerve. Note the difference in latencies, which is typical for B and C fiber inputs, reflecting the difference in relative conduction velocitites. Calibration bars: horizontal, 10 msec; vertical 20 mV. Action potentials retouched.

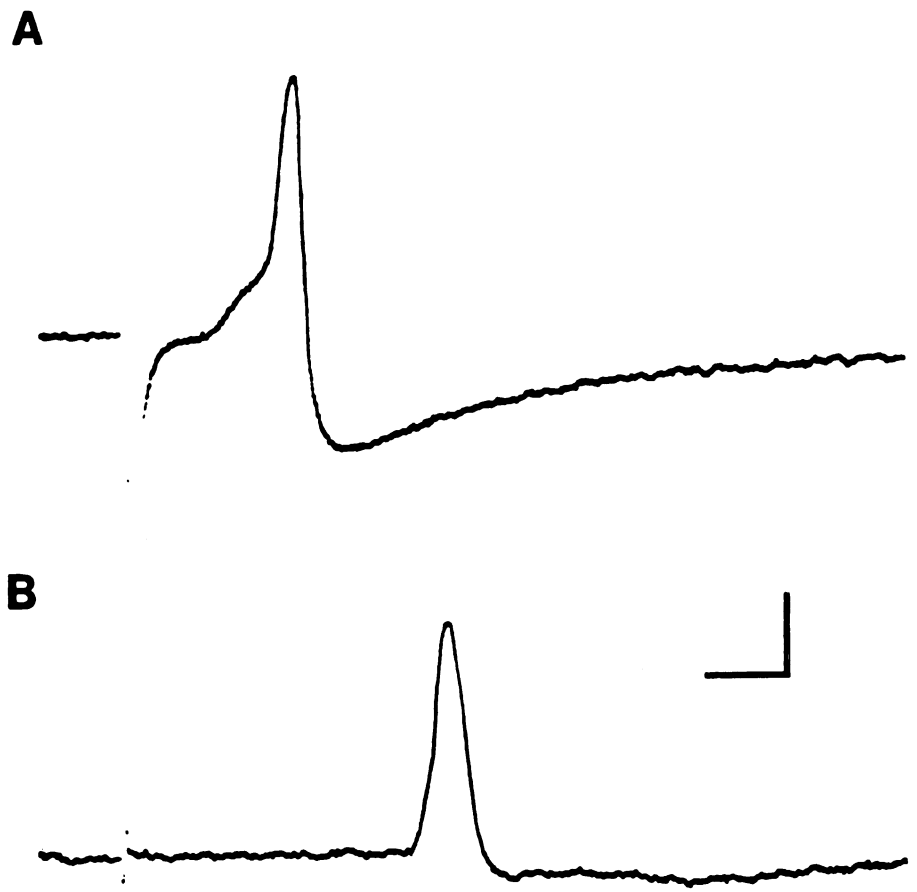

Fig. 5. C cell with appropriate and inappropriate input.

fiber synapses from both B cells and C cells. To distinguish between these possibilities, I have compared the mean numbers of B fibers innervating cells of each class at various intervals after preganglionic lesion. As shown in Figure 6, during the first several weeks both B and C cells aquired new inputs. During the period 6 or more weeks after lesion, the average number of B fibers innervating the C cells (inappropriate synapses) dropped markedly, eventually down to about one-fifth of its earlier value. On the other hand, the average number of B fiber inputs to B cells (appropriate synapses) decreased less rapidly, and to a lesser extent; even by the final time interval, four-fifths of the maximal number of innervating fibers were still detectable. Although there is an overall decrease in the number of B fibers innervating both types of neurons, the withdrawl of synapses does not appear to be random; Both the different time courses, and the different magnitudes of withdrawl of synapses from the two cell classes, indicate that synapse loss is selective.

In four animals the sympathetic trunk was severed, rather than crushed, between the 9th and 10th ganglia. Between 9 and 12 weeks after denervation, the innervation pattern closely resembled that seen in the crush preparations six or more weeks after denervation. Twenty out of 26 B cells (77%) were appropriately reinnervated by B fibers, and 35 of 50 C cells (70%) were appropriately reinnervated by C fibers. Two (8%) of the B cells received C fiber

Figure 6. Selective loss of inappropriate synapses. The mean numbers of B fibers innervating B cells (open bars) and B fibers innervating C cells (filled bars), at various intervals after preganglionic lesion, are compared. For ease of comparison, the data are expressed as percentages (left-hand scale) of the maximal mean number of B fiber inputs attained by each cell class. The right-hand scales give the actual mean numbers of inputs for each cell class, showing the degree of scaling of the normalized data. The error bars represent standard errors ($\pm$ S. E.) of the values.

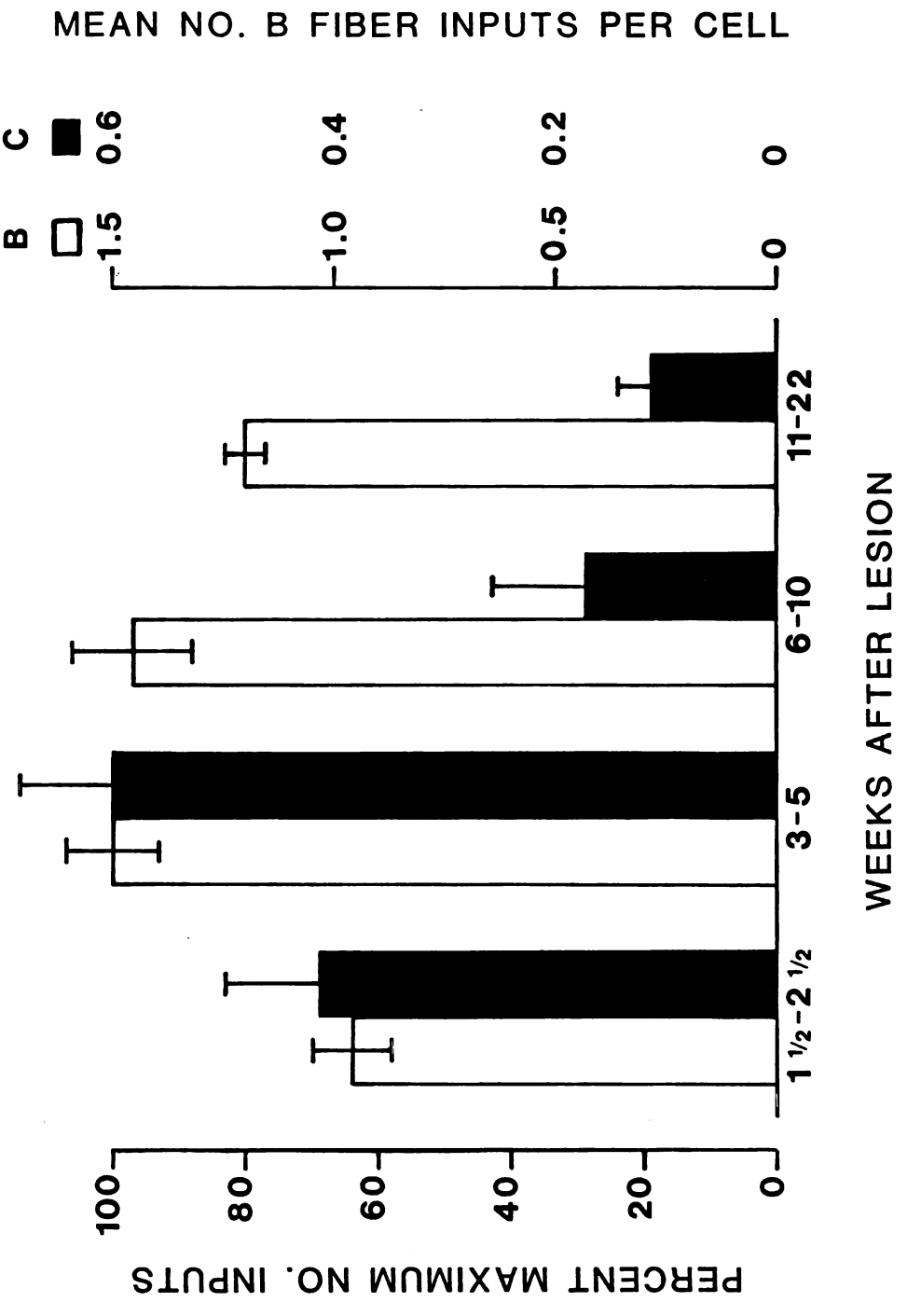

Fig. 6. Selective loss of inappropriate synapses.

input, and 8 (16%) of the C cells received B fiber input. Thus most cells were appropriately reinnervated and relatively few were inappropriately reinnervated. Given this accuracy of reinnervation following nerve cut, it is unlikely that the reestablishment of appropriate synaptic connections depends primarily on axons growing through the old sheaths back to their original target cells.

Timing of Reinnervation by B and C Fibers

That inappropriate inputs develop frequently on C cells, but only infrequently on B cells, suggests that the larger B fibers may reinnervate the ganglia prior to the small C fibers, contacting not only their appropriate B cell targets but many C cells as well; it is known that larger motor axons regenerate more rapidly than smaller ones (Schmidt & Stefani, 1976). In support of this hypothesis, ganglia examined during the first two weeks after denervation showed extensive reinnervation by B fibers, but only sparse reinnervation by C fibers. If B fiber -> C cell synapses were generated more frequently than C fiber -> B cell synapses because the B fibers returned first and encountered uninnervated C cells, then it might be possible to generate more C fiber -> B cell connections by giving C fibers an advantage in reinnervating ganglia. I tested this possibility by crushing the sympathetic trunk anterior to the 7th ganglion in addition to the usual crush between the 8th and 9th ganglia (Lesion 1 + Lesion 3 in Figure 1).

Under these conditions the C fibers needed to grow only a relatively short distance to the 9th and 10th ganglia (2-3 mm to the 10th), whereas the B fibers had several times as far to regenerate, and in addition had to cross two lesion sites instead of one. In 7 preparations examined 3-9 weeks after such a double lesion, 13 out of 74 (18%) B cells received synaptic input from C fibers. This is substantially higher than the 2% incidence observed after the single crush lesion described above. Most of these ganglia had not been reinnervated by B fibers; in a few the B fibers had returned and formed synapses with B cells predominantly, but also with some C cells. These results support the hypothesis that the type of inappropriate connections (C fiber -> B cell or B fiber -> C cell) that arises depends in part upon the relative time of return of the two groups of preganglionic fibers.

When B fibers reinnervate the ganglion in advance of C fibers, it is of interest to know whether they form synapses non-selectively with B and C cells, or if they show some 'preference' for appropriate connections with B cells. I therefore further retarded the return of C fibers by cutting the 7th and 8th communicating rami (marked 'Lesion 2' in Figure 1), as well as crushing the trunk anterior to the 9th ganglion, thereby prolonging the period of reinnervation by B fibers alone. In 8 ganglia examined 1 to 3 weeks after such a lesion, 64 of 78 B cells (82%) but only 33 of 85 C cells (39%) were reinnervated by B fibers. Likewise, in the

ganglia described above, in which the return of B fibers was retarded (Lesion 1 + Lesion 3), the C fibers preferentially reinnervated C cells; although 13 of 74 B cells (18%) were reinnervated by C fibers, 45 of 68 C cells (66%) received C fiber input. These two sets of results thus indicate that from the outset, there is preferential reformation of appropriate connections, which occurs in the absence of competition between preganglionic fiber types.

Competition between Appropriate and Inappropriate Synapses

In the first group of animals described above (Lesion 1 only) I observed the appearance and eventual decline of inappropriate synaptic connections. The fact that inappropriate connections of B fibers began to decline only after appropriate C fiber input had returned to the majority of C cells (compare Figures 4a and b) suggests that the elimination of inappropriate synapses results from some form of competition between synapses. Alternatively, B fiber inputs to C cells might disappear spontaneously in the absence of competition from C fibers. To distinguish these possibilities, I prevented C fibers from reinnervating ganglia as described above (Lesion 1 + Lesion 2) for 6-10 weeks (this usually required resection of the entire 7th & 8th rami). In this period of time, inappropriate B fiber inputs to C cells would have been reduced had C fibers been allowed to return (see Figure 4a, solid bars). However, in ganglia reinnervated by B fibers alone, 54 of 83 C cells (65%)

retained inappropriate inputs (Figure 4a, open bar). Thus the disappearance of B fiber inputs to C cells appears to be contingent upon the return of the normal C inputs, suggesting that a competitive interaction is involved in elimination of inappropriate synaptic inputs. Whereas 65% of C cells received B fiber input, all 29 B cells studied in these ganglia received B fiber input. This is further indication of an inherent preference for appropriate synaptic connections, even in the absence of competition, as discussed above.

Discussion

The results presented here indicate that following experimental denervation, frog sympathetic B and C ganglion cells are ultimately reinnervated each by the appropriate class of preganglionic axons (i.e. B fiber -> B cell; C fiber -> C cell). This specificity of reinnervation appears to result from two different processes. From the outset there is a preferential reformation of synapses of the appropriate type, although synapses may be made with inappropriate targets when these are available. In addition, those inappropriate synapses that do arise are eliminated, apparently as a consequence of some competitive interaction between appropriate and inappropriate fibers. The results of the various experiments from which these conclusions are drawn are summarized in Table 1.

Table 1. Summary of reinnervation experiments.

LESION (see fig. 1)	PURPOSE	APPROPRIATE SYNAPSES		INAPPROPRIATE SYNAPSES		MAJOR CONCLUSIONS
		B fiber → B cell	C fiber → C cell	B fiber → C cell	C fiber → B cell	
Lesion 1 (crush)	Observe reinnervation by both B & C fibers. Will synapses be formed selectively?	95%	see fig. 4b	see fig. 4a	<2%	Inappropriate synapses are formed transiently, and are replaced by appropriate ones.
Lesion 1 (cut)	Determine whether axonal guidance is primarily responsible for selective reinnervation.	77%	70%	16%	8%	Synpses regenerate selectively without continuity of extra-axonal nerve sheaths.
Lesions 1 & 2 1-3 weeks	Retard return of C fibers. Will B fibers preferentially reinnervate B cells?	82%	none	39%	none	Preference for appropriate synapses in the absence of competition between preganglionic fiber groups.
Lesions 1 & 3	Retard return of B fibers. Will C fibers preferentially reinnervate C cells?	none	66%	none	18%	Same as above
Lesions 1 & 2 6-10 weeks	Keep C fibers away. Will inappropriate synapses made by B fibers disappear spontaneously?	100%	none	65% see fig. 4a	none	Despite the preferential formation of appropriate synapses, inappropriate ones are maintained in the absence of competition from the other fiber group.

The Relative Nature of Synaptic Specificity

By making selective lesions, it has been possible to induce the return of either B fibers or C fibers in the absence of the other fiber group to the ganglia which contain both B and C cells. Under these conditions, synapses with appropriate targets (B fiber -> B cell; C fiber -> C cell) are found more often than connections with inappropriate targets (B fiber -> C cell; C fiber -> B cell). This indicates that there is a preference for appropriate connections which is independent of competition between preganglionic fibers. That this is not simply due to the relative numbers of B and C cells in the ganglia is shown by the fact that 'preference' is demonstrated for both B fiber -> B cell and C fiber -> C cell connections (see Table 1).

Several bases for this preference may be postulated. One possibility is that axons are mechanically guided back to former synaptic sites through individual channels bounded by connective tissue and glial cells. Such channels might escape serious damage in the crush lesions used in most of these experiments. Another possibility is that regenerating axons distinguish and follow specific pathways back to their former targets, by means of growth cone-substrate interactions. Alternatively, such guidance mechanisms might serve only to bring axons to the general vicinity of ganglion cells, with the preferential formation of appropriate connections resulting from recognition between axons and gan-

glion cells, perhaps by selective affinity for each other (Sperry, 1963). In considering the role of guidance mechanisms in the establishment of appropriate connections, it should be stressed that B and C cells are intermingled throughout the ganglion. Although satellite cells, fibrocytes, and a collagenous network surround ganglion cells and fiber bundles within the ganglia, it is not known to what extent the individual axons pass along isolated channels. It is unlikely that axons are confined to a rigid network guiding particular axons to particular cells, as shown by experiments reported in Chapter 3, in which intact C fibers were induced to sprout and form functional synapses with B cells denervated by transection of the B fibers; no barrier prevents the sprouting fibers from forming synapses with B cells. The formation of many inappropriate synapses in the present experiments further argues against a rigid guidance mechanism; it also argues against an exclusive, inviolable form of cell recognition.

The results show that in the absence of a 'preferred' synaptic partner, a ganglion cell may receive innervation from an inappropriate preganglionic axon. Furthermore, if the preferred input is kept away, the inappropriate connection is retained: it is not eliminated spontaneously. Various studies of cross-innervation have shown that ganglionic neurons in mammals and amphibians may be innervated by a variety of foreign cholinergic axons (Sargent & Dennis, 1977; Proctor, Frenk, Taylor, & Roper, 1979; McLachlan,

1974; Purves, 1976).

Upon regeneration of the C fibers into ganglia already reoccupied by B fibers, the C fibers form synapses with C cells that have already been reinnervated inappropriately by B fibers. It is interesting that C fibers will synapse with C cells previously reinnervated by B fibers, but rarely will the returning C fibers synapse with B cells previously reinnervated by B fibers. This suggests that the presence of an appropriate connection discourages (or fails to encourage) the formation of an inappropriate one, whereas the converse does not apply. Through a competitive interaction that is not understood, the preferred synapses are retained and the inappropriate ones are eliminated, as judged by physiological criteria. B fiber -> C cell synapses may be lost because the pre- or post-ganglionic neurons recognize these connections as inappropriate. However, 'inappropriateness' per se need not be the reason for their loss; it may be that the B fibers, which have reinnervated most of the B cells and many C cells as well, are 'overextended' and make more synapses than their metabolic machinery can ultimately maintain (Purves, 1976). Under such circumstances, some of the synapses must be lost (although they are maintained for at least 10 weeks when C fibers are kept away). The results show that synapses made by B fibers are not lost randomly: synapses with C cells are eliminated, but those with B cells are not. Regardless of the primary cause of this elimination, there clearly must be something that distinguishes

inappropriate synapses from appropriate ones; synapse elimination is selective.

These findings are consistent with a modified form of Sperry's chemoaffinity hypothesis (Sperry, 1963) in which neurons recognize each other by means of a family of surface molecules. Appropriateness of connection is determined in a relative, rather than in an absolute manner. Thus, if a preferred synaptic connection is not possible, a less preferred connection is acceptable, and better than no connection at all. Cells may have a hierarchy of preference as to which axons they will make connections with. Conversely, the incoming axons may have a range of affinities for potential target neurons. The selectivity may be exerted by either the pre- or post-ganglionic cell, or both.

With the meager information available, one can only speculate on the nature of the competition between appropriate and inappropriate axons. It may be mediated through the post-synaptic cell: for example, the cell may recognize the preferred axon, and maintain post-synaptic specializations only at the site of contact with the preferred axon. Such specializations might include adhesion sites or surface molecules that promote the differentiation of the presynaptic terminal. Alternatively, a diffusible factor released from a restricted region of the post-synaptic membrane might serve to promote presynaptic differentiation. The ganglion cell might then release such a factor to some of its inputs

preferentially. A second possible mechanism for competition would involve the post-synaptic cell only indirectly; the axons could compete directly with each other for synaptic sites, the outcome depending upon relative affinities of the axons for restricted adhesion sites on the post-synaptic cell. Implicit in such a mechanism is that the adhesion sites of B and C cells must differ, and that the preganglionic fibers can distinguish this difference. Finally, recent studies of regeneration of motor axon terminals into former synaptic sites on muscle fiber sheaths devoid of post-synaptic myofibers (Marshall, Sanes, & McMahan, 1977; Sanes, Marshall, & McMahan, 1978) raise the possibility that the competing fibers recognize components of the extracellular matrix surrounding the ganglion cells. Those studies show that components within the muscle fiber basal lamina can direct the precise reinnervation of former synaptic sites, and can direct the differentiation of motor nerve terminals at those sites. If such a mechanism operates in the neuronal system studied here, the competition might then involve recognition by regenerating preganglionic axons of extracellular matrix constituents installed there either by the post-synaptic cells, or the original preganglionic terminals.

Selective Reinnervation in Other Systems

Landmesser and Pilar (1970) reported that two neuronal populations in the pigeon parasympathetic ciliary ganglion

were reinnervated by appropriate preganglionic axons following section and regeneration of the oculomotor nerve. Their data, based on extracellular recording of compound action potentials, do not clearly indicate whether or not transient inappropriate synapses were formed; that technique would not have revealed small (i.e. generating small post-synaptic potentials) inappropriate connections.

Selective reinnervation of neurons also occurs in mammalian sympathetic ganglia (Langley, 1897; Guth & Burnstein, 1961; Nja & Purves, 1977a,b). However, in the guinea pig, Nja and Purves were unable to find evidence for formation of inappropriate connections, even early in reinnervation (Nja & Purves, 1978). The discrepancy between their results and my own could be due to a more effective mechanism of early selection for preferred connections in guinea pigs than in frogs, or to a more rapid elimination of incorrect contacts in the warm-blooded animals. The innervation of mammalian ganglion cells is considerably more complex than that of frog ganglia, and the methods for assessing specificity are less direct. Therefore, if a transient period of relative imprecision exists in the mammal, it might escape detection. The simplicity and manipulability of innervation in frogs, the direct manner in which an appropriate connection may be detected, and a presumably slower rate of regenerative remodeling in the cold-blooded frog, have enabled the resolution of two separate processes that underly selective reinnervation.

Formation and subsequent suppression of two different types of abnormal synaptic input to parasympathetic ganglion cells in the frog heart have been reported. Abnormal synapses between the ganglion cells (Sargent & Dennis, 1981) and synapses by hypoglossal motorneurons onto ganglion cells (Proctor et al., 1979) are both suppressed when the normal vagal preganglionic axons reinnervate the heart and restore synaptic function. In both cases, the abnormal synaptic inputs persist if the normal innervation is kept away, indicating that in this system as well synaptic competition plays a role in the restoration of normal connections.

Amphibia show both selective reinnervation of distinct myofiber types within individual skeletal muscles (Hoh, 1971; Schmidt & Stefani, 1976) and appropriate reinnervation of whole muscles by matching motor nerves (Grimm, 1971). Furthermore, in amphibian muscles, previously-formed inappropriate synapses may be eliminated upon return of the normal innervation (Schmidt & Stefani, 1976; Dennis & Yip, 1978). In contrast, adult mammalian skeletal muscle does not appear to show selective reinnervation (Bernstein & Guth, 1961; Miledi & Stefani, 1969; Frank, Jansen, Lomo, & Westgaard, 1975). Dennis & Yip (1978) propose that these differences between amphibians and mammals might reflect the retention of some embryonic characteristics by adult neurons in the more primitive species. Whatever the reasons, regeneration of appropriate neuronal connections in mammals has so far been convincingly demonstrated only in the autonomic

nervous system.

Implications for Development

The formation of inappropriate synapses and their eventual elimination in favor of appropriate ones may also occur during development of the nervous system. The segmental organization of neuromuscular connections in amphibians and birds appears less precise in embryos than in adults (Lamb, 1976; Pettigrew, Lindeman, & Bennett, 1979) A growing body of evidence (So, Schneider, & Frost, 1978; Rakic, 1977; LeVay, Stryker, & Shatz, 1978; Innocenti, Fiore & Caminiti, 1977) indicates that neurons of the immature CNS project to targets beyond those they will innervate in the adult animal. In at least one case (So et al., 1978) the retraction of an inappropriate projection appears contingent upon the arrival of the appropriate afferent axons. It is generally not known from studies of this type whether functional synapses are formed by the inappropriate projections, although in the studies of neuromuscular connections cited above, and in the study of thalamocortical projections described by LeVay et al., (1978), function was demonstrated.

As described in Chapter 4, I have found evidence that in developing tadpole sympathetic ganglia functional connections between pre- and post-ganglionic neurons are less selective than they are in adults. It may be that the regeneration of connections recapitulates a sequence of

events that takes place during the development of these ganglia. Although regeneration is not completely analogous, the restoration of a more-or-less normal pattern of connections suggests that some of the mechanisms that contribute to the specificity of synapse formation during development do exist in the adult frog.

PARTIAL DENERVATION OF THE FROG SYMPATHETIC GANGLION RESULTS IN A TRANSIENT LOSS OF NEURONAL SPECIFICITY

One form of placticity in the nervous system is the sprouting and formation of new synapses by intact axons following the removal of synaptic input to a neuronal field or peripheral target. This phenomenon has been described in the central, (Liu & Chambers, 1958; Raisman & Field, 1973) the autonomic, (Murray & Thompson, 1957; Courtney & Roper, 1976; Sargent & Dennis, 1977) and the peripheral (Edds, 1953; Weddell et al. 1946) nervous systems. In the central nervous system (CNS) it is believed that the responding axons are of types that form part of the normal complement of synaptic input to the partially denervated target cells (Cotman & Lynch, 1976). In the autonomic and peripheral nervous systems this point is unclear. In frog sympathetic ganglia, two intermingled ganglion cell populations, and their respective sources of preganglionic input, can be distinguished: B cells are innervated by B fibers, and C cells are innervated by C fibers. (Nishi, et al. 1965; Libet, et al. 1968; Francini & Urbani, 1973; see Chapter 2) By selectively cutting the B fibers, I show here that C fibers may be induced to sprout and form synapses with B cells, with which they do not normally synapse. When the B fibers regenerate, they reform synapses selectively with B cells only, and the sprouted synapses can no longer be detected electrophysiologically. Thus, the normal pattern of synaptic connections is restored.

Methods

In experimental animals, the B fibers were cut by transecting the sympathetic trunk anterior to the 7th ganglion. (See Chapter 2 for complete description of surgical and physiological techniques.) During subsequent weeks ($1\ ^{1}/_{2}$ -21 weeks post-operatively) ganglia and associated pre- and postganglionic nerve trunks were dissected out and studied by intracelllar recording. B cells and C cells were distinguished by the size of the cell body (mean diameters, $\pm$S.D.; B cells, 39.7 µm $\pm$3.8 µm; C cells, 26.2 µm $\pm$3.4 µm.) and by the latency of antidromic spike invasion following stimulation of the postganglionic nerve trunk 4-6 mm from the site of intracellular recording in the cell body. B cell latencies were typically 2-6 msec, whereas C cell latencies were 15-40 msec (see Figure 3a, Chapter 2). A 'sprouted' connection was identified when a B cell, identified as above, responded with a fast excitatory postsynaptic potential (Kuba & Koketsu, 1978) to stimulation of the 7th or 8th nerves (which carry only C fibers). In ganglia in which the trunk connective between the 6th and 7th ganglia had regained continuity, presumably due to regeneration of B fibers and associated glial and connective tissues, the B fiber input to ganglionic neurons was determined by stimulating the trunk anterior to the 7th ganglion, but posterior to the site of the original lesion.

Results and Discussion

Recording from normal ganglia confirmed that in Rana pipiens, as in other frog species (Libet, et al. 1968; Skok, 1965; Francini & Urbani, 1973) B cells are innervated solely by B fibers originating anterior to the 7th ganglion and that C cells are innervated exclusively by C fibers entering the trunk at the 7th and 8th segmental levels (see Figure 3a, Chapter 2). In some ganglia, both normal and experimental, a class of neurons not preveously described was distinguished. These cells are characterized in Chapter 2, and were not considered in the present study. In experimental ganglia, in which B fiber input had been removed 1 $^{1}/_{2}$ - 5 weeks prior to recording, nearly half of the B cells (n=68, pooled from 6 ganglia) received synaptic input from one or more C fibers, as in the example of Figure 7 (see Table 2). The C cells in these ganglia appeared normally innervated. The synapses from sprouted C fibers onto B cells usually generated rapidly-rising synaptic potentials, often of sufficient amplitude to fire the post-synaptic cell after a single orthodromic impulse. Some sprouted inputs generated more slowly-rising potentials, possibly due to the synapses being on the axon at some distance from the cell body.

In ganglia examined 6-21 weeks after partial denervation, continuity of the sympathetic trunk was usually reestablished, although the site of the original lesion could often be seen under the dissecting microscope. In 4 ganglia

Table 2. Appearance and regression of sprouted synapses after partial denervation.

Time after cutting B fibers	B cells		C cells	
	% with B fiber input	% with C fiber input	% with B fiber input	% with C fiber input
1 $^{1}/_{2}$ - 6 weeks	0 (n=68)	49 (n=68)	0 (n=40)	100 (n=40)
7 - 21 weeks	92 (n=38)	0 (n=38)	0 (n=22)	99 (n=22)

Figure 7. Physiological evidence of 'sprouted' synapses. a. Antidromic action potential from a B cell which received at least two sprouted C fiber synapses. The short latency is typical of B cells. b & c. Synaptic potentials elicited in this cell upon stimulation of C fibers in the 7th (b) and 8th (c) nerves. The relatively long latencies of these responses are typical for C fibers. The response shown in (c) was of sufficient amplitude to elicit an action potential. Calibration bars: horizontal, 10msec; vertical, 20mV. Action potentials retouched.

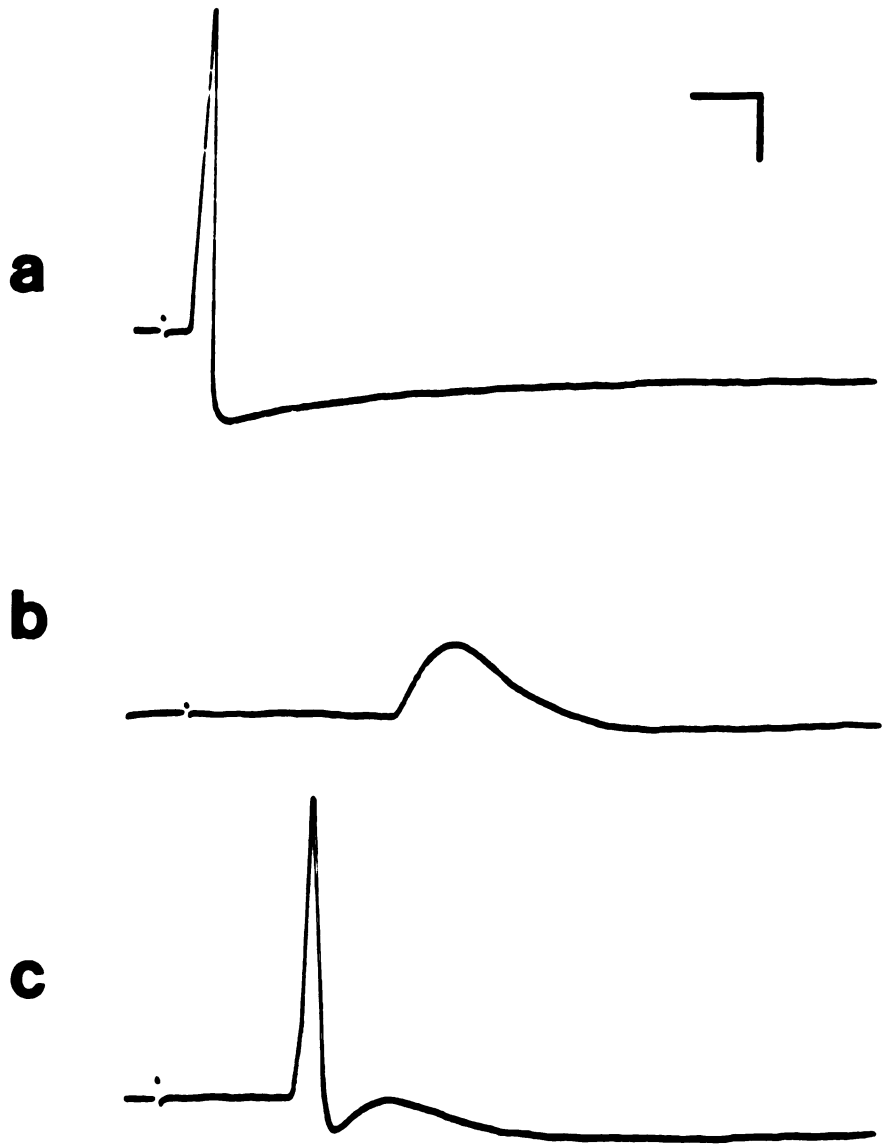

Fig. 7. Physiological evidence of 'sprouted' synapses.

with trunk continuity reestablished, no C fiber inputs to B cells were detected, and nearly all the B cells (n=38) were reinnervated by B fibers. Surprisingly, no C cells innervated by regenerated B fibers were detected either-- C cells (n=22) were innervated by C fibers only (see Table 2).

These results indicate that when B cells are denervated by selectively removing all B fiber input, intact C fibers are induced to sprout and form synapses with the denervated neurons. C fiber -> B cell synapses are not normally found, even though C fibers are in close proximity to B cells. (B cells are often immediately adjacent to C cells, which are innervated by C fibers.) The stimulus for sprouting, whatever its nature, is not specific for one or another type of preganglionic sympathetic fiber. Studies of sprouting in partially denervated mammalian superior cervical ganglia (Murray & Thompson, 1957) suggest a similar conclusion, but the behavioral methods used in those studies could not distinguish whether sprouts innervated new neurons (i.e. neurons they had not preveously innervated prior to partial denervation), or whether the responding axons simply formed additional and more effective connections with neurons they already innervated. One implication of the present results is that the developmental mechanisms that assure proper connections between the pre- and postganglionic neurons of sympathetic ganglia do not depend upon mechanical exclusion of contact between preganglionic fibers and innapropriate ganglion cells: no such mechanical barrier prevents the

formation of innapropriate sprouted synapses.

When the cut B fibers regenerate, they form synapses with B cells and the sprouted synapses are no longer detectable electrophysiologically. Presumably the sprouted synapses are lost as a consequence of a competitive interaction between regenerating B fibers and sprouted C fibers. The basis of this competition is not known, however. C fibers may have a competitve disadvantage because they make synapses with many B cells in addition to their normal connections with C cells, and they are thus 'over-extended' (Purves, 1976). Perhaps the synapses most readily lost under such circumstances are those recently formed through sprouting. An alternative explanation for the loss of sprouted synapses is that they are somehow inappropriate for the B cells, and are recognized as such by either the pre- or post-synaptic neurons. Such inappropriate synapses might be readily lost when preffered inputs from B fibers become available. These alternatives were suggested as possible explanations for results obtained in frog cardiac (parasympathetic) ganglia (Sargent & Dennis, 1981), which were analogous to the present results. Although the present experiments do not distinguish between these alternatives, studies of reinnervation of completely denervated ganglia (see Chapter 2) indicate that appropriateness of competing synapses plays a major role in determining which synapses will be maintained. In any case, it is the sprouted synapses that are lost when the B fibers return, whereas the

normal synapses with C cells are not displaced.

The sprouting described here, which succeeds partial denervation, results in a transient violation of the pattern of specificity found normally in frog ganglia. These anomolous synapses arise rapidly, without implantation of a foreign nerve into the denervated target. If a similar violation of specificity rules occurs in response to lesions of the CNS, it could provide a partial explanation for the impaired function suffered by victims of strokes or CNS injuries. In this preparation the sprouted synapses can be unequivocally distinguished from normal synapses for study with intracellular physiological recording, and also with morphological techniques. The frog sympathetic ganglion is therefore an excellent model system for studying the phenomena of sprouting and competitive synapse elimination.

INNERVATION OF DEVELOPING SYMPATHETIC NEURONS IN TADPOLES

The experiments described in the preceeding sections show that during regeneration, neurons in the sympathetic nervous system of adult frogs form inappropriate synaptic connections. Subsequently, competitive interactions occur which result in the elimination of inappropriate synapses, and the restoration of appropriate connections. It seems likely that the cellular mechanisms underlying these regenerative capacities also play a role in the assembly of appropriate neural connections during embryonic development. To date, there are no published reports describing the innervation of sympathetic neurons in tadpoles. In light of evidence in the CNS and the peripheral nervous system (see Chapter 1) that developing neural connections are less specific than the connections found in adult animals, and in light of the results of regeneration described above, it is of interest to know how the specificity of connections in frog sympathetic ganglia comes about. Does the specificity found in normal adult animals arise from the outset of synaptogenesis, or does it arise through competitive interactions between synapses initially formed non-selectively?

Here I describe a primary study of the innervation of sympathetic ganglia in Rana catesbiana tadpoles. (R. catesbiana tadpoles were used because of their large size, relative to R. pipiens, which were used in the regeneration

experiments described above.) Although a detailed sequence of the development of synaptic connections is not yet available, the observations presented here do reveal some critical features of that development, and should serve as a basis for further study.

Methods

Animals were staged according to Taylor & Kollros (1946), a staging scheme developed for R. pipiens larvae; the development of R. catesbiana conforms adequately. A stage for each specimen could be defined precisely, or within narrow limits spanning 2 stages. Most animals studied were at stages 17-18, by which time the larvae are relatively large, and the hindlimb (a major target of the neurons of the 9th and 10th ganglia) is well-developed and has become functional. Some preparations were studied as early as stages 14-15, and some at stages 21-22, during the period of rapid metamorphosis. Post-metamorphic juvenile frogs were also studied.

The dissection in tadpoles is complicated by several factors: all structures, especially the rami communicantes, are small and extremely fragile; the sympathetic trunk lies directly on the aorta, and is ensheathed by a densely pigmented membrane, making it difficult to see, and increasing the risk of bleeding; there is poor access to the the proximal portions of spinal nerves 8 and 9, which are buried in the dorsal muscle wall; the ganglia are indistinct, and are

enmeshed in connective tissue, thereby hindering cleaning for intracellular recording. Methods were devised to deal with these problems, as detailed below. Nevertheless, many preparations were damaged during the dissection, often to the extent that they could not be used.

Animals were anaesthetized in 0.1% (w/v) Tricaine Methanesulfonate (Sigma) for 5 min. In some instances animals were soaked for 20 min in 0.01% (w/v) melatonin (Sigma), a procedure that causes contraction of melanophores (Bagnera, 1963), thereby improving somewhat the visibility of the sympathetic trunk. A ventral incision was made, and the heart was exposed. Animals were perfused through the heart with a ringer solution of the following composition (mM): NaCl, 120; KCl, 2; $CaCl_2$, 5.0; glucose, 10; HEPES buffer, 4 (pH 7.3). The viscera were dissected away, exposing the dorsal wall of the peritoneum, beneath which lie the kidneys. The kidneys were removed from the dorsal body wall by lifting and cutting the mesentary in which they lie, taking care not to damage the underlying spinal nerves, rami communicantes, and sympathetic chain. The ganglionic chain was severed anterior to the 6th ganglion, and the posterior portion was freed from the aorta. The distal (post-ganglionic) 9th spinal nerve was freed by trimming the mesentary which attaches it to the body wall. (Most recordings were made from the 9th rather than the 10th ganglion because at stages 17-18, there appeared to be little or no connection between the 10th ganglion and the 10th nerve.) To

obtain the proximal (preganglionic) segments of the 7th and 8th spinal nerves, it was necessary to dig into the dorsal muscle wall, by teasing apart the muscle fibers. Care was taken to avoid stretching of the delicate rami communicantes while the spinal nerves and sympathetic trunk were freed. The preparation was mounted in the recording chamber and fitted with suction electrodes, as described for adult preparations. Superficial connective tissue was teased from the 9th ganglion, insofar as possible without destroying the ganglion itself. Ringer solution, as above, was slowly perfused through the dish. The elevated calcium concentration (5.0 mM) served to improve stability of intracellular recording and enhance synaptic transmission. Physiological recordings were made as described for adult ganglia in Chapter 2. Preganglionic trunks were stimulated briefly (1-5 sec), and at low frequencies (not exceeding 0.5 sec^{-1}), to avoid fatiguing the developing synapses, which often transmitted with small quantal content.

Results and Discussion

An obvious way to approach the question of synaptic specificity in tadpole ganglia would be to penetrate neurons, identify them as B or C cells, and determine their source of input, by the methods used for adult ganglia. Unfortunately, in tadpole ganglia, neurons could not be readily identified by antidromic conduction rate and cell body size. In adult bullfrogs (R. catesbiana) B cell diame-

ters range from 30-70μm, and C cell diameters range from 10-40μm (L. and Y.N. Jan, personal communication). In tadpoles a few neurons were as large as 30μm, but most were much smaller (10-20μm). The critical distinction between cell types in adults, used for the reinnervation experiments, was antidromic conduction latency. In the tadpole ganglia, no antidromic action potentials could be recorded in many cells. Perhaps such cells had not yet elaborated axons into the post-ganglionic nerve trunk, or action potentials could not yet propagate into the cell bodies, especially if the neurons were damaged by penetration with an intracellular electrode; alternatively, damage to the rami communicantes sustained during dissection may have blocked propagation. Nevertheless, in at least some preparations, antidromic responses were observed in many cells. The latencies of such responses indicated that the axons of these cells conducted at rates well below 1 m/sec. Conduction rates did not appear to fall into separate classes. This can be seen in Figure 8 which is a compound action potential recorded from the surface of the 9th ganglion of a stage 17 tadpole, upon stimulation of the 9th nerve about 3.5 mm from the cell body. Apparently B cell axons have not yet become myelinated by this stage. Compound antidromic action potentials recorded from ganglia of stage 21-22 (metamorphic) tadpoles had complex waveforms, suggesting that by this period the conduction velocities of B and C post-ganglionic axons have begun to diverge significantly.

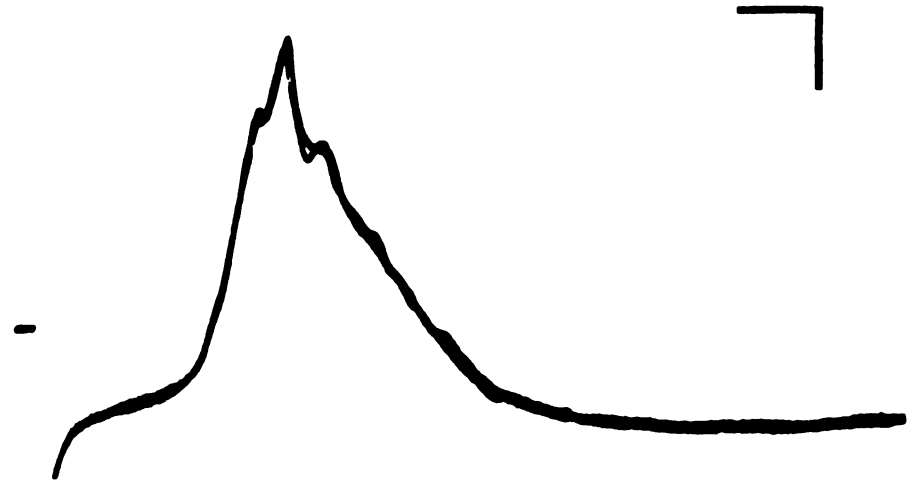

Figure 8. Antidromic compound action potential from the 9th ganglion of a stage 17 tadpole. Two traces are superimposed. The 9th nerve was maximally stimulated approximately 3.5 mm away from the ganglion. No obvious division of postganglionic fibers into separate classes is apparent. Calibration bars: horizontal, 10 msec; vertical, 20 μV.

Both extracellular and intracellular recordings from ganglia of post-metamorphic juvenile frogs showed that B and C populations are clearly distinguishable by the time metamorphosis is complete.

Further studies may yet reveal characteristics by which B and C cells may be distinguished in pre-metamorphic tadpoles, but for the present study I have taken an alternate approach to the question of synaptic specificity in tadpole ganglia: I ask whether neurons can be found that are simultaneously innervated by both B and C fibers. If such a situation exists, it can be inferred that one (though it is not possible to say which) of the synaptic inputs is destined to be eliminated during maturation, since in the adult such simultaneous innervation is virtually never found.

One-hundred thirteen (113) neurons were studied in 15 preparations from stage 17-18 tadpoles. In the majority of neurons input by only one type of preganglionic fiber could be demonstrated. Such neurons received 1-3 presynaptic axons of the same type, just as is found in adults (L. and Y.N. Jan, personal communication). There was a preponderance of input due to B fibers; 76 of the 113 cells received B fiber input exclusively, whereas only 17 received C fibers exclusively. This may suggest that C fibers have not yet innervated many neurons by stages 17-18, but experimental artifacts are also possible: C fibers may have been damaged during dissection, or perhaps the apparent preponderance of

B fiber input is indicative of a sampling error due to the higher success rate of recording from larger cells-- this supposes that even though the conduction velocities of B and C neurons do not differ substantially, the two cell types are partially differentiated, and the majority of cells innervated by C fibers are smaller ones. An effort was made to record from smaller cells, (which were indeed more difficult to record from) but those studied were innervated by B fibers about as often as were larger cells, and the largest cells were sometimes innervated by C fibers. Nevertheless, the smallest cells are not well-represented in the sample.

In 13 of the 113 neurons, synaptic potentials could be elicited by stimulation of either the sympathetic chain, or the 8th (or 7th) nerve, In 5 of these cells, from a comparison of the amplitudes of the synaptic potentials it was clear that they came from different preganglionic fibers, as shown by the examples in Figures 9 and 10. In other cases, stimulation of either nerve trunk elicited responses which were not readily distinguishable, either because they were of similar amplitude, or because the amplitudes of both fluctuated markedly. One possible explanation for these would be if collaterals of a single axon were present in both preganglionic trunks; such appeared to be the case in two instances, as shown by the ability of an impulse in the B fiber pathway to occlude the response due to stimulation of the C fiber pathway, or vice versa, when the two stimuli were delivered within a few msec of each other (collision

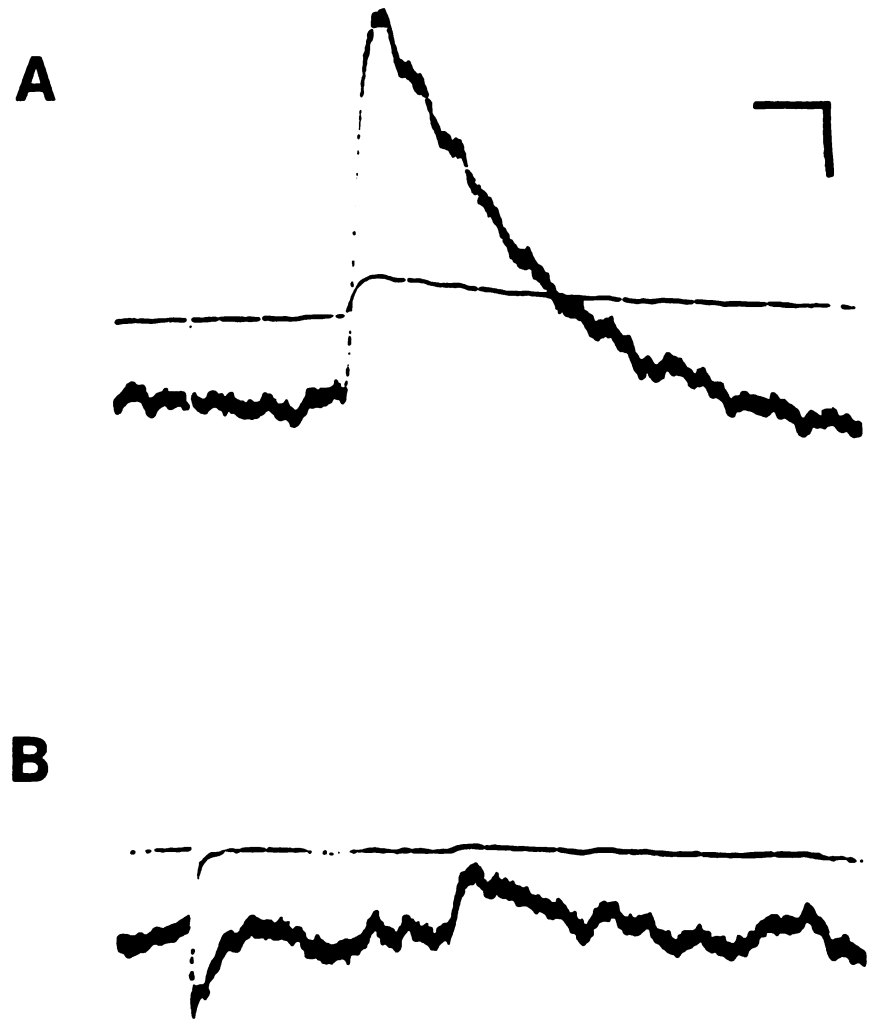

Figure 9. Example of tadpole neuron innervated by both B and C fibers. Both high and low gain recordings are shown. A. Response to stimulation of the 8th nerve. B. Response to stimulation of the sympathetic trunk anterior the 6th ganglion. Calibration bars: horizontal, 10 msec; vertical, 20 mV for the upper traces in A and B, 2 mV for the lower traces.

Figure 10. Another tadpole neuron with both B and C fiber synaptic input. A. Response to stimulation of the 8th nerve. Synaptic potential was of sufficient amplitude to fire the cell. B. Response to stimulation of the anterior sympathetic trunk. This small response was difficult to see without passing hyperpolarizing current into the cell during the synaptic potential, as is shown here. Calibration bars: horizontal, 10 msec; vertical, 20 mV.

test). However, in 3 instances, stimulation of both trunks at an interval such that both responses occurred within 1 msec of each other confirmed that the responses were due to different innervating fibers. Thus, of the 113 neurons studied, 8 were demonstrated by one of the above criteria to receive both B and C fiber input. Three additional cells with responses to stimulation of both preganglionic pathways were lost before a collision test could be performed.

If C fibers were damaged during dissection of some of the preparations, the number of neurons with simultaneous B and C fiber innervation might be underestimated, because some might be left apparently receiving only B fiber input. It is also possible that some of the neurons innervated by only one type of preganglionic axon are actually innervated inappropriately, having yet to be innervated by appropriate axons; without a means of distinguishing B and C cells it is impossible to say to what extent such may be true. Allowing for these possibilities, the 8-10% of neurons receiving both types of input is perhaps a lower limit on the number of neurons that are transiently innervated by innapropriate axons.

On the other hand, the apparent innervation of most cells by one type of preganglionic fiber suggests that B fibers and C fibers innervate predominantly distinct populations of sympathetic ganglion cells, at least by stages 17-18. A reasonable hypothesis is that most of these connec-

tions are appropriate; perhaps this suggests an overall tendency for appropriate synapse formation from the outset, in analogy to the non-competitive 'preference' observed during regeneration after complete denervation in adult frogs. The existence of some inappropriate synapses might then be viewed as a consequence of an exploratory process vital to the search for appropriate synaptic partners.

By what stage of maturation are inappropriate synapses eliminated? Recordings from ganglia of juvenile frogs (stage 25) indicate that by the time metamorphosis is complete, the neurons can be distinguished as B or C cells by their antidromic conduction velocities, and each neuron is innervated solely by appropriate preganglionic axons. Three metamorphic tadpoles (stage 21-22) were examined, and no examples of simultaneous innervation by both B and C fibers were found amoung 24 neurons studied in these preparations. Thus, before the completion of metamorphosis, most or all inappropriate synapses have been eliminated, and the specific pattern of innervation found in mature ganglia has been established.

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