zontal cells and their processes in the outer plexiform layer.

As can be ascertained from Fig. 2, the amine-containing interplexiform cells provide a centrifugal pathway from the inner to the outer plexiform layers of the retina. Such a retinal pathway has not been generally accepted in recent years (*16*). The abundance of processes of the amine-containing interplexiform cells both in the outer and inner plexiform layers of goldfish (Fig. 1a) suggests that these cells may be of more than marginal importance for retinal function. Golgi impregnations have shown that interplexiform cells are found in the retinas of several species (*5*), although they may not contain amines demonstrable by fluorescence microscopy. It must be inferred, therefore, that interplexiform cells are a general feature of vertebrate retinas and that they may employ different neurotransmitters.

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Mechanism of Axonal Transport: A Proposed Role for Calcium Ions

Abstract. *In vitro axonal transport of tritiated protein decreased 40 to 60 percent when neuronal cell bodies were incubated in calcium-free medium, but was not affected when only nerve trunks were exposed to calcium-free conditions. In addition, calcium-45 was transported along axons at a rate similar to that of rapidly transported tritiated protein. These data are interpreted to suggest that calcium ions are involved in the initiation of axonal transport and in the coupling of transported proteins to the transport system.*

Neurons are characterized by numerous cytoplasmic projections—axons and dendrites—that frequently extend for relatively large distances from their cell bodies. A major portion of the macromolecules and organelles required by distant regions of the neuron is supplied by a system known as axonal (or dendritic) transport, which actively moves these materials from their site of synthesis in the perikaryon to their sites of structural and metabolic utilization (*1, 2*). While the mechanisms of axonal transport are not clearly understood, broad outlines of the system have emerged: different materials are transported from as well as toward the cell body at markedly differing rates (*1, 2*); once macromolecules have been synthesized and "loaded" onto the transport system the cell body is no longer required for their continued transport, but the system is dependent on a local supply of metabolic energy (*3*); and the intra-axonal longitudinal networks of microtubules or neurofilaments may be involved in the transport system (*4*).

Models of axonal transport (*5*) have been based on the mechanochemical coupling system of muscle contraction, in which linkage of the proteins actin and myosin is mediated by calcium ions (*6*). Our previous studies (*7*) have indicated that calcium ions are required for axonal transport of proteins in vitro. Calcium-free conditions (that reduced endogenous calcium concentrations to 25 percent of normal) resulted in a decrease of approximately 60 percent in the amount of protein transported along axons. This effect was not due to depression of uptake of the precursor amino acid into the cell bodies nor to a selective action on synthesis or degradation of proteins. We now report that the calcium requirement appears localized in the cell body, and that $^{45}Ca^{2+}$ actively moves along axons at a rate similar to that of rapidly transported proteins. These observations more directly suggest a role for calcium in the mechanism of axonal transport.

Axonal transport was followed along the peripheral axons of dorsal root ganglion neurons from the bullfrog, *Rana catesbeiana*. The sciatic nerves, eighth and ninth spinal nerves, spinal roots, and dorsal root ganglia were dissected together with the spinal cord. In

each experiment, the cord was bisected longitudinally to provide a contralateral control. The incubation chamber, as previously described (*7*), was designed to allow the ganglia, which contain the neuronal cell bodies, to be isolated by silicone grease barriers from their spinal roots and peripheral nerves. With this

Incubation conditions		TCA-insoluble (count/min)		
Experimental	Control	E	C	E/C
A		1. 19,693	49,782	0.39
		2. 27,762	61,748	0.45
		3. 10,694	24,436	0.44
B		1. 4,131	9,189	0.45
		2. 16,234	28,493	0.57
		3. 13,151	22,600	0.58
C		1. 19,709	18,598	1.06
		2. 11,463	12,609	0.91
		3. 22,852	24,061	0.95

Fig. 1. Effect of calcium-free incubation conditions on axonal transport. In each experiment, the eighth and ninth dorsal root ganglia of both hemicord preparations were exposed for 1 hour to [4,5-^{3}H]-leucine (50 μc per milliliter of normal incubation medium; specific activity, 30 to 50 c/mmole; New England Nuclear). The hemicord preparations were then removed from the chamber, cleaned of silicone grease, and returned to fresh incubation medium. Axonal transport at 18°C in normal medium was compared to that in calcium-free medium during an additional 19-hour period under conditions where (A) ganglia and nerve trunks, (B) only ganglia, or (C) only nerve trunks of one preparation were exposed to the calcium-free medium. Normal incubation medium was composed of (in millimoles per liter) NaCl (114), KCl (2.0), $CaCl_2$ (1.8), glucose (5.5), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (2.0), adjusted to *p*H 7.4. Calcium-free medium was supplemented with 1 m*M* EGTA and 4 m*M* $MgCl_2$. The eighth and ninth dorsal root ganglia (*G*), spinal nerves (*SN*), and the sciatic nerve (*Sc*) are drawn to show incubation conditions: hatched areas indicate portion of preparation exposed to normal medium and open areas that exposed to calcium-free medium. After the 20-hour experimental period, spinal and sciatic nerves were cut into 3-mm segments and the amount of trichloroacetic acid (*TCA*)–insoluble radioactivity in each piece was determined (*7*) as a measure of [^{3}H]protein undergoing axonal transport. Total counts per minute of radioactivity in equal lengths of spinal and sciatic nerves from each hemicord preparation are shown for three experiments from each incubation procedure. Radioactivity in the 6-mm segment of nerve adjacent to each ganglion was omitted from the total count because of the proximity of this segment to isotope during the initial 1-hour pulse. The ratio of total radioactivity in the experimental (*E*) to that in the contralateral control (*C*) preparation is *E*/*C*.

arrangement, the ganglia of both hemicord preparations can be incubated in [^{3}H]leucine or $^{45}CA^{2+}$, while the spinal cord, roots, and nerves are maintained in normal (unlabeled) medium. Following a 1-hour exposure of ganglia to the isotope in normal frog Ringer solution (1.8 m*M* in Ca^{2+}), the nerve trunks or the ganglia (or both) of one hemicord preparation were selectively perfused for varying periods at 18°C in nonradioactive, calcium-free medium supplemented with 4 m*M* Mg^{2+} and 1 m*M* ethylene glycol bis(aminoethylether)tetraacetate (EGTA). The contralateral preparation was maintained for similar time periods in normal medium. Axonal transport of protein was assessed by determining the acid-insoluble radioactivity in 3-mm segments of nerve (*7*); $^{45}Ca^{2+}$ was determined in nerve segments dried at 60°C and dissolved in Soluene (Packard Instrument Co., Inc.). Adenosine triphosphate (ATP) concentrations within the ganglia were determined by a fluorometric procedure (*8*).

A marked decrease in the amount of transported protein was seen when ganglia as well as nerves were exposed to calcium-free medium (Fig. 1A). A similar decrease occurred when only the ganglia were perfused in calcium-free medium (Fig. 1B). By contrast, when the peripheral nerves, but not the ganglia, were exposed to calcium-free medium, no effect was observed on the amount of transported protein (Fig. 1C). In all three types of calcium-free incubation conditions there was no apparent difference in the rate of transport relative to that in normal incubation conditions. These findings are consistent with previous observations (*9*) that calcium-free medium does not affect axonal transport if the cell bodies remain in their normal ionic milieu.

Electron microscopic examination was performed to assess the possibility that decreased transport might be secondary to structural disruption of the tissue. No obvious ultrastructural changes in either cell bodies or axons were observed after a 20-hour incubation in calcium-free medium (*10*).

One indication that the general metabolic state of the cell bodies was not affected by calcium-free conditions was the similarity in the ATP concentrations within ganglia exposed to both incubation conditions: 8.8 ± 0.7 μmole of ATP per gram of protein (mean ± standard error of the mean, $N = 9$) after normal incubation; 8.5 ± 0.9 ($N = 9$) after calcium-free incubation. The findings that anoxia or drugs which inhibit generation of energy depress the rate of axonal transport but not the amount of material transported (*3*) further suggest that exposure of ganglia to calcium-free medium did not affect oxidative metabolism, since during these incubation conditions the amount but not the rate of [^{3}H]protein undergoing axonal transport was decreased. The dependence of axonal transport on a local source of energy within the axon (*3*) has been postulated to involve a Ca^{2+},Mg^{2+}-activated adenosine triphosphatase (*11*). Since exposure of axons to calcium-free conditions has no marked effect on the amount or rate

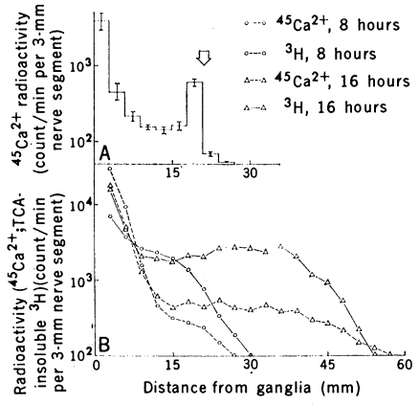

Fig. 2. Axonal transport of $^{45}Ca^{2+}$. (A) Accumulation of $^{45}Ca^{2+}$ proximal to ligature placed on spinal nerve. Transport at 18°C was assessed 19 hours after a 1-hour exposure of ganglia to $^{45}Ca^{2+}$ (600 μc per milliliter of normal incubation medium; specific activity, 9.3 mc/mg; New England Nuclear). After incubation, nerves were cut into 3-mm segments and individually transferred to minivials (Cal Glass for Research). Segments were dried at 60°C and dissolved in 250-μl Soluene per vial. Radioactivity was determined after addition of 5 ml of toluene-based scintillation fluid. Arrow indicates position of ligature. Each bar indicates mean ± standard error of the mean from four experiments. The high levels of radioactivity in the two segments closest to the ganglia are due to direct exposure of that portion of nerve to isotope during the initial 1-hour incubation and to retardation of calcium exchange during the subsequent 19-hour period because of residual silicone grease on the nerve (see Fig. 1 legend). (B) Comparison of axonal transport of $^{45}Ca^{2+}$ and [^{3}H]protein. Transport of $^{45}Ca^{2+}$ was assessed in the absence of ligatures during 8- and 16-hour incubation periods. Incubation conditions and treatment of tissue were as described in (A). Transport of [^{3}H]protein was assessed in the contralateral preparation at the same time periods after a 1-hour exposure of ganglia to [4,5-^{3}H]leucine, as described in the legend to Fig. 1. Results are representative of three separate experiments for each time period.

of transport, provided the cell bodies are maintained in normal incubation medium, it follows that the energy supply within the axons on which transport depends is not affected by the altered ionic conditions.

The finding of a calcium requirement for axonal transport, localized at a site within the ganglia, suggested that calcium ions might serve as ionic links during the loading of proteins onto the transport system. To test the hypothesis that such calcium links might be involved during the transport process as well as during loading, the possible axonal transport of $^{45}Ca^{2+}$ was examined. Such transport was indicated 19 hours after a 1-hour exposure of ganglia to $^{45}Ca^{2+}$ by a pronounced buildup of the isotope proximal to ligatures placed on spinal nerves (Fig. 2A). The rate of $^{45}Ca^{2+}$ transport during 8- and 16-hour incubations at 18°C was similar to the fast transport rate of [^{3}H]protein, approximately 75 mm/day (*12*), as determined at the same temperature in the contralateral preparation (Fig. 2B).

Calcium movement along axons in association with mitochondria would be an expected observation since these organelles concentrate large quantities of calcium (*13*). However, studies have generally shown that mitochondria are transported at rates significantly slower than those observed for rapidly transported proteins (*14*). Calcium-binding proteins present in extracts of neural tissues (*15*) might also account for the axonal transport of calcium, but characteristics of the transport of these proteins have not yet been reported.

The present observations that calcium-free incubation conditions markedly depress the amount of radioactive protein transported from cell body to axon, and that exposure of the ganglia to calcium-free medium is essential for this effect to occur, indicate that calcium ions may be involved in the initiation of axonal transport. In addition, the transport of $^{45}Ca^{2+}$ along axons at a rate similar to that of rapidly transported proteins suggests a mechanism in which calcium ions couple the transported proteins to the transport system, a system that may involve the "sliding filaments" proposed in models of axonal transport (*5*).

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Development of Specific Sensory-Evoked Synaptic Networks in Fetal Mouse Cord-Brainstem Cultures

Abstract. *Neurites of nerve growth factor–enhanced fetal mouse dorsal root ganglion cells can not only establish characteristic sensory synaptic network functions in dorsal regions of attached spinal cord explants, but some of the neurites may grow through the cord tissue in these cultures and make similar functional synaptic connections with specific types of "target" neurons in localized zones within nearby medulla explants.*

In a recent study of fetal rodent spinal cord explants with attached sensory dorsal root ganglia (DRG's), we observed marked augmentation of characteristic sensory-evoked synaptic network discharges in dorsal cord regions following DRG hypertrophy induced by nerve growth factor (NGF) (*1*). The present report demonstrates, for the first time, that some of these NGF-enhanced DRG neurites may grow through the cord tissue and establish similar functional synaptic connections with specific types of "target" neurons in nearby brainstem explants. Earlier electrophysiologic studies showed that complex synaptic interactions can, indeed, develop between neurons in explants of fetal rodent spinal cord and brainstem tissues after growth of neurites across gaps of 0.5 to 1 mm (*2*), but no signs of selective synaptic connections between specific central nervous system (CNS) neurons were detected.

Spinal cord with attached DRG's and meningeal covering from 13- to 14-day fetal mice was cut into cross sections 0.5 to 1 mm thick (Fig. 1). Prior to explantation, a midline section of the spinal cord fragment was made from the central canal through the dorsal cord and meninges; in the younger fetuses only the meninges required cutting since dorsal closure of the cord was not yet complete. This ensured outgrowth of CNS neurites and glial cells, including DRG fibers which have passed through the cord, comparable to dorsal column axons. Previous studies showed that "peripheral-type" neurites (invested by Schwann cells) will not invade separate CNS explants (*3*), and functional bridging of neural explants has so far only been achieved through growth of "CNS-type"