# MEMBRANE PROPERTIES OF NEUROGLIA IN THE OPTIC NERVE OF *NECTURUS*

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SUMMARY

The optic nerve of *Necturus* has proved a useful preparation for the study of glial cell membranes *in vivo* and *in vitro* with anatomical relations to axons intact and isolated following axon degeneration. The glial membrane potential behaves as a selective potassium diffusion potential; there is no evidence of a significant permeability to other naturally occurring ions. The specific membrane resistance of the glial cells is high compared to that of neurones; there are low-resistance intercellular connexions among the cells which permit the passage of both ions and the dye Lucifer Yellow. The cells are readily and reversibly uncoupled by procedures which decrease the intracellular pH. There is no evidence for voltage-sensitive sodium channels in the membrane. Following sodium gain and potassium loss the membrane displays a potassium-dependent strophanthidin-sensitive electrogenic sodium pump. The glial membrane is depolarized by potassium released from active axons as well as by glutamate. The glial depolarization contributes to potentials recorded with surface electrodes. Depolarization by $K^+$ plays a role in the redistribution of $K^+$ which locally accumulates around active neurones and also affects glial metabolism and glucose uptake.

INTRODUCTION

Many functions of cells have been shown to be linked to the behaviour and properties of their external membranes. Thus, transport phenomena, receptors for specific molecules and electric activity are studied in membranes for the insight they provide into the control of physiological processes. In our studies of astroglia the motivation is somewhat different. Measurements of the physiological properties of these cells were begun 'as an initial step in testing various hypotheses and widely held assumptions concerning the role of neuroglia' (Kuffler, 1967). The physiological role of these cells is still rather poorly defined; we study their membrane properties to gain additional clues to possible functions.

The present study of glial cells in *Necturus* optic nerve began about 15 years ago in Stephen Kuffler's laboratory in Boston (Kuffler, Nicholls & Orkand, 1966). The preparation was used because of its anatomical simplicity, large glial cells and capacity for survival when isolated from the animal. The physiological observations made in *Necturus* have provided a model for other vertebrate glial preparations (Somjen, 1979).

For a number of years some of his naïve students thought that Steve's remarkable knack for choosing pregnant biological problems had been transiently inhibited in the early 1960s. However, the varied and interesting studies summarized in the present volume provide ample evidence that this was not the case. The scope of likely glial functions is growing rapidly as work on the physiology of neuroglia increases.

## ANATOMICAL CONSIDERATIONS

### Normal nerve

The optic nerve of *Necturus* is a naturally isolated central-nervous-system tract surrounded by a pia-arachnoid layer containing the blood supply (Bracho, Orkand & Orkand, 1975; Kuffler *et al.* 1966). The nerve is about 100 $\mu$m in diameter and about 1 cm long. It consists of large glial cells and small axons ( < 2 $\mu$m diameter). Intracellular electrodes can readily be inserted to record the membrane potential of the glial cells either *in situ* with the blood circulation intact, or in the isolated desheathed preparation. Since blood vessels do not penetrate the nerve, the pia-arachnoid containing them can be dissected away leaving the glial cells in normal relation with the axons and with the external environment. As seen in Fig. 1, the glial processes course through the nerve forming thin sheets that separate groups of axons into bundles. At places where glial processes come close to one another they form frequent desmosomal type junctions and presumed gap junctions. Specialized junctions are not observed between glia and axons. At the surface of the nerve the glial processes form a *glia limitans* which is interrupted by narrow intercellular clefts. A basal laminal surrounds the nerve.

### All-glial nerves

After eye removal the axons of the optic nerve undergo 'Wallerian' degeneration. During axon breakdown membrane-bound electron-dense inclusion bodies appear in the glial cytoplasm, suggesting that they phagocytize the axonal remnants. There is a striking hypertrophy of the glial cytoplasm whose constituents appear essentially normal except for the addition of some phagosomes and lipid droplets as shown in Fig. 1. In the period 2–3 months after enucleation the optic nerve consists of relatively normal appearing astrocytic glial cells (Orkand, Bracho & Orkand, 1973). This preparation has proved a useful one in which to study the properties of isolated neuroglia (Cohen, 1970; Orkand *et al.* 1973; Salem *et al.* 1975; Tang, Strichartz & Orkand, 1979). Three months to a year after axonal degeneration the glial cells show some signs of disintegration. Increasing numbers of dense inclusion bodies and lipid droplets appear, the peripheral glial processes become narrower and more tortuous, the perinuclear cytoplasm decreases in volume, and the whole nerve becomes smaller in diameter. The glial cells progressively atrophy. Thus the long-term maintenance of the astrocytes seems to depend on the presence of axons.
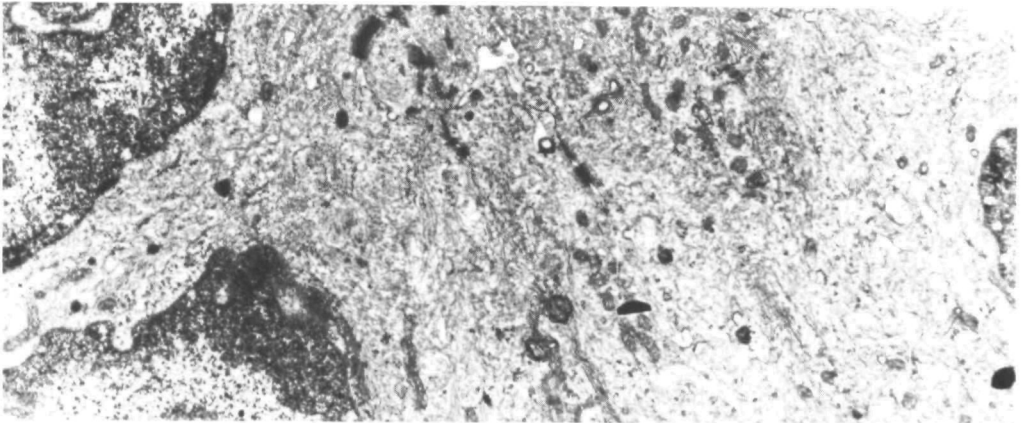
Normal
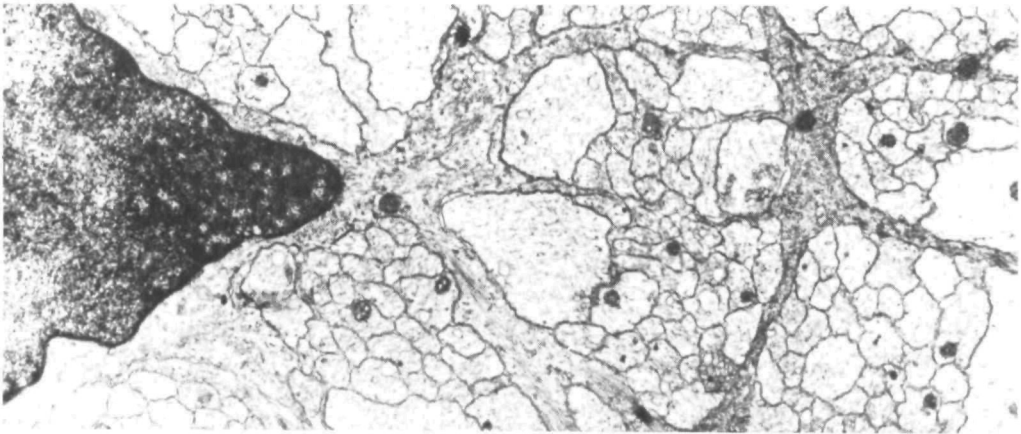


2 μm

All glia

Fig. 1. Electron micrographs of cross sections of normal and all glial optic nerves. In the normal nerve sheets of glial cytoplasm delineate bundles of unmyelinated axons. In the all glial nerves, fixed 65 days after enucleation, there are large masses of glial cytoplasm. The anatomical features of the glial cells, e.g. mitochondria, filaments, desmosomes and nuclei appear the same in both nerves (from Orkand *et al.* 1973).
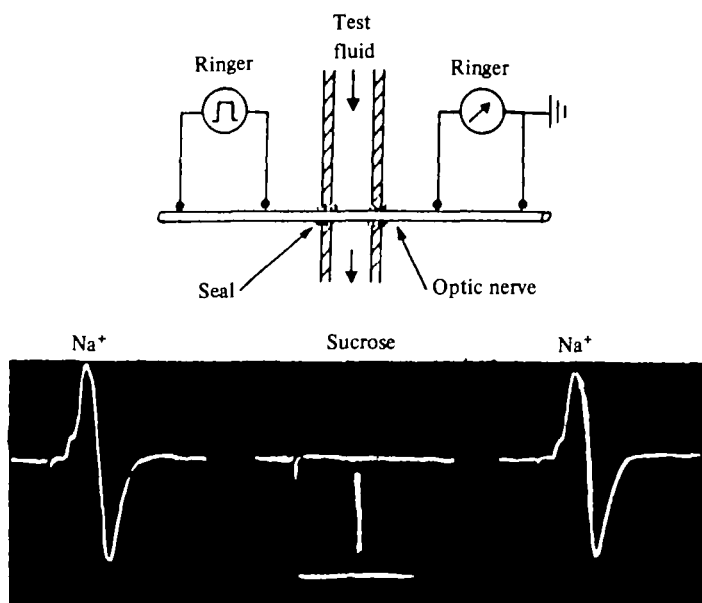
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Fig. 2. Movement of $Na^+$ and sucrose through the optic nerve of *Necturus* and reversible conduction block produced by replacing $Na^+$ with sucrose. Top: scheme of the chamber in which the optic nerve lies. The two side-chambers contain normal Ringer's solution and are sealed off from the narrow central compartment by Vaseline. The test solution flows through the central part of the chamber. Bottom: left record, a compound nerve action potential set up by maximal stimulation in the left chamber and recorded in the right chamber; middle record, 12 s after perfusion with $Na^+$-free sucrose-Ringer; conduction through the middle portion of the nerve was blocked. Right record: recovery was complete 10 s after returning to the normal Ringer solution. Calibrations: 0·5 mV, 40 ms (from Kuffler *et al.* 1966).

### PHYSIOLOGICAL PROPERTIES

*Ionic environment*

Morphometric studies of the *Necturus* optic nerve indicated that only about 10 % of the glial membrane faces the pia-arachnoid space while the remainder is surrounded by a system of narrow, 100–200 Å wide, extracellular clefts separating glial membranes and glia from axons (Dolack, Tang & Orkand, 1979). Physiological experiments have demonstrated that the ionic composition of these clefts under rest conditions is the same as that in the surrounding space (Kuffler *et al.* 1966). That is, the system of extracellular clefts freely communicates with the pia-arachnoid space; there is little if any hindrance to free diffusion for ions and small molecules such as sucrose. Fig. 2 illustrates an experiment which demonstrates the rapid exchange of sodium for sucrose through the cleft system. The dependence of the axon action potential on external sodium was used to indicate the exchange of extracellular sodium. Replacing sodium in the external solution by sucrose rapidly and reversibly blocked action potentials in the axons. Since the resting potential of the glial cells (which depends on a high $[K^+]_i$) was unaffected by this exchange it was concluded that the exchange of sodium occurred by diffusion through the extracellular clefts rather than through the glial cytoplasm. In later experiments (Tang *et al.* 1979) it was found that sodium channel

blockers like saxitoxin rapidly and reversibly diffuse in to block the nerve action potential tials.

Cohen, Gerschenfeld & Kuffler (1968) studied the question of the relative contribution of the blood plasma and the cerebrospinal fluid to the ionic environment of the glial cells. By maintaining *Necturus* in water with elevated $[K^+]$ for a few days it was possible to raise the plasma $[K^+]$ with only a minimal effect on cerebrospinal $[K^+]$. Using the membrane potential of the glial cells as an indicator of $[K^+]_0$ it was found that the $[K^+]_0$ was determined by that of the cerebrospinal fluid rather than that of the plasma. The functional ionic environment of the optic nerve may differ significantly from that in the plasma. Apparently control mechanisms exist which maintain the $[K^+]$ in the environment of the neurones and glial cells within relatively narrow limits despite fluctuations in plasma $[K^+]$.

*Passive electrical properties*

Current injection into a glial cell through an intracellular electrode leads to glial potential change over a considerable length of the optic nerve. With increasing distance from the site of injection the potential decreases in amplitude and has a slower time course. The relation between the amount of current injected, hyperpolarizing or depolarizing, and the recorded steady-state change in voltage is linear for membrane potential changes of up to 100 mV (Kuffler *et al.* 1966; Cohen, 1970). This result indicates that over the range of voltages studied the glial membrane behaves as a linear resistance and capacitor in parallel. In marked contrast to excitable membranes of neurones, the glial membrane conductance is not voltage- or time-dependent. The passive electrical properties of the glial membrane have been determined from steady state measurements of the distribution of membrane potential change produced by intracellular current injection with intracellular potential recording at various distances between the two electrodes (C.-M. Tang, unpublished observations). The optic nerve was modelled as a finite one-dimensional cable. The electrical length constant, $\lambda$, determined in this way is $800 \pm 200 \mu m$. Preliminary estimates of the specific glial membrane resistance are of the order of $40,000 \Omega cm^2$, which is considerably higher than that of neurones.

*Dye-coupling and uncoupling*

The measurements of the electrical length constant of the glial cells show that ionic current readily flows from one astrocyte to its neighbours. More recently, it has been possible to demonstrate that the fluorescent dye, Lucifer Yellow CH, mol. wt. 457, readily diffuses from one glial cell to another (Gutnik, Connors & Ransom, 1981; C.-M. Tang, P. M. Orkand & R. K. Orkand, in preparation). Fig. 3. illustrates the distribution of the dye a few minutes after intracellular injection. It can be seen that a number of glial nuclei are stained following intracellular injection. If the glial cells are perfused with Ringer's solution made acidic (pH 6·5 or below) by either bubbling with $CO_2$ in the presence of 15 mM-$HCO_3^-$ or by replacing an equivalent amount of NaCl with 50 mM sodium propionate the cells uncouple (Spray, Harris & Bennett, 1981). As shown in Fig. 3, injected uncoupled glial cells have very much the same form as cells injected with the protein horseradish peroxidase which is known from other studies not to traverse intracellular gap junctions (Takato & Goldring, 1979). The
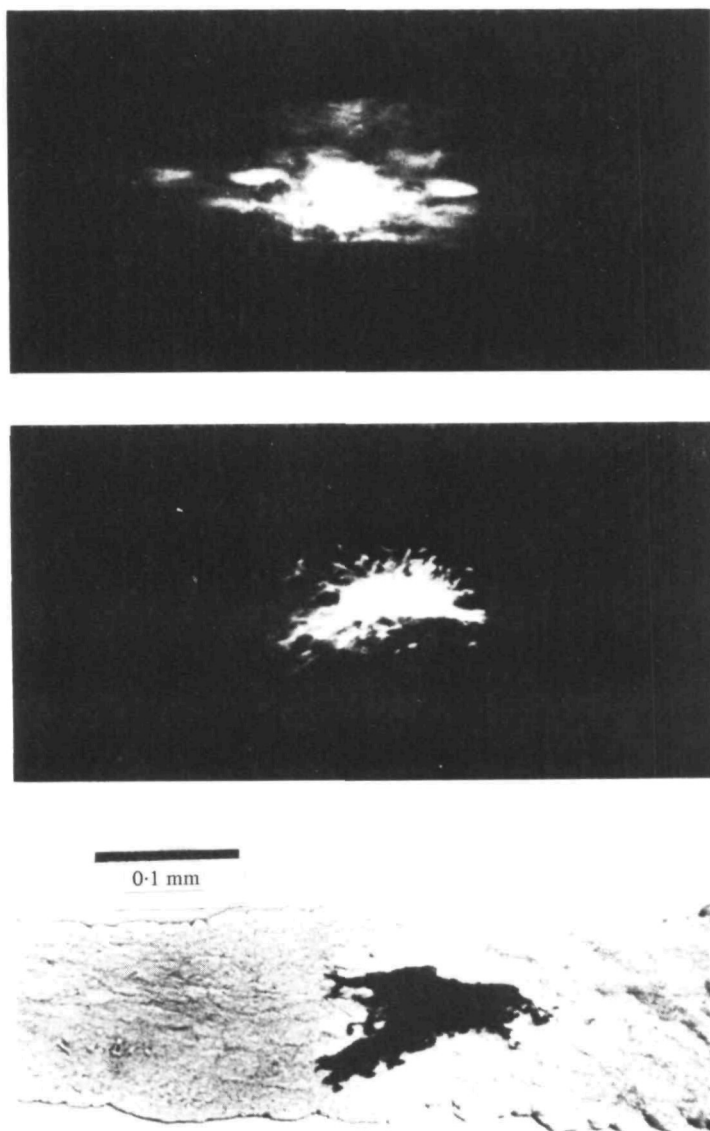
Fig. 3. Intracellular injection of Lucifer Yellow and horseradish peroxidase in normal and uncoupled optic nerve neuroglia. Top: distribution of Lucifer Yellow as viewed with fluorescence microscopy a few minutes after intracellular injection. The dye has diffused readily across cell boundaries as illustrated by the staining of a number of glial nuclei. Middle: after bathing the nerve for a few minutes in 50 mM propionate Ringer, pH 6·5, the Lucifer Yellow is confined to a single glial cell. Bottom: bright-field optics. Distribution of horseradish peroxidase injected intracellularly into a normal optic nerve. The protein is confined to a single glial cell (after C.-M. Tang, P. M. Orkand & R. K. Orkand, in preparation).

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uncoupling of glial cells in acidic media is of particular interest because it suggests that glial cell communication may be interrupted under conditions of severe respiratory acidosis.

### Permeability to monovalent ions

The normal high resting membrane potential (about $-90$ mV) of optic nerve neuroglia arises from the diffusion gradient for $K^+$ across the cell membrane (Kuffler *et al.* 1966; Bracho *et al.* 1975). Over a wide range of $[K^+]_0$ (2–110 mequiv/l) the membrane potential is accurately predicted by the Nernst relation assuming $[K^+]_i$ about 100 mequiv/l (Fig. 4A). Attempts to demonstrate a measurable effect of other naturally occurring monovalent ions (i.e. $Na^+$ and $Cl^-$) on the potential have not been successful. For other monovalent cations the permeability characteristics of the glial membrane are similar to that of the potassium channel of the neurone. The selectivity fingerprint for the glial membrane is shown in Fig. 4B. From the observation that $Tl^+$ is much more permeable than $K^+$ and that $NH_4^+$ is less permeable than $Rb^+$ it is clear that properties other than that of the diameter of the unhydrated cation determine the permeability of an ion in the glial membrane channel.

### Temperature effects

A study of the temperature dependence of the glial membrane potential is of interest not only because it serves as a test of the applicability of the Nernst relation for potassium but also because it can indicate if active transport processes contribute to the resting potential. In those systems where electrogenic transport contributes to the resting potential, a decrease in temperature produces a greater depolarization than that predicted from the Nernst relation (Carpenter & Alving, 1968), The slope of the relation between change in temperature and glial membrane potential is about 3 mV/10 °C for temperatures between 1 and 30 °C, i.e. precisely as predicted for a $K^+$ diffusion potential (Bracho *et al.* 1975). Raising the temperature above 30 °C leads to a progressive decrease in membrane potential presumably because the membrane loses its selective permeability for $K^+$ at these temperatures.

### Sodium channels

Attempts to demonstrate voltage-sensitive sodium channels in vertebrate glial cells have been fruitless. The current–voltage relation of the glial membrane is linear over a range of $\pm 100$ mV, indicating that the overall membrane conductance is not voltage dependent. Furthermore, although the normal optic nerve demonstrates a saturable uptake of saxitoxin, which binds selectively to sodium channels, as well as depolarization in response to low concentration of veratridine, which opens sodium channels, both of these responses disappear when the axons disappear 2 months following enucleation (Tang *et al.* 1979). In normal optic nerves there is a saturable uptake of saxitoxin which is equivalent to a density of sodium channels of about 25 $\mu m^{-2}$ of axon membrane, a value similar to that found in other vertebrate unmyelinated axons. After the axons were allowed to degenerate, and the optic nerve consisted only of glial cells, there was no significant saturable component of saxitoxin binding. Normal nerves are depolarized by concentrations of veratridine of $10^{-6}$ or $10^{-5}$ M. This depolarization is tetrodotoxin or saxitoxin sensitive and appears to arise
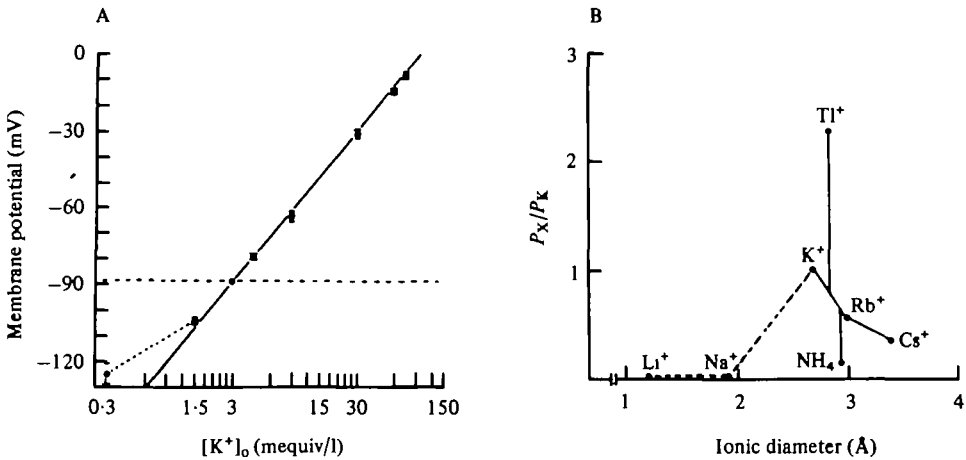
Fig. 4 (A) Relation between the glial membrane potential and $[K^+]_o$ of the fluid bathing optic nerves of *Necturus*. The mean of the resting potentials in Ringer solution (3 mequiv/1 $K^+$) was 89 mV, indicated by the horizontal dotted line. The solid line has a slope of 59 mV for a tenfold change in $[K^+]_o$ according to the Nernst equation. The membrane potential is zero when $[K^+]_i$ equals $[K^+]_o$. $[K^+]_i$ therefore is 99 mequiv/l. The membrane of glial cells can be used as an accurate $K^+$ electrode (from Kuffler *et al.* 1966). (B) Selectivity 'fingerprint' for the glial membrane. Permeabilities of different cations relative to $K^+$. Diameter is for unhydrated cation. Values for $Na^+$ and $Li^+$ were not measurable (from Bracho *et al* 1975).
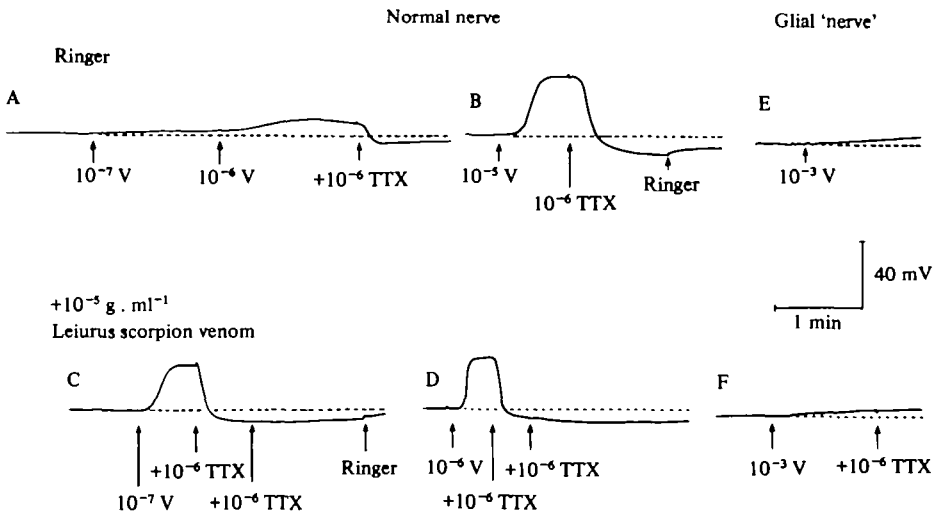


Fig. 5. Effect of veratridine (V) to depolarize the normal (A–D) and all glial (E, F) optic nerve. D.C. recording across a sucrose gap. A, B and E in normal Ringer. In A and B depolarization is blocked by the addition of $10^{-6}$ M tetrodotoxin (TTX). In C, D, and F, $10^{-5}$ g. ml$^{-1}$ Leiurus scorpion venom has been added to the Ringer. This substance potentiates the effect of veratridine in normal nerve (C, D) but not in the all glial nerve (F). TTX has no effect on the small depolarization produced by $10^{-3}$ M veratridine in the all glial nerve. The dashed line shows the base line for no change in membrane potential (from Tang *et al.* 1979).

from the effect of veratridine to open axonal sodium channels (Fig. 5). All glial nerves are not depolarized by such low concentrations. High concentrations of veratridine ($10^{-2}$ to $10^{-3}$ M) do depolarize isolated glial cells. However, this depolarization is not sensitive to the sodium channel blockers and its basis is unknown. If one records intracellularly from a glial cell in a normal nerve during application of veratridine in low concentration a glial depolarization results. This depolarization appears due to a release of potassium from the depolarized axons rather than to a direct effect on the glial membrane.

### Chemical sensitivity

In addition to veratridine, optic nerve neuroglia in the normal nerve are depolarized by exposure to glutamate ($10^{-5}$ M). This glutamate depolarization is still observed in the isolated glial cells. At this time we know little of the properties of glutamate receptors on glial cells or the nature of the permeability changes induced by glutamate in the glial membrane.

### Electrogenic sodium transport

The sodium permeability of the glial membrane is apparently low compared to that in neurones (Kuffler *et al.* 1966; Bracho *et al.* 1975). Nevertheless, there is presumably some inward leakage of sodium which must be balanced by active transport. If a glial nerve is bathed for some hours at low temperature in a potassium-deficient medium the recorded resting potential does decline (Tang, Cohen & Orkand, 1980). This depolarization probably results from a loss of internal potassium (presumably in exchange for sodium) (Keenan & Niedergerke, 1967). When such a potassium-depleted glial cell is exposed to normal potassium the membrane potential will either increase or decrease less than that expected from the normal potassium sensitivity of glial membrane (Fig. 6). Pretreatment of a potassium-depleted glial cell with the sodium-pump inhibitor strophanthidin or replacement of sodium for lithium in the external bathing solution will restore the normal potassium sensitivity. These results suggest that the addition of normal potassium to a potassium-depleted glial cell activates a hyperpolarizing electrogenic pump. The electrogenic pump in glial cells, like that in neurones, appears to be stimulated by an elevated internal sodium and requires external potassium. Under more normal conditions, without pretreatment in potassium-depleted solutions, we have been unable to demonstrate an electrogenic component of the membrane potential either by the addition of strophanthidin or by varying temperature.

### Depolarization with neuronal activity

Nerve impulses lead to glial depolarization as a result of an accumulation of $K^+$, lost from active axons, in the narrow intercellular cleft separating axons from glial cells (Orkand, Nicholls & Kuffler, 1966). As shown in Fig. 7, with a single nerve volley, the glial depolarization rises to a peak of about 3 mV in 50–150 ms and decays with a half-time of about 2 s. Repetitive nerve volleys lead to a nonlinear summation of glial depolarization as expected from the nonlinear relation between glial membrane depolarization and $[K^+]_o$. The observed glial depolarization is consistent with a
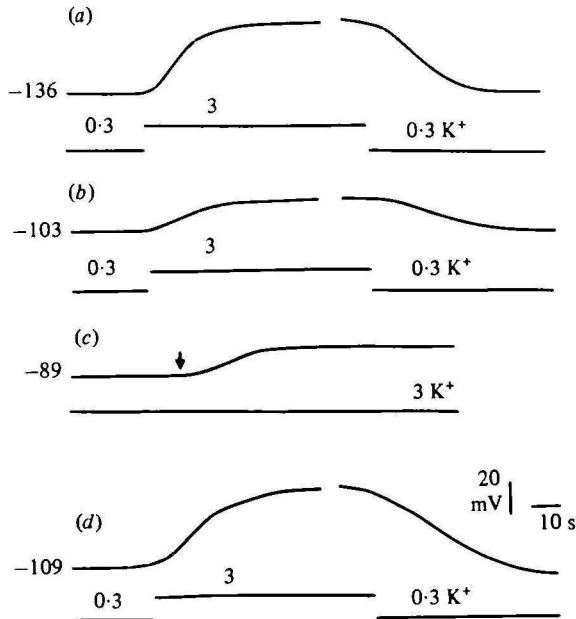
Fig. 6. Tracings of intracellular records from glial cells from all-glial optic nerves in normal Ringer's solution with varying $[K^+]_o$. (a) Freshly dissected nerve. In a nerve soaked in 0·3 mM-K for about 1 min changing to normal 3 mM-K depolarizes the glial cell by about 40 mV. (b–d) Glial nerve soaked for 2 h in 0·3 mM-K at 10 °C prior to placing in chamber in same low $K^+$ solution at room temperature (22 °C). (b) Changing to normal 3 mM-$K^+$ depolarizes the nerve by less than 20 mV (c) The addition of $10^{-4}$ M strophanthidin (↓) after about 1 min in 3 mM-K depolarizes the nerve by about 20 mV. (d) In the presence of $10^{-4}$ M strophanthidin an increase in $[K^+]_o$ from 0·3 to 3 mM depolarizes the nerve by about 40 mV similar to the effect in freshly dissected nerve shown in (a). Initial resting potentials are indicated to left of traces. Gaps in traces 30–60 sec (from Tang et al. 1980).
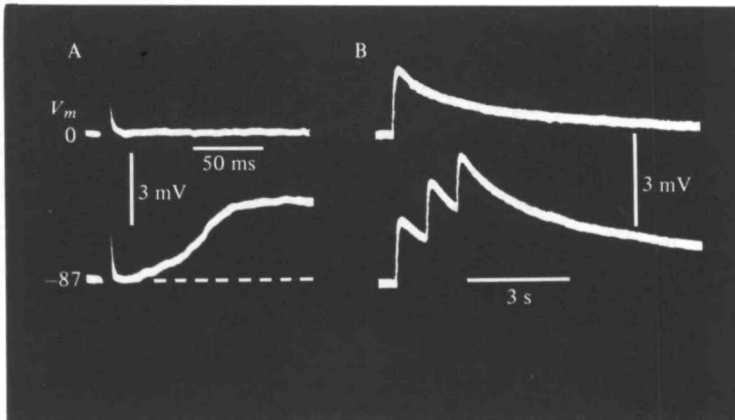


Fig. 7. Depolarization of glial cell produced by nerve impulses in *Necturus* optic nerve. (A) Top record: microelectrode within optic nerve was extracellular, thus $V_m = 0$, and nerve was stimulated by maximal electrical stimulus (shock artifact recorded, nerve action current not measurable). Bottom record: electrode slightly advanced and glial membrane potential of −87 mV recorded. Same electrical stimulus produced a glial depolarization of 3·1 mV, which reached a peak in about 75 msec. (B) Top: record of change in glial membrane potential following nerve stimulation shown on a much slower time scale. Bottom: stimulus is repeated 3 times at 1 s intervals and there is summation of glial depolarizations (from Orkand et al. 1966).
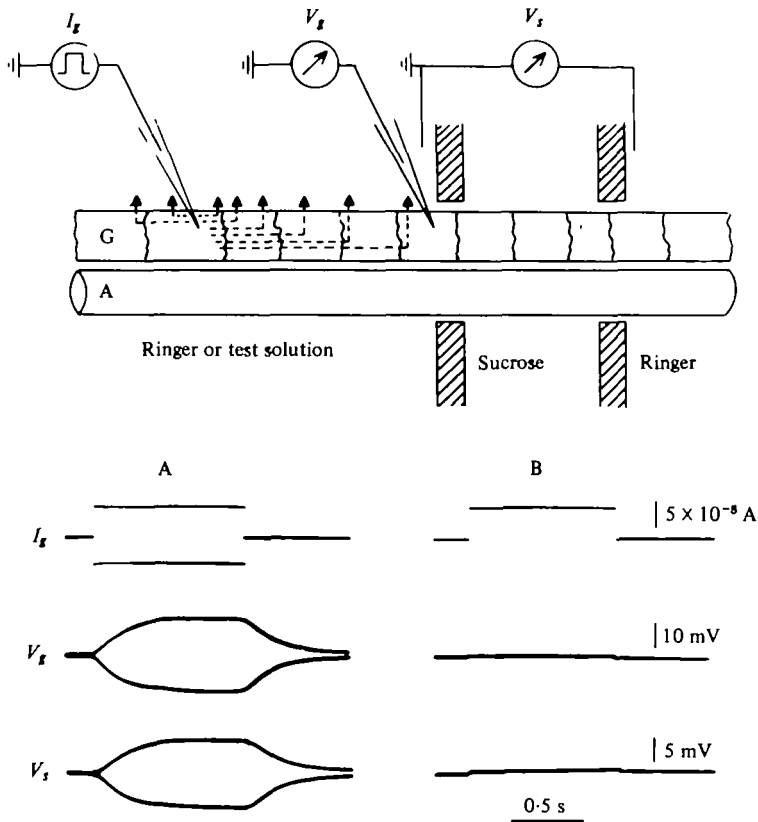
Fig. 8. Top: schematic representation of experimental arrangement. The desheathed optic nerve of *Necturus*, here represented by a single axon (A) and a row of glial cells (G), was placed in a sucrose gap chamber consisting of three compartments. Two microelectrodes were inserted into glial cells in the left-hand compartment. The one distant from the central compartment, or gap, was used for passing current pulses $(I_g)$. In the same compartment, less than 90 $\mu$M from the gap, resulting changes in glial membrane potential $(V_g)$ were recorded. Changes in surface potential were recorded simultaneously between two other electrodes, one on each side of the gap $(V_s)$. Since glial cells are electrically coupled to each other, current injected into one cell spreads to neighbouring cells, causing their membrane potentials to change. Bottom: (A) simultaneous potential changes recorded with an intracellular electrode $(V_g)$ in a glial cell near the sucrose gap and with extracellular leads across the gap $(V_s)$. The currents, monitored in the top trace $(I_g)$, were injected into a glial cell 1020 $\mu$M from the gap. Note similar time courses of the potentials, but $V_s$ is about one-half of $V_g$ (see calibration). (B) Same conditions as in (A), but current electrode was advanced into the intercellular space just outside the glial cell. Note the absence of significant potentials when current does not cross glial membrane (from Cohen, 1970).

potassium efflux from the axons of about $1_p M/cm^2$ into a maximum intercellular cleft width of about 250 Å. Since the introduction of potassium selective electrodes the increase in $[K^+]_o$ with nerve impulses has been directly measured in the amphibian spinal cord (Sykova *et al.* 1976). Glial depolarization with neuronal activity is readily observed in the optic nerve under conditions of natural stimulation of the retina with light (Orkand *et al.* 1966). Glial depolarization by raised $[K^+]_o$ may participate in the proposed role of glial cells to equilibrate $[K^+]_o$ in various regions of the nervous system (Orkand *et al.* 1966; Gardner-Medwin, 1981).
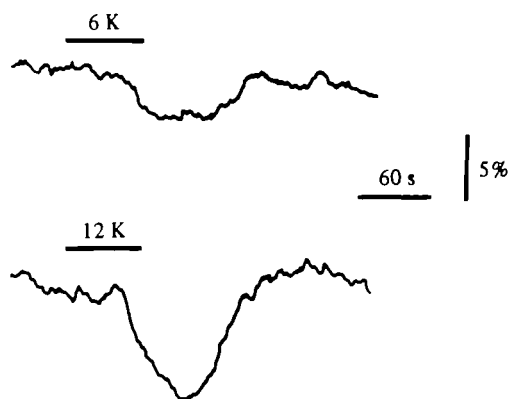
Fig. 9. Fluorescence decrease in all glial optic nerve when the K$^+$ is raised from normal 3 m-equiv/l to 6 or 12 mequiv/l. Optical technique is based on the observation that NADH fluoresces when excited with light of 355 nm and emits light with a peak at 480 nm; NAD$^+$ does not. Decrease in fluorescence results from decrease in NADH (from Orkand *et al.* 1973).

*Contribution to surface recording*

When an optic nerve is placed across a sucrose gap a significant fraction (30–60%) of the change in a glial membrane potential produced either by passing current across the glial membrane or by changing [K$^+$]$_0$ may be recorded extracellularly across the gap. (Fig. 8). The glial contribution to the surface potential is a function of the relative volume of neuronal and glial tissue and the ease with which current spreads through the glial syncytium (Cohen, 1970). This result is of interest for the interpretation of electrical recordings made from the surface of active nervous tissue. As indicated above, the glial membrane potential undergoes slow potential changes with changes in [K$^+$]$_0$ due to K$^+$ efflux from active neurones. With maximal axonal stimulation the potassium concentration might increase to a level of 20 mM leading to a glial depolarization of almost 50 mV (Orkand *et al.* 1966; Sykova & Orkand, 1980). During the recovery phase after such stimulation the axons undergo a marked hyperpolarization due to activation of their electrogenic pump while the glial depolarization slowly subsides (Tang *et al.* 1980). Under these conditions the surface recorded potential is a complex function of the opposing potential changes going on in both neurones and glia.

*Control of glial metabolism*

Hypotheses for a functional role of neuroglia in the behaviour of the nervous system usually require coordination between neuronal and glial activity. Therefore, it is of some interest that it has been possible to demonstrate that increases in [K$^+$]$_0$ of the same order as that which occurs as a result of potassium efflux from active neurones are sufficient to alter metabolic activity in glial cells. In the all-glial optic nerve, it was possible to demonstrate (Fig. 9) that the levels of reduced pyridine nucleotide are decreased in intact cells by the addition of potassium to the external bathing solution (Orkand *et al.* 1973), and that glucose uptake is increased (Salem *et al.* 1975) What remains is the more difficult task of defining which metabolic reactions are

ffected and demonstrating how these changes contribute to the functional activity of the glial cells.

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