tion with D_2O was replaced by that with H_2O.

In the presence of D_2O, calcium is not released and there is consequently no contractile response. These results are similar to those of Ashley and Ridgway (*9*) when they used hypertonic sucrose.

Results were reproducible with some minor variations. In some instances, both the flash and tension were more intense for a brief period during the recovery period than in the control whereas in others recovery was not complete. Such an increase in tension during recovery had been found in frog atrial muscle (*4*). In a few experiments the D_2O did not completely eliminate either the tension or the light flash.

The possibility that D_2O inhibited the luminescent reaction of aequorin and calcium was considered. By visual means, the aequorin flash in preparations exposed to $CaCl_2$ in the presence of more than 80 percent D_2O was compared to that in H_2O; no difference was observed. The kinetics of the reaction in D_2O should be studied, as should the site and mechanism of D_2O action.

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15. Supported by NIH grant NS 10075 and general research support from Boston University Medical School. We thank J. R. Blinks and O. Shimomura for samples of aequorin, L. B. Cohen, H. Grundfest, J. W. Hastings, and A. Szent-Györgyi for loan of apparatus, and H. M. Fishman, G. Katz, and S. Steinberg for assistance.
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17 January 1972

Radioautography of the Optic Tectum of the Goldfish after Intraocular Injection of [^{3}H]Proline

Abstract. *Radioautography of the optic tectum of the goldfish, performed after injection of [^{3}H]proline into the contralateral eye, effectively resolves several distinct layers of retinal synapses. Silver grains are found unilaterally over nerve tracts containing efferent fibers from the tectum, a result that suggests intercellular migration of labeled molecules. The low background and high specific grain density obtained with [^{3}H]proline radioautography indicate the usefulness of this technique for the elucidation of neuroanatomical connections in the visual system.*

Newly synthesized proteins that are labeled with radioactive amino acid precursors in neuronal cell bodies are transported to nerve terminals by the process of axonal flow (*1*). The goldfish visual system has proved useful for studies of this process; this is true because the optic tracts are completely crossed, and the labeled proteins that are formed in retinal ganglion cells after intraocular injection of radioactive amino acids migrate to a distinct region of the contralateral optic tectum (COT) (*2, 3*). Radioactive protein appears in the COT, 4 to 6 hours after injection of [^{3}H]leucine into the eye; this time course represents rapid axonal transport of newly synthesized protein (*4*). In addition, labeled protein that appears in the ipsilateral optic tectum (IOT) and throughout the brain represents [^{3}H]leucine that has escaped into the circulation and has been taken up by the brain and incorporated into protein. The systemic background labeling thus limits the usefulness of radioautography as a method of identifying nerve endings to which the labeled proteins migrate or of detecting possible intercellular movement of labeled molecules.

We previously measured the relative efficacy of 18 amino acids (*3*) in selectively labeling proteins that are synthesized in the retinal ganglion cell of the goldfish and transported to the optic tectum. [^{3}H]Proline and [^{3}H]-asparagine, injected into the eye, were both more efficient than [^{3}H]leucine in labeling substances recovered in the COT fraction that was insoluble in trichloroacetic acid (TCA), and these amino acids were much less active than [^{3}H]leucine in labeling the brain systematically (that is, in labeling TCA-insoluble material in the IOT). While the ratio of the radioactivity in protein recovered from the COT to that in the IOT was less than 2 : 1 with [^{3}H]leucine, the ratio was from 17 : 1 to 70 : 1 with [^{3}H]proline, depending upon the specific activity of the proline. Studies in visual systems of the chick (*5*) and mouse (*6*) have confirmed the usefulness of labeled proline as a marker for the study of axonally transported proteins. Proline's effectiveness in part may be due to the fact that it is poorly incorporated into the brain from blood (*7*) and is metabolized into glutamic and aspartic acids, which do not become highly labeled because they are present in high concentrations relative to other amino acids (*8*). When COT protein that is synthesized after [^{3}H]proline injection is hydrolyzed in acid, more than 90 percent of the radioactivity can be accounted for as proline (*3*).

Three goldfish, weighing 8 to 10 g, were each injected in the right eye with 40 μc of L-[2,3-^{3}H]proline (45.7 c/mmole) in a volume of 5 μl. After 24 hours, brains were removed and fixed in Bouin's solution, dehydrated with butyl alcohol, and embedded in paraffin. Sections for radioautography were dipped in Kodak NTB3 emulsion (*9*), and were developed 6 to 13 days later and stained with hematoxylin-eosin. Other sections were treated with phosphotungstic acid–hematoxylin or Bodian's stain to facilitate classification of the tectal layers according to Leghissa (*10*). Cross sections through midtectal regions were selected for study, and mediolateral areas of similar thickness were used for counting grains. At least 7000 grains were counted per brain.

Regions typical of those counted are indicated in Fig. 1A. At low magnification (Fig. 1A), a dark band of silver grains restricted to the COT is readily identified, but few exposed grains are seen in the IOT, even at higher magnification (Fig. 1B). There are several distinct layers of grains in the COT (Fig. 1C), which receives fibers from the injected eye. The highest concentrations of grains are seen in a discrete area (S) medial to the marginal fiber (MF) layer, in the external plexiform (EP) layer containing retinal axons and nerve endings, in the internal plexiform (IP) layer, and in the periventricular (PV) layer. These areas appear to correspond to retinal synaptic regions

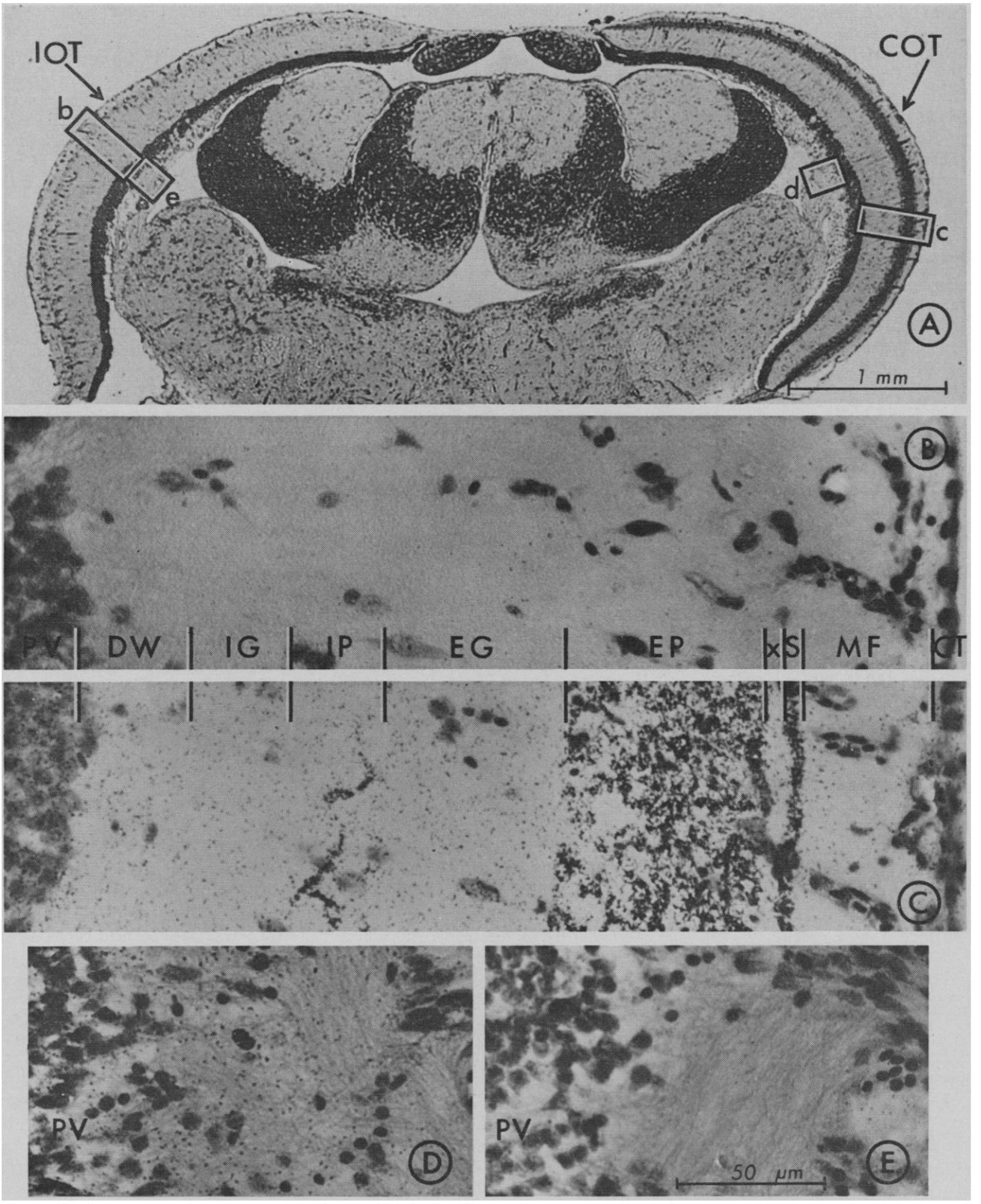

Fig. 1. Radioautogram of a cross section of a goldfish brain. The section was obtained 24 hours after intraocular injection of [^{3}H]proline and was exposed for 13 days, developed, and stained with hematoxylin-eosin. Areas b, c, d, and e in A are typical of those analyzed and presented in Fig. 2 and are seen at higher magnification in B, C, D, and E, respectively. Most of the exposed silver grains are found over the contralateral tectum (*COT*) which receives retinal ganglion cell axons from the injected eye. Tectal layers in B are *CT*, pial connective tissue; *MF*, marginal fiber; *S*, "outer" synaptic; *X*, undefined; *EP*, external plexiform; *EG*, external portion of central gray; *IP*, internal plexiform; *IG*, internal portion of central gray; *DW*, deep white; and *PV*, periventricular.

detected by axonal degeneration techniques (*11*). In addition, the efferent fiber tracts of the COT are labeled unilaterally (Fig. 1, D and E).

Grain counts over the tectal layers (Fig. 2) reflect the high concentration of radioactivity in layers S, EP, IP, and PU, which are known (*10, 11*) to receive optic-tract terminals. The pial connective tissue (CT) contains considerable radioactivity on both sides of the brain. This appears to be in protein formed from labeled proline that arrives systemically. Grains found in the central gray (IG and EG) layers and deep white (DW) layer may reflect the existence of nerve endings not previously resolved by degeneration studies.

Alternatively, these grains may represent labeled protein being transported along retinal axons that terminate in deeper layers. It is also possible that they are the result of intercellular movement. Support for the latter idea is found in the fiber tracts medial to the periventricular layer (Fig. 1, D and E), which contain nonretinal afferent fibers and efferent fibers from tectal cell bodies (*10*). The small but significant number of grains found over these nerve fibers (Fig. 2, layer EF) on the side contralateral to injection could indicate some transsynaptic movement of labeled molecules. Since the region of the efferent fibers is in close proximity to the radioactivity in the COT, it is possible that the labeling seen in the efferent fibers represents nonspecific diffusion through the brain substance. The sharp delineation of radioactive bands apparent in Figs. 1 and 2 militates against the presence of large diffusion gradients of the labeled materials through the brain.

If transsynaptic flow or nonspecific diffusion has occurred, the question remains whether the radioactivity in the efferent fiber tract represents passage of intact protein or of free proline. We previously observed that, several hours after intraocular injection, there was a small but significant difference between the amount of radioactive proline in TCA extracts of the COT and the smaller amount in TCA extracts of the IOT; the difference was perhaps due to breakdown of presynaptic labeled proteins. Free labeled proline that appeared in the COT, as a result of either axonal flow of free amino acid or degradation of axonally transported protein, could migrate transsynaptically. If this proline were incorporated into protein postsynaptically, it could account for grains in the deep tectal layers or in the efferent fiber tracts from the COT. However, when acetoxycycloheximide was injected intracranially to block tectal protein synthesis, efferent fibers from the COT remained labeled. This suggests that labeled macromolecules, rather than their soluble breakdown products, are transported, whether by transsynaptic migration or by some other transmembrane process.

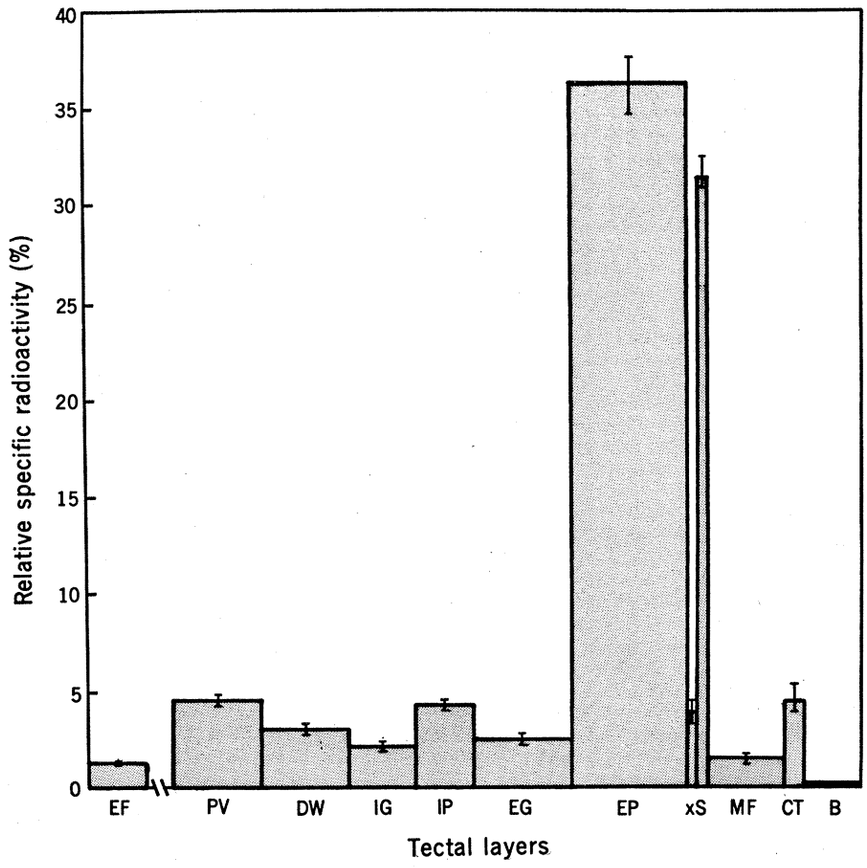

Fig. 2. A histogram of radioactivity in tectal layers. Cross sections from three brains were exposed to emulsion for 6 days, and grains were counted in the tectal regions indicated in Fig. 1. Column height is mean specific radioactivity, expressed as percentage of the sum of grain densities in layers counted in each brain (*16*); the bars indicate the range. The abscissa shows the approximate width of the tectal layer (from *PV* to *CT*). Grain density of the background (*B*) outside of the section was 0.2 percent, relative to all regions counted, while no IOT layer had a grain density exceeding 0.3 percent except the connective tissue layer, where the density was 2.4 percent (*EF*, efferent fibers; other abbreviations given in legend to Fig. 1).

The question of possible transsynaptic flow of protein has been raised (*12, 13*), and varying conclusions have been reached. The use of [^{3}H]leucine in some studies (*2, 13*) may have obscured a small amount of interneuronal migration of labeled protein. Evidence for transsynaptic flow of labeled molecules following injection of [^{3}H]proline has been reported in the mouse visual system (*6*).

Regardless of the mechanisms involved, [^{3}H]proline incorporation provides a biochemical technique that complements axonal degeneration (*11*) and electrophysiological mapping (*14*) for the elucidation of anatomical relations in the visual system. In the poikilotherm, [^{3}H]proline radioautography may be of additional interest. The demonstration of specific reconnection of the retinal ganglion cell axons in the optic tectum following section of the optic nerve (*15*) suggests a high degree of chemical coding of these cells, which could be mediated by specific trophic factors that are transported axonally.

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4. We estimate the rate of rapid axonal flow in the optic tract of the goldfish to be 70 to 100 mm/day at 20°C (*3*). Additional radioactive protein appears in the brain after 5 to 6 days and represents the arrival of "slow" axonal flow from the retina (*2*).
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16. In each section studied, the tectal layers were defined and grains were counted continuously from the connective tissue to the periventricular nuclei. Four sections were counted for each fish. The number of grains counted in each tectal region was divided by the cross-sectional area examined, density values obtained from all regions were summed for each brain, and the percentage of the total density that each layer represented was calculated. This method normalized the data for variations in the total radioactivity reaching the tectum in different fish.

17. We thank G. E. Callaway for excellent technical assistance. Supported by NIH training grant MH-07417 (to J.H.N.), NIH postdoctoral fellowship 1 FO2 NS50407-01 (to E.A.N.), NIH grant MH-12506; and NSF grant GB-5125X.

14 December 1971; revised 18 January 1972 ■

A Mutation Influencing the Transportation of Manganese, L-Dopa, and L-Tryptophan

Abstract. *Transportation of manganese,* L-*dopa, and* L-*tryptophan was slower through the tissues of intact pallid mice than through those of black C57Bl/6J mice, presumably because of the influence of the gene* pallid.

Since both circumstantial (*1*) and direct evidence (*2*) indicate links between the metabolism of manganese and that of biogenic amines, the search for a fundamental connection is relevant to studies of parkinsonism (*1*) and chronic manganese poisoning (*3*). Such a connection would be one reflected by a well-defined gene. We studied the effects of the mutant gene *pallid* on the transportation of manganese and of L-dopa (L-dihydroxyphenylalanine) into the brain for the following reasons: (i) a neurological syndrome of pallid mice (*4*) was prevented in offspring by feeding large amounts of manganese to mothers during pregnancy (*5*); (ii) pallid mice seemed insensitive to the cerebral effects of L-dopa, a precursor of catecholamines (*6*). (iii) In contrast, black C57Bl/6J mice, which do not have the gene *pallid*, had shown neither unexpected manganese requirements nor unusual responses to L-dopa.

Male pallid mice were obtained by first crossing pallid (*pa/pa*) females to C57Bl/6J (*Pa/Pa*) males and then backcrossing the F_1 males (*Pa/pa*) to pallid (*pa/pa*) females because inbreeding of pallid mice reduced their viability. Control black (*Pa/Pa*) males were obtained from the C57Bl/6J stock. All mice were reared on Purina Laboratory Chow unless specified otherwise.

Radioisotopes of manganese have been injected intraperitoneally into mice consuming Purina; they accumulate preferentially in the liver, disappear thereafter from that organ and from the intact body at fairly rapid, characteristic rates (*7–10*). On diets deficient in manganese, normal animals had shown a slow rate of loss of intraperitoneally injected radioactive manganese ($^{54}Mn^{2+}$) from the intact body and a retention of the tracer in the liver so that the remainder of the body received relatively less isotope (*7, 8*). If pallid mice distribute and eliminate ^{54}Mn at slower rates than do black mice, this should be demonstrable with a variety of manganese intakes. Therefore, pallid and black mice on either a low manganese diet (milk, 0.025 μg of Mn per milliliter) or a high manganese diet (Purina Laboratory Chow, 30 μg of Mn per gram) were compared after receiving carrier-free $^{54}Mn^{2+}$ intraperitoneally. The intact, living pallid mice showed a significantly slower rate of loss of ^{54}Mn from the whole body under both diets (Table 1). Upon removal of the abdominal viscera and the brain, the remaining carcasses of pallid mice had lower concentrations of ^{54}Mn tracer than did those of black mice, on both stock and milk diets. The reverse was true for the livers, as shown by the ratios of ^{54}Mn in the carcass to that in the liver (Table 1). Thus, the pallid mice exhibited the signs of slower manganese transportation found in manganese deficiency (*7, 8*).

An accepted criterion of manganese deficiency is a decreased concentration of natural metal (^{55}Mn) in tissues. Selected tissues from animals fed Purina were analyzed by a nondestructive modification of neutron activation analysis, after extensive calibration with the destructive method (*11*). Relative manganese deficiency was pro-

Table 1. Whole body loss and organ distribution of $^{54}Mn^{2+}$. The half-time of the whole-body loss of ^{54}Mn was calculated in each mouse for the first 7 days after isotope injection (*7*); the animals were killed between days 9 and 7. Parentheses indicate numbers of animals. Results are expressed as means ± standard error. Student's *t*-tests were applied only on corresponding pallid and black mice. Differences between black and pallid mice were minimized with high intakes of $^{55}Mn^{2+}$.

Diet	Half-time (days)		Ratio of carcass to liver concentration (count/min)	
	Black	Pallid	Black	Pallid
Milk (27 days)	6.1 ± 0.20 (12)	8.2 ± 0.33 (12)*	6.7 ± 0.98 (3)	3.6 ± 0.44 (4)*
Purina (9 days)	4.1 ± 0.16 (8)	7.1 ± 0.31 (9)*	6.5 ± 0.98 (5)	2.4 ± 0.14 (5)*
Milk + $^{55}Mn^{2+}$ (55 μg/ml, 27 days)	4.1 ± 0.10 (3)	4.8 ± 0.10 (3)†	18.6 ± 0.50 (3)	15.7 ± 1.01 (3)†
Purina + $^{55}Mn^{2+}$ (180 μg/g, 9 days)	3.9 ± 0.24 (4)	6.6 ± 0.31 (4)*	7.4 ± 0.94 (3)	5.1 ± 0.27 (4)†
Purina + $^{55}Mn^{2+}$ (0.2 mg, i.p., daily, 9 days)	1.8 ± 0.12 (5)	1.8 ± 0.13 (4)	20.0 ± 2.42 (5)	25.4 ± 6.45 (3)

* $P < .01$; † $P < .05$.