*J. exp. Biol.* (1981), **95**, 135–151
*With 8 figures*
*Printed in Great Britain*



# AXON/SCHWANN-CELL RELATIONSHIPS IN THE GIANT NERVE FIBRE OF THE SQUID

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SUMMARY

This communication summarizes the experimental evidence obtained in the giant nerve fibre of the tropical squid *Sepioteuthis sepioidea*, on the nature of the mechanism responsible for the long-lasting effects of axonal excitation on the membrane potential of the Schwann cell. In these nerve fibres the propagation of a train of nerve impulses by the axon is followed by a prolonged hyperpolarization of the Schwann cell which can be reproduced, or modified, by the external application of cholinergic compounds. The presence and exact localization of the different components of the acetylcholine system directly involved in such Schwann-cell responses was detected by means of pharmacological, histochemical and chemical procedures. Thus, the results of the experiments herein discussed revealed that, under physiological conditions: the Schwann cell is able to synthesize, store and release acetylcholine; and that it has acetylcholine receptors of the nicotinic type, and acetylcholinesterase enzyme activity in its plasma membrane. On the other hand, the axon has low acetyltransferase activity and acetylcholine concentration in its axoplasm, and a high acetylcholinesterase activity in its axolemma. It was also found that acetylcholine hyperpolarizes the Schwann cell by increasing its relative permeability to the potassium ion. The distribution pattern of the acetylcholine system indicates that it operates as a feedback mechanism for the regulation of the Schwann-cell membrane potential and ionic permeability following axonal excitation.

Since their description by Young (1936) the giant nerve fibres of the squid have been extensively used for exploring fundamental mechanisms of nerve function. The giant fibres and synapses constitute a fast conducting system that synchronously drives the muscle fibres of the mantle during the fast swimming behaviour of the squid.

The ultrastructural studies of these nerve fibres, carried out by Villegas & Villegas (1960, 1963, 1964, 1968) in the squids *Dorytheutis plei* and *Sepioteuthis sepioidea* from the Caribbean Sea and *Dosidicus gigas* from the Pacific Ocean, revealed several features of their functional organization. It was found that the Schwann-cell layer of the giant fibre is formed by several Schwann cells arranged in a single row around the axon. Each Schwann cell is 0·2–0·8 $\mu$m thick in the squid *D. plei* (Villegas & Villegas, 1963), while in the *S. sepioidea* and *D. gigas* it is about 1·5–6 $\mu$m in thickness. The Schwann

layer is crossed by tortuous channels that represent intercellular spaces between two neighbouring cells or between two processes of the same cell (Villegas & Villegas, 1963). Such intercellular channels are permeable and allow the diffusion of particles as large as those of thorium dioxide (Villegas & Villegas, 1964). However, these pathways are not empty spaces but are occupied by a material which gives positive reaction to histochemical tests for mucopolysaccharides and binds, to a certain extent, the thorium dioxide micelles (Villegas & Villegas, 1968).

On the other hand the studies on the permeability and electrical potential profile of these nerve fibres (Villegas & Barnola, 1961; Villegas *et al.* 1962, 1963), identified the axolemma as the excitable membrane, the axolemma-Schwann cell space as its immediate extracellular environment, and the Schwann cell extracellular channels as low resistance pathways through which potassium ions diffuse away from the surface of the excitable membrane (Frankenhaeuser & Hodgkin, 1956).

The present communication reviews the experimental evidence obtained in the giant nerve fibre of the squid *S. sepioidea* (Villegas *et al.* 1963, 1965, 1968; Villegas, 1968, 1972, 1973, 1974, 1975; Villegas & Villegas, 1974, 1976; Rawlins & Villegas, 1978; Villegas & Jenden, 1979; Heumann *et al.* 1981), on the functional relationships between the axon and its satellite glial cell, the Schwann cell. The experiments to be discussed herein deal with: (1) the effects of axonal excitation on the membrane potential of the Schwann cell; (2) the cholinergic properties of the Schwann cell response to axonal excitation; (3) the localization of the acetylcholine system in the giant nerve fibre, and (4) the mechanisms of axon-Schwann cell signaling in these nerve fibres.

## EFFECTS OF AXONAL EXCITATION

### *Nerve-impulse conduction*

Simultaneous intracellular recordings of the axon and Schwann-cell electrical potentials in the giant nerve fibre of *S. sepioidea* (Villegas *et al.* 1963), revealed that the Schwann cells have lower resting potentials ($\sim -40$ mV) than the axon they surround ($\sim -60$ mV), and that their membrane potential remains apparently unmodified (within $\pm 2$ mV) during the passage of an action potential or during the injection of a pulse of depolarizing current into the axon (Villegas *et al.* 1963). A similar electrical independence between the neuronal and glial cell membrane potentials in the central nervous system of the leech was reported by Kuffler & Potter (1964). In addition, they were able to show that the glial-cell membrane behaves passively even when its electrical potential is displaced over a wide ($\sim 200$ mV) range, and that neurones deprived of glia continue to conduct impulses (Kuffler & Potter, 1964). However, they found that the glial cells have higher resting potentials than the neurones they surround; about $-75$ mV compared with $-50$ mV.

Further studies on the physiological properties of glial cells, carried out by Kuffler, Nicholls & Orkand (1966) in the optic nerve of the mudpuppy *Necturus* and of the frog, showed that the glial cells in these preparations also have high resting potentials. In addition, they found that the glial membrane behaves like a perfect potassium electrode, even at external potassium concentrations lower than the physiological values (Kuffler *et al.* 1966).

On the other hand, further studies on the membrane properties of the squid Schwann cell carried out in *S. sepioidea* giant nerve fibres (Villegas, Villegas & Gimenez, 1968; Villegas, 1968) showed that under resting conditions the membrane potential of the Schwann cell is determined by the ionic concentration gradients and permeabilities, mainly of potassium, and to a minor extent of other ions. It was also found that the intracellular potassium in excess of the membrane potential is maintained constant by a cardiac glycoside-sensitive, active electrogenic potassium transport towards the interior of the cell, which seems to contribute to lower the membrane potential below the potassium equilibrium potential (Villegas *et al.* 1968; Villegas, 1968). Recently, it has been found that the glial cells associated with the medial giant axon in the ventral nerve cord of the crayfish *Procambarus clarkii* have lower resting potentials than the axon ($\sim -42$ mV compared with $\sim -85$ mV) and are sensitive to cardiac glycosides (Lieberman, Villegas & Villegas, 1980).

*Nerve-impulse train conduction*

In the squid nerve fibre, Frankenhaeuser & Hodgkin (1956) have shown that the conduction of nerve-impulse trains is accompanied by an accumulation of potassium ions in the vicinity of the excitable membrane. Potassium diffuses from the surface of this membrane through a low-resistance unspecific layer. Similarly, it has been shown that in the optic nerve of *Necturus* (Orkand, Nicholls & Kuffler, 1966) and in the central nervous system of the leech (Baylor & Nicholls, 1969) the passage of a volley of nerve impulses by the fibres is followed by a depolarization of the glial cells. Both the amplitude and the time course of the glial depolarizations depend on the frequency of action potentials and the duration of the train. If stimulation is maintained, surprisingly large glial membrane depolarizations of up to 48 mV can be seen, and at the end of the train a residue of this large potential may persist for 30 sec or longer (Kuffler & Nicholls, 1966). Such glial depolarizations have been accounted for by potassium leakage from the excited axons (Orkand *et al.* 1966; Baylor & Nichols, 1969).

On the other hand, simultaneous intracellular recordings of the Schwann cell and axon electrical potentials in the giant nerve fibre of the squid *S. sepioidea*, similar to those illustrated in Fig. 1 (Villegas, 1972, 1978), have shown that the propagation of a train of nerve impulses by the axon is accompanied by a transient depolarization of the Schwann cell, followed by a prolonged hyperpolarization of this cell which outlasts for several minutes the duration of the trains (see Fig. 7: Villegas, 1972). Similar results are obtained by successively measuring the electrical potentials of several of the Schwann cells by brief impalements from inside the axon, before and after the conduction of nerve impulse trains (Villegas, 1972, 1973, 1975).

The long-lasting hyperpolarizing effects of the nerve impulse trains were reproduced by the application of a series of depolarizing voltage-clamp pulses to the axon, as shown in Fig. 2. Thus, it was considered that although a rise in the potassium concentration in the intercellular clefts due to the outward potassium current from the depolarized axon, could only account for the initial depolarizing phase of the Schwann cell response, the possibility exists that it could also be triggering the mechanism responsible for the long-lasting hyperpolarizing phase of the response (Villegas, 1972). However, it was found that increasing 3 or 40 times the external potassium concentration during an interval equivalent to that of the nerve impulse trains, or voltage-

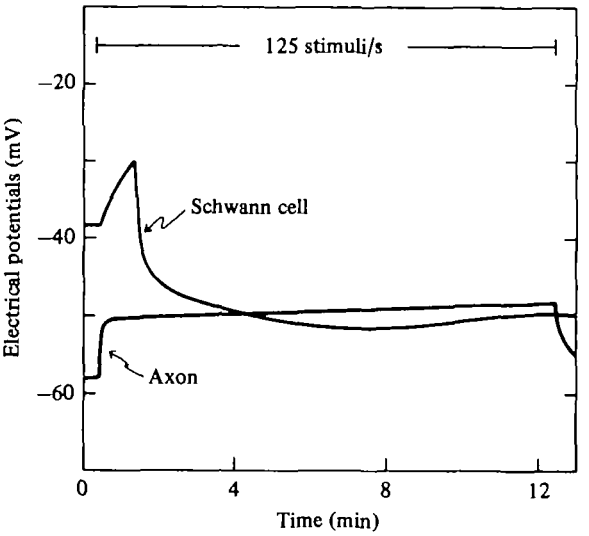


Fig. 1. Time course of the Schwann-cell electrical potential changes following repetitive stimulation of the axon, recorded intracellularly and simultaneously from a single Schwann cell and its neighbouring giant axon in *S. sepioidea*. Due to the inertia of the pen recorder used for the original record, no action potentials can be observed in the axon–electrical potential trace. (After Villegas, 1978.)

clamp pulses, had no appreciable hyperpolarizing after-effects on the Schwann cells in unstimulated nerve fibres (Villegas, 1972).

These findings were considered as suggesting that some form of intercellular coupling could be present between Schwann cells and the axon. The presence of certain membrane specializations described by Villegas & Villegas (1968) in axons of different squid species were confirmed in freshly dissected *S. sepioidea* nerve fibres. Such membrane specializations were first referred to as local thickenings of the inner leaflet of the axolemma and were compared to the postsynaptic densities, and also to the opaque axoplasmic material underlining the nodes of Ranvier (Villegas & Villegas, 1968). More recently, the axolemma thickennings were found to be part of a more complex structural pattern whose typical features are: trilaminar substructure of the axolemma, attachment of dense material to the inner leaflet of this membrane, and a narrowing to disappearance of the axon/Schwann-cell interspace (Villegas, Villegas & De Weer, 1972; Villegas & Villegas, 1976).

It was not known if these structural specializations were directly related to the axon/Schwann-cell interactions described above. However, the fact that they had been compared with the postsynaptic densities involved in the chemical mechanisms of intercellular communication called attention to the evidence provided by Nachmansohn *et al.* (Boell & Nachmansohn, 1940; Nachmansohn, 1959; Brzin *et al.* 1965) on the presence of cholinesterases on the surface of these giant nerve fibres, which later were found to be non-cholinergic (Webb, Dettbarn & Brzin, 1966; Miledi, 1967). Thus, it was considered worth while to explore the possible existence of chemical coupling mechanisms between the axon and the Schwann cell.

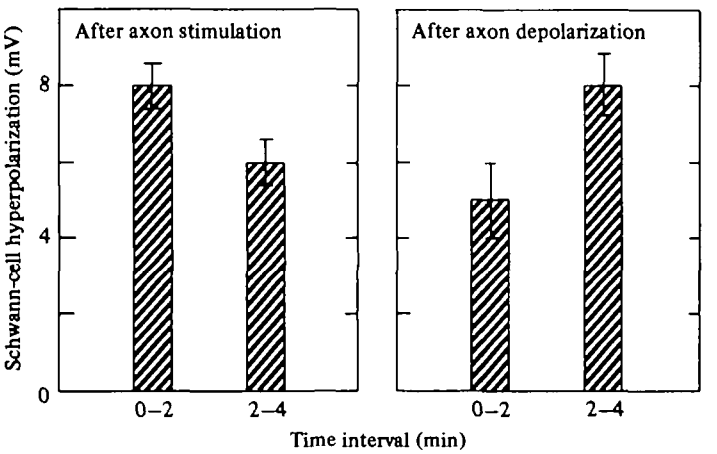


Fig. 2. Magnitude of the Schwann-cell hyperpolarizations following axonal excitation. The values are the mean ± S.E. of mean of the observations obtained (15–32) during each 2 min interval. Left graph: results obtained in eight nerve fibres stimulated repetitively for 1 min, at 250 stimuli $s^{-1}$ during 0·5 s each second. Right graph: results obtained in four voltage-clamped nerve fibres. The axon membrane potential was held at resting level. From this value it was stepped 100 mV in the depolarizing direction (to about $E_{Na}$) for 50 ms each second a total of 50 times. (After Villegas, 1972.)

## CHOLINERGIC PROPERTIES OF THE SCHWANN CELL RESPONSE

### *Effects of eserine and* D-*tubocurarine*

In a first series of experiments (Villegas, 1973), the effects of eserine (physostigmine) and D-tubocurarine on the axon and Schwann cell membrane potentials were determined in the giant nerve fibre of *S. sepioidea.*

Eserine was used to investigate whether cholinesterases are directly involved in the mechanism responsible for the long-lasting Schwann-cell hyperpolarizations. Eserine is known to inhibit cholinesterase activity specifically (Nachmansohn, 1949). However, at concentrations higher than those needed to inhibit the esterases, eserine appears to also antagonize the action of acetylcholine (Dettbarn & Davis, 1963).

Fig. 3 summarizes the observations made on the effects of nerve-impulse trains on the Schwann-cell membrane potential in nerve fibres immersed in sea-water solutions containing different concentrations of eserine. This figure shows that $10^{-9}$ M eserine appears to prolong the effects of the nerve impulse trains on the Schwann-cell electrical potential, whereas $10^{-7}$ M eserine decreases both the amplitude and the duration of the Schwann-cell potential changes, and $10^{-4}$ M eserine completely abolishes the long-lasting effects of axonal stimulation. No changes of the resting and action potentials of the axon were noticed even at the highest concentration of eserine used in these experiments (Villegas, 1973).

To investigate whether the effects of eserine described above may actually be ascribed to an interference of this drug with cholinergic mechanisms present in these nerve fibres, D-tubocurarine was used. Curare alkaloids compete with acetylcholine for specific receptors in the cell membrane (Jenkinson, 1960). However, at high concentrations curare also has slight inhibitory action on cholinesterase (Changeux, 1966).

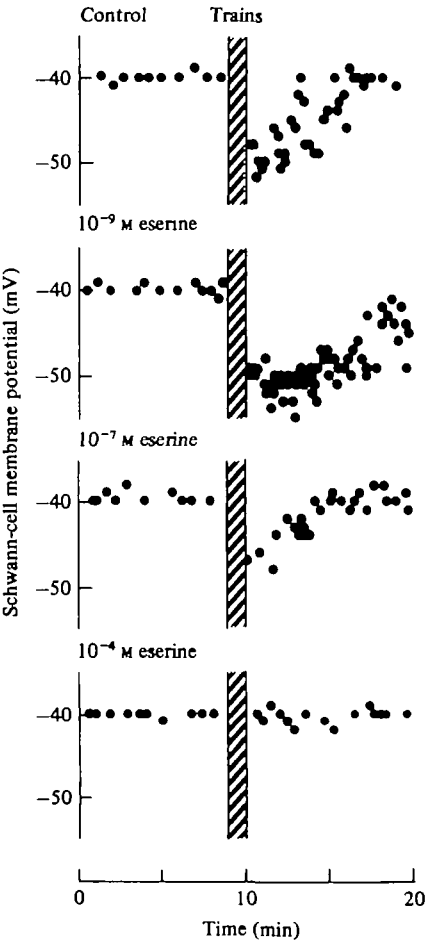


Fig. 3. Effect of eserine on the hyperpolarization of the Schwann cell following conduction of nerve impulse trains by the axon. The electrical potentials have been plotted as a function of time. Each graph corresponds to the results obtained in a different nerve fibre. Each point represents the potential difference measured in a different Schwann cell. At zero time eserine was added to the external sea-water medium. During the interval indicated by the vertical bar stimuli were delivered to the axon at 125 $s^{-1}$. (After Villegas, 1973.)

Fig. 4 summarizes the effect of different concentrations of D-tubocurarine on the after-effects of nerve impulse trains on the Schwann cell membrane potential. The graphs show that D-tubocurarine blocks the Schwann-cell hyperpolarizing response to axonal excitation. Its effect appears to be a function of the concentration of the drug, being maximal at concentrations above $10^{-9}$ M, and unnoticeable at $10^{-12}$ M. Even at the highest concentration used in these experiments, D-tubocurarine had no appreciable effect on the resting and action potentials of the axon (Villegas, 1973). Fig. 4 also shows that the addition of D-tubocurarine to the external medium produces a transient hyperpolarization of the Schwann cells in the unstimulated nerve fibre, both in the presence and in the absense of the giant axon (see Figs. 3–7: Villegas, 1973). This unexpected experimental finding has been considered as indicating the presence of

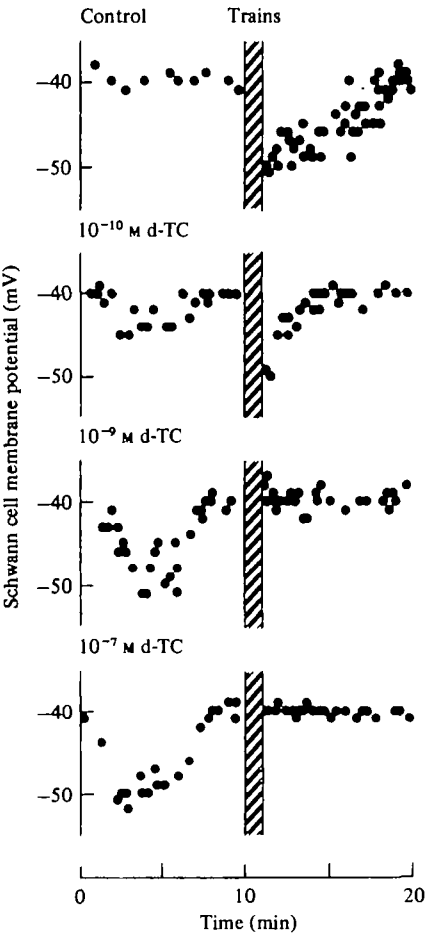


Fig. 4 Effect of D-tubocurarine (d-TC) on the Schwann-cell membrane potential of the resting nerve fibre and on the hyperpolarization of the Schwann cell following conduction of nerve-impulse trains by the axon. Each graph corresponds to the results obtained in a different nerve fibre. Each point represents the potential difference recorded in a different Schwann cell. At zero time D-tubocurarine was added to the bathing solution. During the interval indicated by the vertical bar the axons were stimulated at 125 $s^{-1}$ (After Villegas, 1973.)

curare-sensitive receptors in the Schwann-cell membrane. The high sensitivity of these receptors was considered as strongly suggesting that they are similar to acetylcholine receptors.

*Effects of acetylcholine and carbamylcholine*

The above possibility was tested in a series of experiments to determine the effects of acetylcholine and carbamylcholine on the axon and Schwann-cell membrane potentials in *S. sepioidea* nerve fibres.

Carbamylcholine (a stable analogue of acetylcholine) was used to investigate whether the long-lasting effects of nerve impulse train conduction on the Schwann-cell membrane potential can be reproduced by the external application of the cholinergic transmitter substance.

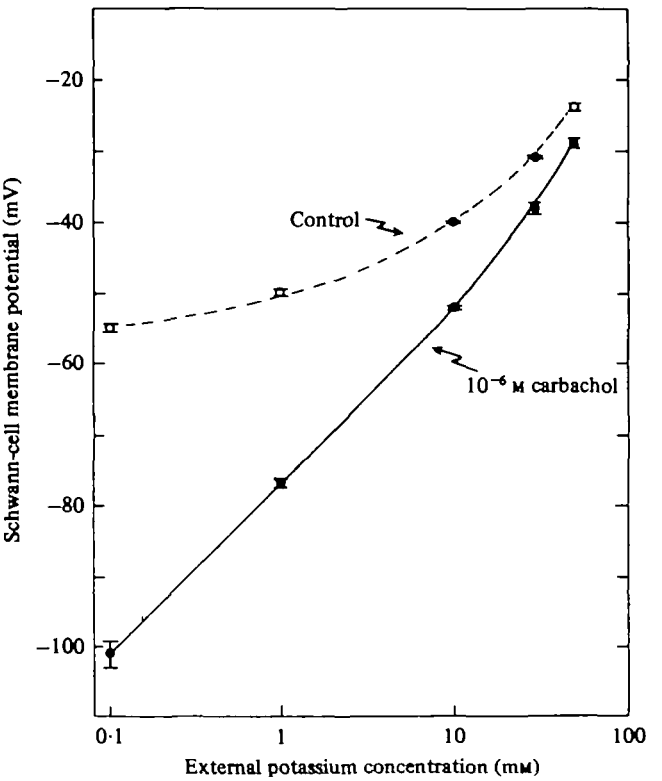


Fig. 5. Effect of carbamylcholine on the relationship between Schwann-cell membrane potential and external potassium concentration. ○, Measurements made in control sea-water solutions; ●, measurements made in sea-water solutions containing carbachol. Values are mean ±S.E. of mean. Results obtained in twenty nerve fibres. (After Villegas, 1974.)

Fig. 5 shows that not only the Schwann cells become hyperpolarized in the presence of $10^{-6}$ M carbamylcholine, but that their membrane potential follows more closely the behaviour of a potassium electrode than in the absence of carbamylcholine. This experimental finding has been considered as indicating that the external application of the cholinergic transmitter substance increases the relative permeability of the Schwann cell membrane to the potassium ion.

Although acetylcholine has a higher affinity than carbamylcholine for specific receptors in the cell membrane (Kasai & Changeux, 1971), it is rapidly hydrolysed by the acetylcholinesterase present in the tissues which can be inhibited by eserine (Nachmansohn, 1949). Thus, to test whether the external application of acetylcholine and carbamylcholine to the resting nerve fibre reproduce the effects of axonal excitation on the Schwann-cell membrane potential, nerve fibres were exposed to $10^{-9}$ M eserine or D-tubocurarine prior to the application of these agents. It was found that eserine prolongs the Schwann-cell hyperpolarizations induced by $10^{-7}$ M acetylcholine, whereas D-tubocurarine reversibly blocks the hyperpolarizing responses to $10^{-6}$ M carbamylcholine (see Figs. 2–3: Villegas, 1974). No such effects are produced by the external application of 50 nM tetrodotoxin, which is known to specifically blo

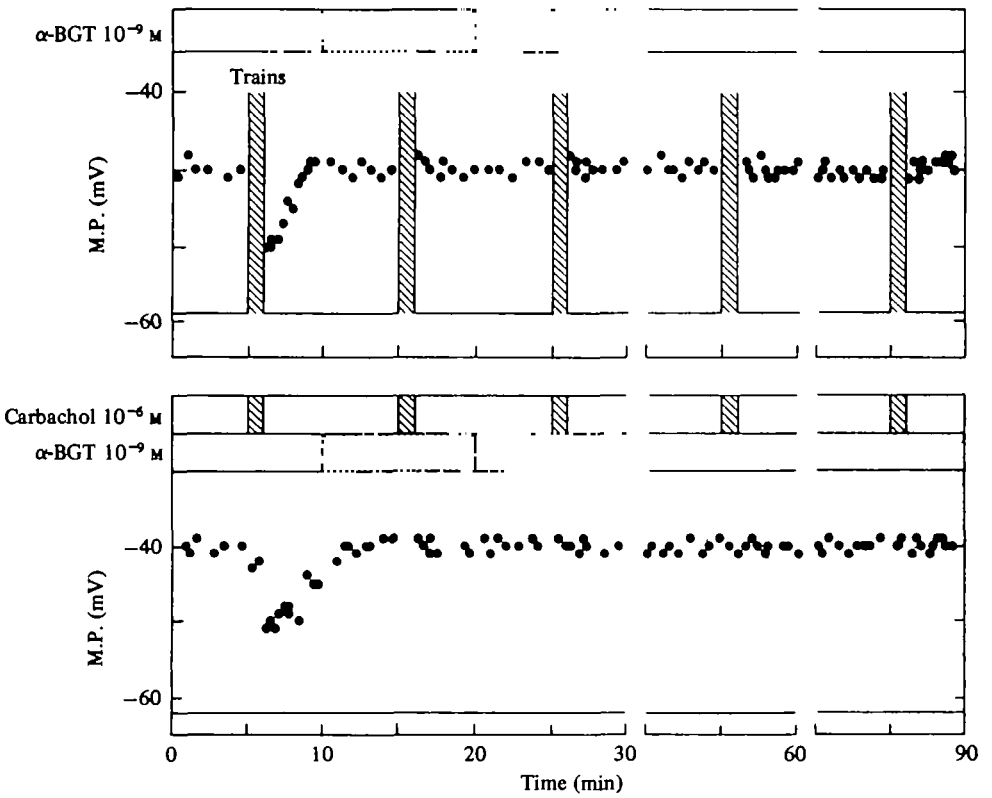


Fig. 6. Effect of α-bungarotoxin (α-BGT) on the hyperpolarization of the Schwann cells following conduction of nerve impulse trains by the axon or the external application of carbamylcholine. The electrical potentials appear plotted as a function of time. Each point corresponds to the potential difference recorded in a different Schwann cell. The membrane potential of the axon (line) was also monitored. During the intervals indicated by the vertical bars (upper graph) the axon was stimulated at 125 $s^{-1}$, or (lower graph) carbachol was added to the external medium (horizontal striped bar). During the intervals indicated by the horizontal stippled bars, $10^{-9}$ M α-bungarotoxin (α-BGT) was present in the bathing solution. (After Villegas, 1975).

the pathways for sodium diffusion across the Schwann-cell membrane in these nerve fibres (Villegas, 1974; Villegas *et al.* 1976).

These experimental results have been considered as indicating that the external application of the cholinergic transmitter substance reproduces the long-lasting hyperpolarizing effects of axonal excitation on the Schwann cell membrane potential, inducing an increase in the relative permeability of the Schwann cell membrane to the potassium ion (Villegas, 1974).

*Characterization of the acetylcholine receptors*

To characterize the membrane site which is sensitive to cholinergic agents, the effects of α-bungarotoxin, nicotine and muscarine on the Schwann-cell membrane potential were determined in the giant nerve fibre of *S. sepioidea* (Villegas, 1975).

The α-toxin derived from the venom of the elapid snake *Bungarus multicinctus* behaves as an apparently irreversible blocking agent of the response of various

cholinergic preparations to cholinergic agonists (Lee & Chang, 1966; Changeux, Kasai & Lee, 1970). Since it has no known action on the acetylcholinesterase, α-bungarotoxin is considered as being a specific, irreversible, reagent for the physiological receptor site of acetylcholine.

Fig. 6 shows that the external application of $10^{-9}$ M α-bungarotoxin for 10 min suppresses the Schwann-cell hyperpolarizations, induced by the nerve impulse trains or the addition of carbamylcholine ($10^{-6}$ M), even after washing the nerve fibres for 60 min in toxin-free sea-water solutions.

The Schwann-cell membrane can be protected against the irreversible action of α-bungarotoxin by addition of $10^{-5}$ M D-tubocurarine to the bathing solution before, during and after the period of exposure to the toxin (see Fig. 4: Villegas, 1975). Thus, within 10 min of re-immersion in toxin-free sea-water solution a complete recovery of the Schwann-cell response to carbamylcholine ($10^{-6}$ M) was observed. This finding has been considered as indicating that carbamylcholine, D-tubocurarine and α-bungarotoxin react with the same sites in the cell membrane.

Nicotine and muscarine, cholinergic agonists which react preferentially with their respective type of cholinergic receptor, were used to further characterize the physiological reacting sites for acetylcholine in the Schwann-cell membrane. At an external concentration of $10^{-6}$ M nicotine, but not muscarine, reproduces the hyperpolarizing effects of acetylcholine and the nerve impulse trains on the Schwann-cell membrane potential in unstimulated nerve fibres. None of these agents had any appreciable effects on the membrane potentials of the axon at the concentrations used in these experiments (Villegas, 1975).

These experimental findings have been considered as confirming the presence of acetylcholine receptors of the nicotinic type in the Schwann cell membrane (Villegas, 1973, 1974), and strongly suggest that acetylcholine is also present in these nerve fibres.

## LOCALIZATION OF THE ACETYLCHOLINE SYSTEM

### *Acetylcholinesterase*

The above findings made it interesting to investigate the presence and exact distribution of the cholinesterase in these giant nerve fibres.

The ultrastructural localization of the acetylcholinesterase in peripheral nerves had been described in the rat sciatic (Schlaepfer & Torack, 1966) and in the nerves of the lobster walking legs (De Lorenzo, Dettbarn & Brzin, 1969). It appeared from these works that the main sites of the enzymic activity are the axon surface, on or near the axolemma, and in the axon/Schwann-cell interspace. Thus, using a modification for electron microscopy of the histochemical method of Koelle & Friedenwald (1949), experiments aimed at demonstrating the pattern of enzyme distribution were carried out in *S. sepioidea* giant nerve fibres (Villegas & Villegas, 1974).

The end-product of the histochemical reaction appeared distributed in foci along the axolemma, attached to its axoplasmic leaflet. At these regions the trilaminar substructure of the axolemma and a narrowing of the axon/Schwann-cell interspace were also present. Only random deposits of end-product were observed in the axoplasm and in the Schwann-cell layer. The material treated with iso-OMPA (mainly

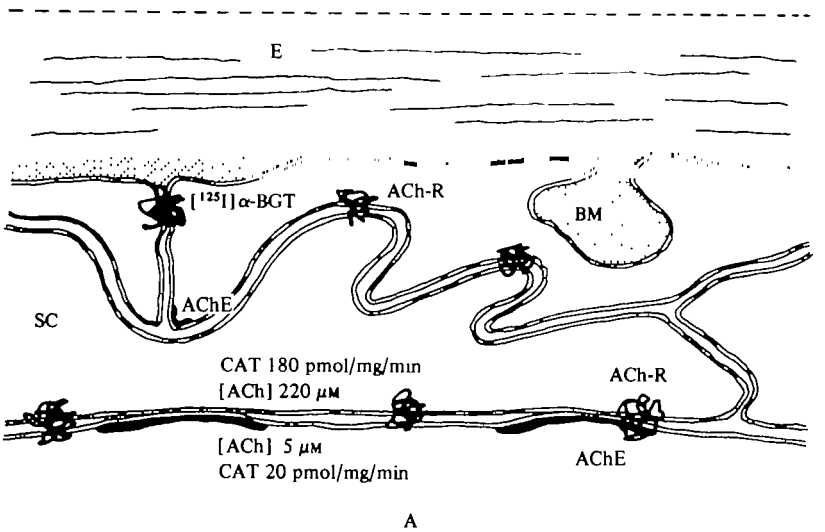


Fig. 7. Distribution pattern of the acetylcholine system in the giant nerve fibre of the squid. The end-product of the histochemical reaction for the localization of the enzymic activity of acetylcholinesterase appears attached to the inner leaflet of the axon plasma membrane (axolemma) in focal zones of this membrane characteristically displaying the trilaminar substructure and a narrowing of the axolemma-Schwann cell space. A few deposits of the end-product are observed in the Schwann cell, attached to the walls of the membranous channels crossing the Schwann layer. The Schwann-cell acetylcholine receptors labelled with radioactive [$^{125}$I]α-bungarotoxin are located mainly on the axon/Schwann-cell boundary, and also on those regions of the Schwann cell rich in extracellular channels. The average intracellular concentrations of acetylcholine and the rates of acetyltransferase enzymatic activity measured in these nerve fibres are also indicated. (After Villegas & Villegas, 1974; Rawlins & Villegas, 1978; Villegas & Jenden, 1979, Heumann *et al.* 1981.)

inhibitor of butyrylcholinesterase) showed exactly the same enzymic distribution. No positive reaction was observed when eserine or BW 284 C51 (an inhibitor of acetylcholinesterase) was used. These results have been considered as showing the presence and exact localization of acetylcholinesterase in the giant nerve fibre of the squid (Villegas & Villegas, 1974). They also showed the possible relationship between the enzyme and the sites of special structural arrangement present at the axon/Schwann-cell boundary, which might serve as sorts of intercellular junctions between the axon and the Schwann cell (Villegas & Villegas, 1976) (Fig. 7).

*Acetylcholine receptors*

The subcellular location of the acetylcholine receptor sites was studied with electron microscopy autoradiography of [$^{125}$I]α-bungarotoxin-binding sites, in *S. sepioidea* giant fibres. Paired samples of each fibre were exposed to the radioactive toxin for similar periods of time, a nerve half in the presence and the other half in the absence of $10^{-4}$ M D-tubocurarine in the test solutions (Rawlins & Villegas, 1978).

No signs of radioactivity were observed in the autoradiographs of the nerve halves which were pre-incubated in D-tubocurarine sea water, exposed to the radioactive toxin in its presence and then post-incubated in toxin-free D-tubocurarine sea-water solution to wash away unbound toxin. In the autoradiographs of the nerve halves exposed to the radioactive toxin alone, grains corresponding to bound [$^{125}$I]α-bungarotoxin were located mainly towards the axon/Schwann-cell boundary. They were also found over the rest of the Schwann-cell layer and accumulated specially over those regions rich in extracellular channels (Rawlins & Villegas, 1978).

These findings have been considered to confirm the presence of acetylcholine receptors in the Schwann-cell plasma membrane (Villegas, 1973, 1974, 1975), and further indicate that they are located on the cell surfaces facing the neighbouring axon and the adjacent Schwann cells, as appears schematically illustrated in Fig. 7.

*Acetylcholine*

The presence of acetylcholine receptors in the Schwann-cell membrane and of acetylcholinesterase on the axon surface made it necessary to investigate whether the Schwann cells and/or the axon in these nerve fibres also contain acetylcholine.

Acetylcholine and choline were identified, and their concentrations measured, by means of gas chromatography/mass spectrometry, in extracts obtained from giant nerve fibres of *S. sepioidea*. Samples of stellar nerve devoid of giant fibre, intact giant nerve fibre, extruded axoplasm, and axoplasm-free giant nerve-fibre sheaths were similarly analysed (Villegas & Jenden, 1979).

The results showed the presence of acetylcholine in the giant and small nerve fibres of the stellar nerve. They indicate that the Schwann cells of the giant nerve fibre have a high intracellular acetylcholine concentration, which is about 40 times that of the axoplasm. These experimental findings settled the point on the presence of acetylcholine in the giant nerve fibre of *S. sepioidea* (Villegas, 1975). They also indicate the Schwann cells themselves as the main source of the acetylcholine released following the conduction of nerve impulse trains in these nerve fibres (Villegas & Jenden, 1979). The distribution pattern of acetylcholine in the giant fibre is schematically illustrated in Fig. 7.

*Acetylcholine synthesis*

The question still remained of whether the acetylcholine present in the Schwann cells of *S. sepioidea* is synthesized within them (Villegas & Jenden, 1979). Experiments aimed at detecting and measuring acetyltransferase enzymic activity were thus carried out on these nerve fibres (Heumann, Villegas & Herzfeld, 1981).

Homogenates obtained from pooled samples of whole stellar nerve, intact giant nerve fibre, extruded axoplasm, axoplasm-free giant nerve-fibre sheaths, and small nerve fibres, were assayed by a modified radiochemical test (Hamprecht & Amano, 1974). The results showed that the giant nerve has acetyltransferase enzyme activity, which is mainly localized in the cells of the periaxonal sheaths. The rate of acetylcholine synthesis in the Schwann cells is about 3–9 times that found in the axoplasm and close to the level of enzyme activity found for the intact giant nerve fibre (Heumann *et al.* 1981).

These experimental findings complete the description of the acetylcholine system in the giant nerve fibre of *S. sepioidea*, shown schematically in Fig. 7.

## AXON/SCHWANN-CELL SIGNALLING

As discussed above, the propagation of a single nerve impulse by the squid axon has no appreciable effects on the membrane potential of its satellite Schwann cells (Villegas *et al.* 1963; Villegas, 1972). However, the conduction of a train of nerve impulses is accompanied by a transient accumulation of potassium ions in the intercellular clefts of the nerve fibre (Frankenhaeuser & Hodgkin, 1956). This induces a transient depolarization followed by a prolonged hyperpolarization in the Schwann cells which outlasts, by several minutes, the duration of the train of impulses (Villegas, 1972, 1973, 1975, 1978).

It is obvious that in these nerve fibres potassium leakage from the excited axon is also a signal for its satellite glial cell, as was previously demonstrated in the central nervous systems of amphibia and the leech. (Orkand *et al.* 1966; Baylor & Nicholls, 1969; Kuffler & Nicholls, 1966, 1976), However, from the above evidence it is also clear that repetitive stimulation of the axon can activate the cholinergic mechanism responsible for the long-lasting Schwann-cell hyperpolarizations following axonal excitation (Villegas, 1978).

The distribution of the cholinergic system in these nerve fibre indicates that as a consequence of the nerve impulse trains, the Schwann cell itself releases acetylcholine. This reacts with the receptors on the Schwann-cell membrane, inducing an increase in relative potassium permeability (Villegas, 1974), which results in a long-lasting hyperpolarization of this cell. The inactivation of the acetylcholine released from the cells is carried out by the acetylcholinesterase present in the axolemma and in the Schwann-cell plasma membrane. Thus, the activation of such a feedback mechanism by the effects of axonal excitation, transforms the low resting membrane potential Schwann cell into a high membrane potential glial cell, which follows more closely the behaviour of a potassium electrode (Villegas, 1974), at least for a period outlasting for several minutes the duration of the axon impulse trains.

The nature of the axonal signal which triggers the Schwann-cell hyperpolarizing responses is still unknown. However, as described above (Fig. 1), the initial phase of the Schwann cell response is depolarizing. This is better seen by blocking the delayed hyperpolarizing cholinergic component (Fig. 8). Fig. 8 shows that in a nerve fibre pre-incubated in $10^{-8}$ M $\alpha$-bungarotoxin the conduction of a train of nerve impulses by the axon is accompanied by a depolarization of the Schwann cell which closely follows the duration of the train, as do the changes in potassium concentration in the intercellular clefts described by Frankenhaeuser & Hodgkin (1956) in other squid species. Thus, it could be that the depolarizing effect of potassium leakage from the axon on the Schwann cell is the triggering signal for the cholinergic response of this cell. However, increasing the external concentration of potassium, during an interval equivalent to that of the nerve impulse trains, has no appreciable hyperpolarizing after-effects on the Schwann cells in unstimulated nerve fibres (Villegas, 1972). This indicates that other factors are also needed to trigger the release of acetylcholine from the Schwann cells (Villegas, 1972, 1978).

The presence of the cholinergic system in the Schwann cell of *S. sepioidea* is not a unique phenomenon. The Schwann cells in transected nerves and denervated neuromuscular junctions of the frog (Katz & Miledi, 1959; Birks, Katz & Miledi, 1960; Miledi & Slater, 1968; Denis, 1962; Dennis & Miledi, 1974; Bevan, Grampp &

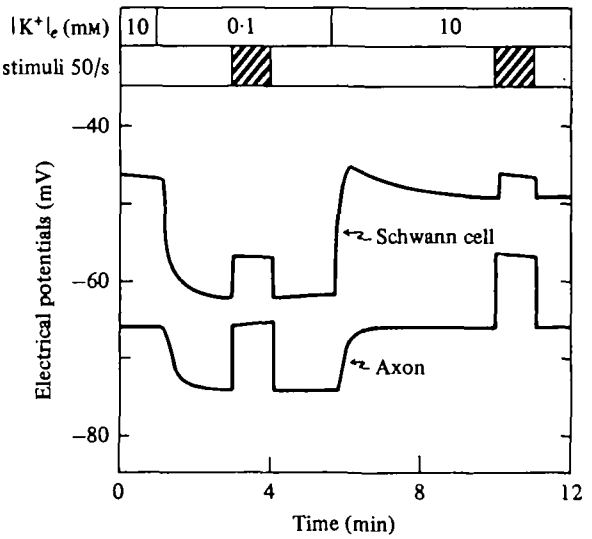


Fig. 8. Time course of the Schwann-cell potential changes following repetitive axonal excitation, as recorded in a single nerve fibre pre-incubated in $10^{-8}$ M α-bungarotoxin. During the intervals indicated in the upper horizontal bar the external concentration of potassium was changed. During the interval indicated by the horizontal striped bar the nerve fibre was stimulated. Due to the inertia of the pen recorder utilized for the original records of the intracellular potentials, no action potentials can be observed in the axon-electrical potential trace (J. Villegas, V. G. Reale & D. W. Herzfeld, unpublished results.)

Miledi, 1973, 1976; Tucek *et al.* 1978) can synthesize choline acetyltransferase and acetylcholine and release acetylcholine. However, such cells are normally in intimate association with cholinergic axons while Schwann cells in the squid are in contact with a non-cholinergic one. Nevertheless, more recently (Lieberman *et al.* 1980) it has been found that the satellite glial cells associated with the medial non-cholinergic giant axon in the ventral nerve cord of the crayfish *P. clarkii*, are sensitive to the external application of different cholinergic compounds. Thus, superfusion of the cells with $10^{-7}$ M acetylcholine, carbamylcholine or nicotine caused a 15–20 mV hyperpolarization from resting level (−42 mV). Muscarine ($10^{-6}$ M) had no effect on the glial cell potential. D-Tubocurarine ($10^{-9}$ M) prevented or reversed the effect of nicotine. The effects of these compounds were completely reversible, and none of them had any obvious effect on the membrane potential of the neighbouring axon (Lieberman *et al.* 1980). It has also been reported in this glial cell–axon preparation that the external application of ouabain to the resting nerve fibre is able to cause the release of acetylcholine, inducing a glial hyperpolarization sensitive to D-tubocurarine (Smiley & Lieberman, 1981).

The similarities observed between the satellite glial cells in the squid and crayfish giant nerve fibres pose the question of whether they form the basis of a generalized function of acetylcholine, and other putative neurotransmitter substances in the functional coupling of these low resting membrane potential glial cells to the usually silent giant axons they surround. In the squid the giant nerve fibres are fast-conducting motor elements responsible for the synchronous firing of the mantle muscle fibres. The medial giant nerve fibres in the crayfish, on the other hand, innervate the muscles responsible for the tail-flip fast-swimming response, and are also fast conducti

fibres for synchronous activation of the motor axons. Thus the possibility exists that only when massive motor responses (such as the fast-swimming behaviour of the squid and crayfish) are started, the membrane potential of the satellite glial cells around the excited axons is driven towards the higher potential level observed in the glial cells of the central nervous system of other species of invertebrates and vertebrates.

It is clear that the interactions between the Schwann cells and the axon in the squid nerve fibre are not simple. Without a complete understanding of the mechanisms involved, the role of the Schwann cell in the normal functioning of the nerve fibre cannot be assesed. Nevertheless, the experiments reviewed here indicate the presence of an acetylcholine system in these nerve fibres, which operates as a feedback mechanism on the Schwann cell for the regulation of its membrane potential and ionic permeabilities following repetitive axonal excitation.

The author wishes to dedicate the present communication to the memory of the late Professor Stephen W. Kuffler, whose contribution to the understanding of neurone–glial relationships, and whose critical and humble attitude in the discussion of experimental evidence has always encouraged our own research.

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