A glial–neuronal–glial communication system in the mammalian central nervous system

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Previous studies have demonstrated that when tritiated proline \(^{3}H\)Pro is injected into the dorsal column nuclei (DCN) of cats, it labels macroglial cells, but fails to label neurons at the injection site. (Tritiated leucine \(^{3}H\)Leu) in contrast, labels both neurons and some glial cells.) Despite the failure of \(^{3}H\)Pro to label DCN neurons, labeling is still observed in DCN terminal targets. This result suggests that glial cells are involved in the translocation of \(^{3}H\)Pro-labeled molecules from one part of the brain to another. The purpose of the present experiment was to use electron microscopic autoradiographic techniques to characterize the labeling produced in internal arcuate fiber tract axons arising from DCN neurons 24 h after injections of \(^{3}H\)Pro (or \(^{3}H\)Leu, for comparison) into DCN. It was reasoned that, if the translocation of \(^{3}H\)Pro-labeled molecules from DCN to its targets is indeed carried out by glial cells, then only glial elements associated with the fibers should be labeled following \(^{3}H\)Pro injections of DCN. If, on the other hand, the translocation involves an initial transfer of \(^{3}H\)Pro-labeled molecules into neuronal perikarya followed by axonal transport, then only axoplasmic elements along the fiber pathway should be labeled. Injections of \(^{3}H\)Pro into DCN labeled axoplasmic elements in samples of axons from the internal arcuate tract both ‘near’ (0.5-0.8 mm) and ‘far’ (2-4 mm) from the injection site at about an equal absolute density. However, glial elements associated with the axons were also labeled in both samples, but much more densely in the ‘near’ than in the ‘far’ axons. Injections of \(^{3}H\)Leu labeled axoplasm more densely than did \(^{3}H\)Pro (by a factor of 4 in the ‘far’ samples). Glial labeling by \(^{3}H\)Leu near the injection site was much less than that of \(^{3}H\)Pro, but, ‘far’ from the injection, the levels of \(^{3}H\)Leu and \(^{3}H\)Pro glial labeling were comparable. Taken together with the results of other studies, these data support the existence of a previously unrecognized system of communication between glial cells and neurons. In this putative system (Fig. 9), molecules containing both \(^{3}H\)Leu and \(^{3}H\)Pro are transferred from glial cells into adjacent neuronal soma and transported down the length of the axon where, all along the way, some of them are transferred from the axon into adjacent glial processes. The system is more readily apparent when \(^{3}H\)Pro is used because of its avid and preferential uptake by glial cells. Potential functions of such a system are unknown, but could be trophic, protective and/or informative.

INTRODUCTION

Amino acids injected into brain tissue are taken up by neuronal perikarya where they are incorporated into larger molecules and transported via axons to their terminals. Autoradiographic studies of the projections of groups of neurons rely upon this conceptualization of incorporation and transport.

The results of several studies on the incorporation and transport of \(^{3}H\)proline(\(^{3}H\)Pro)-labeled molecules are not consistent with this general conceptualization. In these studies, light and electron microscopic autoradiographic techniques were used to compare the labeling patterns following injections of \(^{3}H\)Pro and \(^{3}H\)Leucine \(^{3}H\)Leu into the central nervous system. At the light microscopic level, it was found that wherever \(^{3}H\)Pro was injected, it was incorporated nearly exclusively by small cells, whereas \(^{3}H\)Leu was incorporated by both small and large cells. Although the \(^{3}H\)-labeled small cells were unidentifiable at the light microscopic level, at the electron microscopic level, they proved to be macroglial cells (astrocytes/oligodendrocytes), at least for the dorsal dolumn nuclei (DCN) of the cat.

This result was surprising, because, despite the failure of \(^{3}H\)Pro to label neurons in DCN, there was...
at least some labeling at all of the terminal targets of these neurons. In some targets, such as the inferior olive (IO), the labeling was in fact quite dense (even though the ‘terminal’ labeling produced by $[^{3}H]$Pro injections was invariably less dense than the labeling produced by comparable $[^{3}H]$Leu injections).

These data suggest that the mechanisms by which $[^{3}H]$Pro-labeled molecules are translocated from one part of the brain to another involve macroglial cells and differ from the mechanisms by which $[^{3}H]$Leu-labeled molecules are transported.

Using the DCN to IO pathway as a guide, there are a number of possible ways in which glial cells could be so involved. As shown in Fig. 1A, it may be that the translocation of $[^{3}H]$Pro-labeled molecules from DCN to IO is carried out entirely by glial cells (see p. 16 of ref. 37). Although this possibility seems remote because injections of $[^{3}H]$Pro directly into axonal tracts (e.g., internal capsule, internal arcuate, dorsal columns of spinal cord, pyramidal tract) that label glial cells at the injection site fail to label the targets (or sources) of the axons in those tracts, it still may be the case that the initial incorporation process requires an interaction between neuronal perikarya (or dendrites) and glial cells, but that the rest of the translocation sequence is then carried out by glial cells.

As shown in Fig. 1B, another possibility is that $[^{3}H]$Pro-labeled molecules at the incorporation site are continuously transferred from glial cells into adjacent neuronal perikarya where they are then immediately transferred down the axon via axonal transport mechanisms. (Such a process would leave the neuronal perikarya virtually empty of labeled protein at any given point in time.)

One way to test these alternative possibilities is to compare the labeling patterns associated with the axons extending between DCN and IO (i.e., the internal arcuate fibers) following injections of $[^{3}H]$Pro or $[^{3}H]$Leu into DCN. If the translocation of $[^{3}H]$Pro-labeled molecules from DCN to IO is indeed carried out by glial cells (Fig. 1A), then only glial elements associated with the internal arcuate fibers should be labeled following $[^{3}H]$Pro injections of DCN. If, on the other hand, the translocation involves an initial transfer of $[^{3}H]$Pro-labeled molecules into neuronal perikarya followed by axonal transport (Fig. 1B), then only axonal elements along the internal arcuate pathway should be labeled.

Accordingly, the purpose of the present study was to use quantitative electron microscopic autoradiographic techniques to characterize the differences in $[^{3}H]$Pro and $[^{3}H]$Leu labeling patterns associated with the internal arcuate pathway between DCN and IO following injections of $[^{3}H]$Pro or $[^{3}H]$Leu into DCN.

**MATERIALS AND METHODS**

Four adult cats of either sex, each weighing about 3 kg were used. The cats were initially anesthetized with sodium thiamylal (i.v.) and maintained on halothane gas. Two cats received single injections (0.2 $\mu$L; 20 $\mu$Ci/$\mu$L in saline) of $[^{3}H]$Leu (L-[3,4,5-3$H$]leucine; spec. act., 146.5 Ci/mmol) into the left cuneate nucleus. Two other cats received single injections (0.2 $\mu$L; 20 $\mu$Ci/$\mu$L in saline) of $[^{3}H]$Pro (L-[2,3,4,5-3$H$]Pro; spec. act., 139.1 Ci/mol) into the right cuneate nucleus. Injections were made through a glass micropipette (tip diameter about 75 $\mu$m) at a rate of 0.02 $\mu$L/min.

Twenty-four hours after surgery, cats were anesthetized with sodium thiamylal (i.v.) and perfused

![Diagram](image-url)
transcardially with 500 USP units of sodium heparin and 100 μl of 0.9% NaCl at room temperature fol-
lowed by one liter of 4 °C fixative (2.5% (w/v) para-
formaldehyde and 0.5% (v/v) glutaraldehyde with 
4% sucrose in 0.1 M phosphate buffer, pH 7.2).
Transverse slabs of medulla (4 mm thick) were taken
by hand with a razor blade (Fig. 2A). Cores of tissue
were then removed from DCN, internal arcuate fi-
bers (Fig. 2B) and IO with a beveled 20- or 21-gauge
needle57, as described in a previous paper57.
The core samples were stored in fixative at 4 °C for
30 min, rinsed in cold 1% OsO4 with 1.5%

Fig. 2. Flow chart of the sampling and quantification methods. Cores of tissue along the internal arcuate fiber pathway (B) are re-
moved from slabs of the medulla (A). The cores are embedded in plastic (C). Semi-thin sections (D) are removed for light microscopic
autoradiography. The face of the core is then trimmed (pyramid in C) and thin sections (E) are cut for electron microscopic autoradi-
ography. Groups of 12 adjacent photomicrographs (F) are taken of bundles of labeled internal arcuate fibers. A point-intersect grid
with 50% probability circles at each intersection is used to estimate area occupied by each tissue element (G). This same circle is used
to determine the location of each grain in the same micrographs (H). The arrow in H points to a grain that is located over an astrocytic
soma element. Bar (H) = 2 μm.
K₃Fe(CN)₆ and 4% sucrose in 0.1 M phosphate buffer, rinsed in cold saline, dehydrated in a series of ethanols, infiltrated with Epon–Araldite and embedded in flat molds (Fig. 2C). The slabs (Fig. 2A) were stored for several days in 30% sucrose fixative, frozen, cut into 20 μm thick serial sections and processed for light microscopic autoradiography (Fig. 3). Using these 20 μm thick serial sections as a guide, each cylindrical, plastic-embedded core sample was trimmed to the transverse level where it contained its best sample of labeled internal arcuate fibers. Several 2 μm thick sections were then cut from the core face (approximately 1 mm diameter) and prepared for light microscopic autoradiography (Fig. 2D).

The 20 μm thick sections from the slabs and the 2 μm thick, plastic-embedded sections from the cores were examined to select which cores would be processed further for quantitative electron microscopic autoradiography. Two selection criteria were used. First, the core had to be located within the internal arcuate fibers at measurable and varied distances from the edges of the incorporation site. Second, the labeling over the 2 μm thick, plastic-embedded sections had to be dense enough when viewed under the light microscope to indicate that adjacent thin sections would produce quantifiable labeling at the electron microscope level. Eleven cores satisfied these criteria (Fig. 3A–D, solid circles).

As shown in Fig. 3, each of the selected cores was placed into one of two groups according to its distance from the edge of the incorporation site. One group was ‘near’ the incorporation site (Fig. 3A,C); the other group was ‘far’ from the incorporation site (and close to IO; Fig. 3B,D). The edge of the incorporation site was defined in the 20 μm thick sections under 500 x darkfield light microscopic examination as the region beyond which perikarya were no longer more than about 1.5 times as densely labeled as the adjacent neuropil. Cores in the ‘near’ group were 0.5–0.8 mm from the edge of the incorporation site. Cores in the ‘far’ group were located 2–4 mm from the edge of the incorporation site.

Using the light microscopic autoradiographic labeling patterns of the 2 μm thick core sections as a guide, the core faces were trimmed into approximately 0.5 x 0.5 mm pyramids for thin sectioning (Fig. 2C,E). Serial thin sections, silver to gold interference color, were collected onto formvar-coated, single-hole grids (Fig. 2E) and coated with Ilford L-4 emulsion using the expanded loop method. The electron microscopic autoradiographic procedures were the same as previously described. Exposure times were 19–36 weeks.

Sections with low background noise were scanned. When a region containing fibers covered with silver grains was located (there were large areas with no grains), a sample field of 12 adjacent electron micrographs was made at a magnification of 3000 in a 2 x 6 or 3 X 4 pattern, whichever best fit the dimensions of the fiber bundle (Fig. 2F). (Bias in selection of tissue to be analyzed was minimized once the first of the 12 micrographs in a field was taken because the remaining micrographs were sequentially adjacent to this first micrograph rather than selected under the influence of preconceived or favored labeling patterns.) A total of 48 sample fields were so obtained. The reliability of the labeling patterns in most of these sample fields was qualitatively assured by determining that the labeling pattern over the same fiber field in serial sections was comparable to that observed over the photographed sample field (e.g. Fig. 6).

Some sample fields from each of the 4 sample groups (i.e. Pro-near; Pro-far; Leu-near; Leu-far) were chosen for quantitative analyses. To assure sample independence, those chosen for each group were required to be located in different areas of the same core and to originate from more than one core. In addition, approximately equal numbers of samples were chosen from two animals. This selection provided 8 sample fields for the Leu-near, 10 for the Leu-far, 7 for the Pro-near and 12 for the Pro-far groups.

These fields were quantitatively analyzed as follows: first, tissue elements were divided into the following 7 compartmental sources of radioactivity: (1) macroglial soma and astrocytic processes; (2) oligodendrocytic soma/myelin junctional area; (3) myelin; (4) myelin/axoplasmic junctional area; (5) axoplasm; (6) synaptic terminals; (7) neuronal soma and dendrites. Second, counts of silver grains (‘grain counts’) over and area estimates (‘point counts’) of each of these compartments were calculated for each sample field.

For the silver grain counts, a half-radius (HR) circle calculated to give a 50% or greater probability of containing the radioactive source was centered
Fig. 3. Locations of samples used for data collection are shown by the filled circles in A–D. (Open circles show the location of samples not used for the present experiments.) A shows the location of 'near' and B shows the location of 'far' samples after $[^3]$HLeu injections in two cats (shaded area in A shows one of the injections). C and D show 'near' and 'far' samples, respectively, after $[^3]$HPro injections. E-H are autoradiographs of parts of sections A-D above. E and F show 'near' and 'far' samples, respectively, after a $[^3]$HLeu injection; G and H, after a $[^3]$HPro injection. Bar (H) = 0.5 μm.
over each silver grain (Fig. 2H). For the samples in this study (100 nm thick sections, tritium, Ilford L-4 monolayer emulsion coating, D-19 developer and a final print magnification of 17,900), the appropriate HR circle diameter for counting grains on the prints was 9.1 mm. The area occupied by each of the 7 tissue sources was calculated by point count planimetry using the 9.1 mm HR circle instead of intersecting points\(^4\) (Fig. 2G). (Each ‘point’ (i.e. circle) in the figure represents 1.4 \(\mu m^2\) of tissue.) Because the counts for some of the 7 sources were low, it was necessary to collapse them into 3 compartments. These 3 compartments were as follows: ‘Glia’ (sources 1, 2 and 3 above), 'Myelin/Axoplasm' (source 4 above) and ‘Axoplasm’ (sources 5, 6 and 7 above). The only compartment in which this procedure obscured some of the findings was in the glial compartment from the \(^{3}H\)Pro samples near the injection site. See legends for Figs. 4 and 5 and Discussion.

The numbers from these 3 compartments were then used for the following analyses:

Absolute grain densities (no. of grains/100 \(\mu m^2\)) were calculated to provide a rough estimate of the specific activity of the compartments in each group of samples. Differences between the samples were evaluated using \(t\)-tests of significance (\(P = 0.05\)). Such calculations and evaluations are presented in Fig. 4.

In order to provide an overall image to compare the relative density of labeling over each of the 3 compartments in each of the 4 sample groups, ‘relative proportional grain densities’ were calculated. A ‘relative proportional grain density’ consisted of a ratio of the proportion of grains (\(P_g\)) to the proportion of ‘points’ (\(P_p\)) for each compartment within each sample group. \(P_g = \) no. of grains in a compartment/total no. of grains in all compartments. \(P_p = \) no. of points in that compartment/total no. of points in all compartments. These relative densities are illustrated in Fig. 5.

A 3-part analysis was then performed to determine whether the distribution of labeling over the 3 compartments differed among the sample groups. First, \(\chi^2\)-tests of significance were performed on the point counts to determine whether the relative amount of area occupied by the 3 compartments was comparable among the 4 sample groups. Since no statistically significant differences were found, the relative areal distributions were considered to be comparable. Second, \(\chi^2\)-tests were performed on grain counts to determine whether samples within the same group drawn from different animals differed from each other. Since no significant differences between animals were found, the samples from different animals were pooled. Finally, \(\chi^2\)-tests were performed on grain counts to determine which sample groups differed in the distribution of radioactivity in the 3 compartments. (See first column of Fig. 5.)

Once it was determined that the overall relative distribution of radioactivity between a pair of sample populations differed significantly, an analysis was done to determine which of the 3 compartments provided this difference (Fig. 5, columns 2, 3 and 4). This determination was made by first calculating ‘normalized grain densities’ for each compartment within each sample population and then performing \(t\)-tests. ‘Normalized grain density’ consisted of the ratio of the density of labeling in one compartment (no. of \(G_c/\)no. of \(P_c\)) of a sample group to the total density of labeling over all compartments (no. of \(G/\)no. of \(P\)) in that sample group. No. of \(G_c = \) the number of grains in a compartment of a given sample population; no. of \(P_c = \) the number of points in that compartment; no. of \(G = \) the total number of grains in that sample population; no. of \(P = \) the total number of points in that sample population.

RESULTS

Absolute grain densities are summarized in Fig. 4. These densities provide a rough estimate of the specific activity of each of the compartments in each of the 4 sample populations. Proportional labeling patterns are summarized in Fig. 5. These patterns provide an estimate of the relative distribution of grains across the different compartments in the tissue from each of the 4 sample populations. Examples of autoradiograms are provided in Fig. 6 (serial sections from a Pro-near sample), Fig. 7 (comparison of Leu-near with Pro-near samples) and Fig. 8 (comparison of Leu-far with Pro-far samples).

As can be seen in all of these figures, neuronal and glial compartments were labeled in all of the samples. There were differences between the samples, however, in the absolute and relative labeling of these compartments that varied as a function of both distance and amino acid.
Distance: leucine. The absolute grain densities and proportional labeling distribution for the \[^3\text{H}\]Leu samples changed little over distance, the patterns being statistically identical in samples taken near and far from the injection site. In both sets of samples, most labeling occurred over axoplasmic compartments (64-70\%), some over the junctional myelin/axoplasm compartment (18-24\%) and the rest over glial elements (Fig. 5). Similarly, as shown in Fig. 4 (rows 1, 2 and 5), neither the overall absolute grain density nor the grain densities of the glial, myelin/axoplasmic or axoplasmic compartments changed significantly over distance.

Distance: proline. Unlike the \[^3\text{H}\]Leu samples, both the absolute grain densities and the proportional labeling distribution for the \[^3\text{H}\]Pro samples changed significantly over distance. Proportionally, whereas 76\% of the labeling near the \[^3\text{H}\]Pro injection site was over glial elements (predominantly glial cell soma, see legend for Fig. 5), this percentage fell significantly to 28\% in samples taken far from the injection site, a significantly larger proportion there (57\%) having shifted to the axoplasm (Fig. 5, rows 3, 4 and 6).

These proportional changes were accompanied by significant decreases in the absolute grain densities of glial and to a lesser (but still significant) extent of myelin/axoplasmic compartments (Fig. 4, rows 3, 4 and 6). The specific activity of the axoplasmic compartment remained constant (insignificantly lower) across distance. Thus, the proportional increase in labeling of the axoplasmic compartment that is evident when comparing rows 3 and 4 in Fig. 5 is due not to an increase in the specific activity (absolute labeling) of the axoplasm, but rather to an absolute loss of radioactivity (i.e. decrease in the specific activity) predominantly from the glial compartment.

Proline versus leucine. There were significant differences in the absolute grain densities and proportional labeling distributions between the \[^3\text{H}\]Pro and \[^3\text{H}\]Leu samples both near and far from the injection sites (Figs. 4 and 5, rows 1-4 and 7 and 8).

Near the injection site, the \[^3\text{H}\]Pro samples were about 2.5 times (significantly) more densely labeled than the \[^3\text{H}\]Leu samples. This difference was due to the significantly higher specific activity of the glial compartment in the \[^3\text{H}\]Pro samples (Fig. 4). These absolute differences produced a proportional labeling pattern in which glial and myelin/axoplasmic components were relatively more densely labeled, and axoplasmic compartments relatively less densely labeled in the \[^3\text{H}\]Pro than in the \[^3\text{H}\]Leu samples (despite the fact that the axoplasmic compartments contained comparable absolute amounts of \[^3\text{H}\]Pro- and \[^3\text{H}\]Leu-labeled proteins). (See Fig. 5, compare A and C; Table 1, row 3; see also Figs. 6-8.)

Far from the injection site, the \[^3\text{H}\]Pro samples were about 3 times (significantly) less densely labeled than the \[^3\text{H}\]Leu samples. This difference was due to...
the significantly higher specific activities of the axoplasmic and myelin/axoplasmic compartments in the $[^3]$HLeu samples (the specific activity of the glial compartments being comparable). These absolute differences produced a proportional labeling pattern in which glial compartments contained relatively more $[^3]$HPro- than $[^3]$HLeu-labeled proteins (despite the fact they were of comparable specific activity) and axoplasmic and myelin/axoplasmic compartments were relatively comparably labeled (despite the fact the absolute density of $[^3]$HLeu-labeled proteins was greater than that of $[^3]$HPro-labeled proteins in these compartments).

The change in the differences between the $[^3]$HLeu and $[^3]$HPro samples near and far from the injection site thus appeared to be due to two changes in the pattern of radioactivity as a function of distance from the injection site. First, there was a major absolute...
Fig. 6. Serial electron microscopic autoradiographs of $[^3]$HPro-labeled fibers near the injection site. Sections A-D show consistent labeling of axoplasm and an oligodendrocytic soma. Bar (D) = 5 μm.

loss of $[^3]$HPro-labeled proteins from the glial compartments over distance, so that, far from the injection site, they were labeled at absolute level comparable to that of the $[^3]$HLeu-labeled proteins both near and far from the injection site. Second, there were small increases in the absolute density of $[^3]$HLeu-labeled proteins and small decreases in the absolute density of $[^3]$HPro-labeled proteins in the axoplasm as a function of distance from the injection site. These changes over distance resulted in the axoplasmic compartments far from the injection sites containing significantly more $[^3]$HLeu-labeled proteins than $[^3]$HPro-labeled proteins.

DISCUSSION

Potential sources of axonal labeling

$[^3]$HLeu. After an injection of $[^3]$HLeu into DCN, its neuronal perikarya are densely labeled by the in-
Fig. 7. Electron microscopic autoradiographs from samples near the injection site after a $[^3H]_{Leu}$ injection (A) and a $[^3H]_{Pro}$ injection (B). Note differences in grain distribution (primarily axoplasm in A; primarily glia in B) and overall grain density (greater in B). Compare with Fig. 8. Bar (B) = 3 $\mu$m.
Fig. 8. Electron microscopic autoradiographs from samples far from the injection site after $[^{3}H]_{\text{Leu}}$ injections (A,B) and $[^{3}H]_{\text{Pro}}$ injections (C-E). Note similarity in grain distribution (primarily axoplasm), but difference in overall grain density (denser in $[^{3}H]_{\text{Leu}}$ samples) for the two sets of autoradiographs. Compare with Fig. 7. Bar (E) = 2 $\mu$m.

corporation of $[^{3}H]_{\text{Leu}}$ into their proteins. In the present experiment, most of the $[^{3}H]_{\text{Leu}}$-labeled proteins were located in the axoplasmic compartment of samples from the internal arcuate tract containing axons of the labeled DCN neurons (Figs. 4 and 5). These results are predictable from the commonly known mechanism of molecular translocation in which such proteins originating in neuronal perikarya are transported from the soma down the axon towards its terminals (e.g. 'axon transport').

$[^{3}H]_{\text{Pro}}$. In contrast, labeling patterns after $[^{3}H]_{\text{Pro}}$ injections are consistent with a different mechanism of molecular translocation. Although, in the present study, the axoplasmic compartment of internal arcuate fibers near the injection site contained statistically comparable absolute amounts of
\[^{3}\text{H}]\text{Leu}\text{- and}^{[3}\text{H}]\text{Pro}-\text{labeled molecules}\text{ (Fig. 4, last column, rows 1, 3 and 7), previous evidence has demonstrated that only glial cells in the DCN injection site densely incorporate}^{[3}\text{H}]\text{Pro}\text{. Thus, the route by which}^{[3}\text{H}]\text{Pro}-\text{labeled proteins arrived in axoplasm of the near samples must have involved a mechanism other than the initial uptake and incorporation of}^{[3}\text{H}]\text{Pro}\text{ into proteins by DCN neurons.}

It is unclear how \[^{3}\text{H}]\text{Pro}-\text{labeled proteins could have been transferred from glial cells into the axoplasm of the near samples. One possible route is through myelinated processes into adjacent axoplasm. The paucity of labeling over the myelin compartment in the \[^{3}\text{H}]\text{Pro}\text{ samples near the injection site (see legend for Fig. 5), however, suggests that this route was unlikely (unless the myelin failed to retain labeled molecules or they entered the axon through nodes). Instead, other astrocyte or oligodendrocyte processes, such as those adjacent to neuronal perikarya at the injection site seem more likely. Thus, it appears that}^{[3}\text{H}]\text{Pro}-\text{labeled proteins were transferred from glial cells into neuronal perikarya before being transported down the axon.}

The data from samples taken far from the injection site expand this conclusion. In far samples, the axoplasmic compartment contained nearly 4 times more \[^{3}\text{H}]\text{Leu}\text{- than}^{[3}\text{H}]\text{Pro}-\text{labeled proteins, indicating that, in addition to differences in the translocation mechanisms, the overall kinetics of the uptake, incorporation, transfer and transport for}^{[3}\text{H}]\text{Pro}\text{ differ from those for}^{[3}\text{H}]\text{Leu.}

Unlike the well-recognized transport mechanism used by most of the \[^{3}\text{H}]\text{Leu}-\text{labeled molecules, only limited evidence supports the existence of the alternative translocation mechanism suggested above by data from the}^{[3}\text{H}]\text{Pro}\text{ samples. Although the transfer of molecules from Schwann cells into adjacent neuronal axoplasm has been documented quite clearly in invertebrates}\text{, only minimal evidence exists demonstrating a transfer from glial cells into neuronal soma in the vertebrate central nervous system. Nicholson et al. reported}\text{ that horseradish peroxidase intracellularly injected into turtle cerebellar radial glial (RG) cells was ‘transferred to Purkinje, and other, cells close to the RG processes’. It is possible, therefore, that the molecules being transferred from glial cells to neurons in DCN are labeled proteins. The results do not rule out the alternative possibility, however, that, instead of proteins, the molecules being transferred and incorporated into neuronal perikaryal proteins are breakdown products of labeled glial proteins.}

Another interpretation of the data obtained after \[^{3}\text{H}]\text{Pro}\text{ injections involves consideration of two facts. First, the methods used in this experiment preclude visualization of free amino acids (because virtually all free amino acids were washed out during the predominantly paraformaldehyde fixation)\text{. Second, evidence exists for the synthesis and post-translational modification of proteins within vertebrate axoplasm}\text{. Thus, instead of}^{[3}\text{H}]\text{Pro}-\text{labeled proteins being transported down the axon, the neurons in DCN might take up injected free}^{[3}\text{H}]\text{Pro}\text{ and transport it down the axon where only then it is incorporated into newly synthesized or posttranslationally modified proteins.}

Although plausible, existing evidence fails to support this interpretation. First, even though the results of a number of studies suggest that some free amino acids (particularly putative amino acid neurotransmitters, but also Leu and Pro) are translocated down the axon either by anterograde transport with the fast transport system\text{ or diffusion}\text{, there is limited evidence to the contrary regarding Leu or Pro}\text{. In addition, two studies in this laboratory fail to support such translocation. In one study, protein synthesis inhibitors (e.g. puromycin) used in conjunction with}^{[3}\text{H}]\text{Pro}\text{ injections in DCN prevented the appearance of labeling over terminals in the IO}\text{. In the other study, when glutaraldehyde was used as the sole fixative to bind free amino acids, it failed to increase neuronal labeling even at postinjection survival times as short as 20 min (and unpublished observations).}

Second, although ‘highly suggestive, but not conclusive’ evidence for de novo synthesis of protein in axoplasm exists\text{, such synthesis could involve}^{[3}\text{H}]\text{Pro}\text{ that had been freed by proteolysis from transported proteins}\text{ rather than unincorporated free}^{[3}\text{H}]\text{Pro}.\text{ In support is the fact that DCN neurons injured by the injection needle become intensely labeled by}^{[3}\text{H}]\text{Pro}\text{ (see Fig. 10 on p. 2325 of ref. 57). Such labeling suggests that DCN neurons are able to incorporate free}^{[3}\text{H}]\text{Pro}\text{ (if damage makes the}^{[3}\text{H}]\text{Pro}\text{ accessible to the perikarya synthetic organelles). It seems illogical that a putative axonal syn-}
thetic mechanism would be capable of incorporating \[^3H\]Pro while its perikaryal mechanism would not. In addition, ribosomes have been observed only rarely in axoplasm. Thus, the machinery for protein synthesis in axoplasm does not appear to exist.

Third, transfer RNAs (tRNAs) capable of posttranslational addition of free amino acids to existing proteins are known to exist within peripheral axons (rat sciatic nerve) and central nervous system whole nerves (inclusive of both axons and glial cells; rat optic nerve). To explain the present data, however, the tRNA mechanism would also have to demonstrate posttranslational addition of \[^3H\]Pro to protein within central nervous system axons, and account for both the absence of tRNA activity in the perikarya of the axons studied in the present experiments (i.e. no perikaryal labeling in DCN) and the inability of this process (axonal incorporation by tRNAs) to allow adequate labeling in many of DCNs (and other nuclei's) terminal targets.

Thus, although limited, the evidence so far favors the interpretation of glial–neuronal transfer of \[^3H\]Pro-labeled molecules at the injection site rather than neuronal uptake and transport of free \[^3H\]Pro.

**Potential sources of glial labeling**

Another result of this study was that proteins in glial compartments surrounding axons near the injection site were strikingly more densely labeled by \[^3H\]Pro than \[^3H\]Leu (Fig. 4, column 2, rows 1, 3 and 7). Far from the injection site, in contrast, glial compartments contained comparable amounts of \[^3H\]Leu- and \[^3H\]Pro-labeled proteins (Fig. 4, column 2, rows 3, 4 and 8).

Labeled proteins in glial compartments of any samples could have been derived from one or more sources. These sources include: (a) the extracellular space (via the blood or diffusion from the injection site), (b) via transfer from glial cells at the injection site, and/or (c) via transfer from axoplasm of internal arcuate fibers.

**Transfer through extracellular space from blood.** A portion of the injected labeled amino acid may be immediately lost to the blood, circulated and returned to the extracellular space. Some labeling in all compartments, especially in the \[^3H\]Leu samples, could have involved this route. If so, such labeling would have occurred throughout the tissue. There was, however, no labeling over large portions of the tissue (see Materials and Methods).

**Transfer from extracellular space via diffusion from injectionsite:** Since ‘near’ samples were taken from regions quite close to the injection site as defined by arbitrary criteria (see Materials and Methods), one possibility is that the ‘near’ sample region, but not the ‘far’, was actually part of the effective injection site. In other words, glial incorporation in the ‘near’ samples could have involved the same mechanisms as those presumably occurring within the injection site. This possibility is considered in further detail below.

**Transfer from glial cells at injection site.** Electron microscopic studies have shown that junctions exist between glial cells that would permit exchange of small molecules but including proteins or their breakdown products. Although there is debate regarding neuron-to-glia transfer of amino acids, evidence exists for transfer of large enzymes such as horseradish peroxidase. Thus, another possibility (considered further below) is that some axonally transported proteins (derived from glial cells at the injection site for \[^3H\]Pro-labeled proteins, but from either glial cells or neuronal soma for \[^3H\]Leu-labeled proteins) are transferred from internal arcuate axons into adjacent glia.

Although it is difficult from currently available evidence to form definitive conclusions regarding which of the sources discussed above are the most likely for the labeled proteins in glial compartments of the different samples, tentative suggestions are possible.

\[^3H\]Pro samples near injection site. Biochemical evidence indicates that when equal amounts of Leu or Pro are injected into goldfish brain, more Pro than Leu is available for incorporation because Leu is more likely than Pro to be carried off by the blood.
Therefore, one possibility is that, shortly after comparable injections of \(^{3}\)HLeu or \(^{3}\)HPro, higher \(^{3}\)HPro than \(^{3}\)HLeu concentrations existed within the extracellular space at the DCN injection site. If greater diffusion distances are a result of higher concentration, the \(^{3}\)HPro injection site would then be larger than that of \(^{3}\)HLeu. In addition, other biochemical evidence indicates that extracellularly available Pro is more efficiently incorporated into protein than is Leu (by a factor of 5 when the amino acids are injected into the goldfish brain\(^{59}\), and by a factor of 3 when they are injected into the cat dorsal column nuclei\(^{13,22}\)). It thus seems likely that \(^{3}\)HPro-labeled proteins in the glial compartments of samples near the injection site were derived in large part from the efficient uptake and incorporation of \(^{3}\)HPro from the extracellular space.

\(^{3}\)HLeu samples near injection site. If labeling in glial compartments involved diffusion through extracellular space or through glial processes, one would expect a decrease in labeling density with distance from the injection site. Such a decrease did not occur for \(^{3}\)HLeu-labeled proteins, as both the relative and absolute labeling patterns remained unchanged over distance (Figs. 4 and 5). Thus, by elimination, the source of \(^{3}\)HLeu-labeled proteins in glial compartments is more likely to have been derived from nearby axoplasm than from adjacent glial cells or diffusion of \(^{3}\)HLeu through extracellular space from the injection site. This conclusion is further supported by the presence of considerable labeling in the myelin/axoplasmic compartment of both near and far \(^{3}\)HLeu samples (Fig. 4, column 3, rows 1, 2).

\(^{3}\)HPro samples far from injection site. Since there was a substantial decrease in density of \(^{3}\)HPro-labeled proteins in glial compartments as a function of distance, both diffusion of \(^{3}\)HPro through extracellular space from the injection site and glia-to-glia transfer of \(^{3}\)HPro-labeled proteins remain as possible sources of glial \(^{3}\)HPro labeling in far samples.

If such mechanisms were involved, single injections of \(^{3}\)HPro into one part of the internal arcuate pathway would produce labeling of glial compartments for some distance along its length. Other experiments in the authors' laboratory have shown that such injections produce only local labeling of glial cells\(^{8,15}\). Thus, \(^{3}\)HPro-labeled proteins in glial compartments of far samples, like \(^{3}\)HLeu-labeled pro-

teins in glial compartments of both near and far samples, are more likely to be derived from nearby axoplasm than from either other glial cells or extracellular space. As discussed above for the transfer of \(^{3}\)Hlabeled molecules from glial cells into neuronal perikarya in DCN, the molecules transferred from axoplasm into adjacent glial processes in near and far samples could be \(^{3}\)Hlabeled proteins or \(^{3}\)Hlabeled free amino acids produced by breakdown of proteins in the axoplasm.

In sum, the present results, when considered with the results of other studies, support the following tentative hypotheses (Fig. 9).

\(^{3}\)Hlabeled molecules. Some exogenous leucine is rapidly cleared\(^{59}\) and the rest is incorporated predominantly by neurons, but also by some macroglial cells\(^{57}\). Labeled neuronal proteins are then transported down the axon, during which time some of the proteins are transferred into adjacent glial processes. The fate of the few \(^{3}\)HLeu-labeled proteins located in glial cells at the injection site is unclear, but may follow the same route as \(^{3}\)HPro-labeled proteins summarized below. This scenario is depicted in Fig. 9A.

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**Fig. 9.** Diagram of postulated routes by which \(^{3}\)HLeu- and \(^{3}\)HPro-labeled molecules injected into DCN are translocated away from DCN. See text for further details.
$[^3]H$Pro-labeled molecules. In contrast, when proline is made available extracellularly, it is cleared more slowly than leucine$^{59}$, most of the proline being avidly taken up and incorporated by macroglial cells$^{57}$. Labeled glial proteins are then continuously transferred into perikarya of adjacent neurons for transport down the axon, a small portion being transferred into adjacent glial processes along the way. This scenario is depicted in Fig. 9B.

These experimental results indicate that molecules transferred from glial cells to neuronal perikarya at the injection site are transported down the axon (most likely as proteins). Careful kinetic studies will be necessary, however, to determine how or with what transport group(s)$^{3,26}$ such proteins are transported. Previous experiments in this laboratory and those of others have demonstrated that transport of $[^3]H$Pro-labeled proteins is less sensitive to colchicine than transport of $[^3]H$Leu-labeled proteins$^{7,11,61}$. Although complex to interpret, such results support a general conclusion that the mechanisms of axonal transport of glially transferred molecules differ from those of the other molecules, possibly in the manner in which microtubules are involved. Further work is needed, however, to generate more detailed and testable hypotheses regarding the nature of these differences.

Technical considerations. One possibility that should be considered is whether the different labeling patterns reflect errors or some peculiarity in sampling procedures rather than real differences in the incorporation and transport patterns of $[^3]H$Leu- and $[^3]H$Pro-labeled molecules. Although this possibility cannot be discounted completely, a number of considerations suggest it is unlikely. First, a substantial effort was devoted to controlling and assuring the validity of the sampling procedures (see Materials and Methods). Second, supporting data exist from other experiments in which different techniques were used. For example, the difference in density between $[^3]H$Leu and $[^3]H$Pro samples near the injection site is supported by biochemical data demonstrating that $[^3]H$Pro is more efficiently incorporated into proteins than $[^3]H$Leu$^{13,22}$. Similarly, the density difference between $[^3]H$Leu and $[^3]H$Pro samples far from the injection site is supported by light microscopic anatomical data demonstrating that terminal targets of neurons within $[^3]H$Pro injection sites are less densely labeled than are targets of neurons within $[^3]H$Leu injection sites$^{59}$. And, finally, the denser labeling in $[^3]H$Leu samples taken far than taken near the injection site is supported by studies on axoplasmic transport rate components$^{3,26,27}$. The reasoning is as follows: far samples were located about 3 mm away from the center of the DCN injection site. Since the $[^3]H$Leu injection had been made 24 h previously, it is most likely that far samples contained an accumulation of a substantial amount of the pulse of $[^3]H$Leu-labeled proteins conveyed by fast transport rates (30-240+ mm/day) as well as some of those conveyed by slow component B (3-6 mm/day). In contrast, it is most likely that near samples (located about 0.65 mm from the injection) contained only some of the most slowly transported proteins (0.7-1.1 mm/day), the wave of material that had been transported at faster rates having already gone by.

Another interpretative concern involves the source of axonal labeling in $[^3]H$Pro samples. Much of the discussion above regarding this issue is based upon previous evidence from this laboratory demonstrating that neurons throughout most of the DCN injection site fail to incorporate significant amounts of $[^3]H$Pro$^{57}$. It was the case in those experiments, however, that a small proportion of DCN neurons did in fact incorporate modest amounts of $[^3]H$Pro. Thus, it is possible that $[^3]H$Pro-labeled molecules in the axoplasm of the fiber samples from the present study were derived directly from these modestly labeled DCN neurons rather than from glial cells as depicted in Fig. 9A.

Although this possibility cannot be discounted completely, 3 considerations suggest it is unlikely. First, the few DCN neurons that incorporated $[^3]H$Pro were confined to parts of DCN that do not contain neurons projecting to the IO$^{56}$. Second, in other light microscopic studies$^{10}$, DCN injections confined to regions where no neurons had been labeled in the electron microscope experiments still produced terminal labeling in the IO. Third, if $[^3]H$Pro- and $[^3]H$Leu-labeled molecules had been transported by the same axonal transport mechanisms, differences in labeling patterns between $[^3]H$Pro samples taken near and far from the injection site should have been, but were not, similar to those observed between near and far $[^3]H$Leu samples.
Conclusions

When taken together with results of other studies, the present data support the possible existence of a heretofore unrecognized system of communication between glial cells and neurons. In this system, molecules are transferred from glial cells into adjacent neuronal perikarya and transported down the length of the axon where, all along the way, some of them are transferred from the axon into adjacent glial processes. Such a system appears to exist throughout the central nervous system of vertebrates.\textsuperscript{15}

Even though the proteins involved in this system seem to incorporate $[^{3}\text{H}]\text{Leu}$ as well as $[^{3}\text{H}]\text{Pro}$ (see below and Fig. 9), one or more of the special qualities of Pro make the system more readily apparent when $[^{3}\text{H}]\text{Pro}$ is used. Pro is a non-essential, unusual amino acid (the only one with a rigid ring), present in most proteins and traditionally associated with collagen, whose presence in a polypeptide chain causes a kink or bend.\textsuperscript{50} When injected into brain tissue, it, apparently unlike Leu, is retrogradely\textsuperscript{35,51} and transneuronally\textsuperscript{5,28} as well as anterogradely transported. Recently, Pro has been suggested as having neurotransmitter functions\textsuperscript{24-78}.

Potential functions for a putative glial-neuronal-glial communication system are limited mainly by one’s imagination. One possible interpretation that might help integrate some of these diverse items of information is as follows: glial cells avidly take up exogenous Pro molecules for the immediate purpose of inactivating Pro’s putative transmitter action. Once inside the glial cell, however, Pro molecules are then put to multiple use, one of which involves incorporation into proteins destined for transfer into adjacent neurons (either their perikarya or terminals). The transferred molecules would be transported either retrogradely or anterogradely. Once transported, they would be transferred back out again for selective incorporation into adjacent glial cells and subsequent transfer into neurons (i.e. transneuronal transport; see discussion in ref. 28).

Given this interpretation, retrograde and transneuronal transport of $[^{3}\text{H}]\text{Leu}$-labeled molecules would be possible, but would not occur because the injected $[^{3}\text{H}]\text{Leu}$ had not originally been avidly taken up by glial cells. Thus, the fact that a putative glial-neuronal-glial communication system is more readily apparent when visualizing it with $[^{3}\text{H}]\text{Pro}$ than with $[^{3}\text{H}]\text{Leu}$ may simply be a consequence of Pro’s putative neurotransmitter function and glial affinity. It is likely that some of the proteins in this system contain Leu, since recent biochemical results\textsuperscript{13,22} demonstrate that $[^{3}\text{H}]\text{Leu}$ and $[^{3}\text{H}]\text{Pro}$ are incorporated into a wide range of proteins in DCN.

Thus, there are few substantive clues as to the possible function(s) of a putative glial-neuronal-glial communication system. Many compatible functions can be envisioned. One class of possible functions is trophic (i.e. nutritive), involving transfer of proteins from a glial pantry into neuronal soma. Another class is protective, since tentative evidence exists for a glia-to-neuron transfer and transport of heat shock proteins\textsuperscript{4,29,74}.

Yet a third possible function is informative, in which glial cells associated with different parts of a neuron could use the neuron as a conduit to communicate with each other for various purposes. One such purpose, for example, would be to provide a way for glial cells associated with neuronal soma to influence various aspects of the myelination or synapse formation of the axons of those neurons. Such influence would be important not only during development\textsuperscript{19,23,34,71}, but also during adulthood when it could provide a means for appropriate modification of the conductive or synaptic efficiency of a system\textsuperscript{62}.

In sum, it is clear that more work is needed, not only to support, refute or add to the potential functions discussed above, but also to support the existence of this putative glial-neuronal-glial system and characterize further the mechanisms by which it operates.

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REFERENCES


15 Contos, N., Molinari, H.H. and Berkley, K.J., Variation in the cellular incorporation of [3H]-leucine and [3H]-proline in the nervous system as a function of neuron-glial cell relationships at the incorporation site, in preparation.


35 Heacock, A.M. and Agranoff, B.W., Reutilization of precursor following axonal transport of [3H]proline-labeled...


