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Axonal Transport of Macromolecules
I. Protein Migration in the Central Nervous System

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Summary. The avian visual system has been used to study the transport of proteins and their precursors along the optic tract. Various labeled compounds were injected into a single eye of new hatched chicks. The radioactivity of components in the optic lobe that was contralateral to, and innervated by, the injected eye was compared to radioactivity in the ipsilateral lobe, not innervated by the treated eye. Proteins migrating from the ganglion cells of the retina to the optic tectum seemed to be relatively stable and may be rich in proline and glycine. Microtubular protein migrated at a rate similar to nonmicrotubule soluble protein, and slower than particulate protein. With the exception of γ-aminobutyric acid, transport of free amino acids occurred to only a minor extent. Following monocular injection of tritiated fucose, a rapid asymmetry in the specific activities of protein from contralateral and ipsilateral lobes, was established. Thus the more rapidly migrating proteins may be attached to glycosidic residues. The carbohydrate moeity of these glycoproteins is attached in the nerve cell body, prior to their axonal transport to the optic tectum. There was no evidence for transneuronal transfer of protein as in no case was a differential in specific activity observed in labeled protein from paired cerebral hemispheres.

Key Words: Axon flow — Protein migration — Avain visual system — Optic nerve

Introduction

The movement of materials along neuronal axons was first established by Weiss and Hiscoe (1948) and has since been well documented. More recently interest has focused especially on the axonal transport of proteins (Karlson and Sjostrand, 1968; McEwen and Grafstein, 1968; Kidwai and Ochs, 1969).

A convenient model for such studies is the avian visual system where the optic nerves are totally decussated (Cowan, Adamson and Powell, 1961). Each optic tract of the bird supplies solely the contralateral optic lobe. Thus, after injection of radioactive precursor into a single eye, isotopically labeled compounds may migrate to the contralateral lobe. Any radioactivity appearing within the ipsilateral optic lobe must be due to blood-borne or diffused radioactive materials. Increased specific activity of the contralateral optic lobe over the paired ipsilateral lobe within a single animal must be attributed to a specific migratory process (Taylor and Weiss, 1965; Elam and Agranoff, 1970). The ipsilateral lobe thus

serves as an internal control to the contralateral lobe. Utilizing this system we have investigated the synthesis of proteins within the ganglion cells of the retina and their migration along the axon, and the nature of these proteins.

Experimental

Day old chicks of white Leghorn strain Kl37 were intraocularly injected with 10—20 μl of an aqueous solution containing various radioactive chemicals. Chicks were lightly anesthetized with fluothane and injection was carried out into either eye with a 1/2'' No. 30 hypodermic needle. At various times after this procedure, chicks were killed by decapitation and cerebral hemispheres and optic lobes rapidly dissected out on ice and weighed. The specific activity of protein was assayed in brain regions and the amount of unincorporated label was determined.

Protein Assay

Brain regions from the chicks were homogenized in 5 ml cold 15% (w/v) trichloracetic acid (TCA) and centrifuged at 0°C at 600 g for 10 min. The clear supernatant constituted the acid soluble precursor pool and was retained. The precipitate was resuspended in 5 ml 5% trichloracetic acid, heated to 90°C for 10 min to hydrolyze nucleic acids and any amino-acyl transfer RNA present, then cooled, and recentrifuged. This precipitate was washed twice more with 5 ml cold 5% trichloracetic acid and the final precipitates from optic lobes or cerebral hemispheres were dissolved in 2.5 or 10 ml, respectively, of 0.1 N NaOH at 40°C. Samples of this solution were taken for determination of protein (Lowry *et al.*, 1951) and of protein-bound radioactivity after neutralization with Beckman Bio-Solv solubilizer No. 2. Radioactivity in 0.5 ml of the acid soluble precursor pool was assayed by adding 10 ml standard scintillation solution. Protein bound radioactivity in 2 ml of the neutralized protein solution was counted by adding 15 ml of standard scintillation solution. Standard scintillation solution consisted of a 2:10 (v/v) solution of Beckman Bio-Solv solubilizer No. 3 and toluene containing 0.4% 2-phenyl-5-(biphenyl-2-yl)-1,3,4 oxidiazole and 0.008% 1—4 bis (5-phenyl-oxazole-2-yl) benzene. Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer at an efficiency of 28% for ^{3}H and 73% for ^{14}C.

Amino Acid Analysis

Chicks were injected in a single eye with 20 μC of a mixture of 15 ^{14}C-amino acids (New England Nuclear, Inc., Boston, Mass.). This contained (μC), Ala 1.6; Arg 1.4, Asp 1.6; Glu 2.5, Gly 0.8, His 0.3, Ile 1.0; Leu 2.8; Lys 1.2, Phe 1.6; Pro 1.0; Ser 0.8, Thr 1.0; Tyr 0.8; Val 1.6, in a volume of 25 μl. After 2 days chicks were killed and cerebral regions were dissected out. Amino acid analysis of hydrolysed labeled protein from paired optic lobes was carried out in a Beckman Spinco Model 120 amino acid analyser as described by Roberts (1963).

Preparation of Soluble Neurotubule Fraction

Chick optic lobes were homogenized in 30 vol. 0.32 M sucrose and centrifuged at 3000 g for 10 min in a Sorvall RC-2 centrifuge. The resulting supernatant was

then centrifuged at 100,000 g for 90 min in a Spinco L2 centrifuge. The final supernatant (soluble protein) was mixed with an equal volume of an aqueous vinblastin solution (1.6 mg/ml) to a final vinblastin concentration of 10^{-3}M and centrifuged at 10.000 g for 40 min (Marantz *et al.*, 1969). The supernatant and precipitate were dialysed extensively against 0.05 M sodium phosphate buffer at pH 7.4. The dialysed samples were then assayed for protein and radioactivity as described above.

Each data point presented is the mean of at least three experimental animals, separately analyzed. Regions contralateral to the injected eye are referred to as C and ipsilateral regions are named I. Probability of the ratio of paired sets of data being greater than unity was calculated by Students' one-tailed T-test.

Results and Discussion

Time Course of Protein Transport

Thirty minutes after injection of 1 μC ^{14}C-leucine into a single eye, no significant difference in the specific activity of proteins from paired optic lobes was detectable. There was also close correspondence of the TCA soluble counts within the two lobes (Table 1). Four hours after injection, protein within the optic lobe

Table 1. *Incorporation of radioactivity into protein of paired chick brain optic lobes after monocular injection of 1 μC (U-^{14}C)-leucine (163 mC/m-mole)*

C = lobe contralateral to injected eye
I = lobe ipsilateral to injected eye

Time After Injection (h-hours; d-days)	Acid Soluble Counts/min/10 mg Tissue			Protein Bound Counts/min/mg Protein		
	C	I	C/I	C	I	C/I
0.5 h	250	269	0.9 ± 0.2	483	460	1.1 ± 0.1
4h	46	33	1.4 ± 0.3	1048	501	2.1 ± 0.3*
2d	39	31	1.3 ± 0.2	870	236	3.2 ± 0.5*
3d	—	—	—	800	230	3.5 ± 0.6*
17d	—	—	—	430	68	6.3 ± 0.9*
35d	—	—	—	208	35	5.9 ± 1.3*

* $P < 0.05$

contralateral to the injected eye had a markedly higher specific activity than that within its ipsilateral partner. This radioactive differential was maintained for up to 35 days after administration of isotope. The equivalent decline of total counts in both optic lobes after 17 days suggested that the turnover rate of some proteins that migrate along the optic tract is relatively slow. At no time was a significantly different degree of labeling found in proteins from paired cerebral hemispheres (Table 2). The avian cerebral hemispheres receive no direct innervation from the optic tract (Cowan *et al.*, 1961). There was then no evidence of major transneuronal migration of proteins from the optic lobes to the corresponding cerebral hemispheres which they innervate.

Table 2. *Incorporation of radioactivity into protein of cerebral hemispheres after monocular injection of 1 μC (U-^{14}C)-leucine (163 mC/m-mole)*

Time After Injection	Counts/min/mg Protein Contralateral Hemisphere C	Ipsilateral Hemisphere I	C/I
4h	233	237	0.98 ± 0.04
2d	139	136	1.02 ± 0.03
21d	75	78	0.96 ± 0.03
35d	41	40	1.02 ± 0.06

The transport of TCA-soluble components may also occur. Four hours after intraocular injection of various amino acids, significant asymmetry between TCA-soluble supernatants from paired optic lobes was found for GABA, lysine (Table 3) and leucine (Table 1). However this was always lower than the differential for incorporation into protein within the paired lobes. Furthermore the total number of counts within protein rapidly exceeded TCA-soluble counts except in the case of the inert amino acid analogue α-aminoisobutyric acid. Thus within 4 hours, considerable migration of protein had occurred, and this could not be attributed to migration of TCA-soluble precursors. No asymmetry was found for the metabolically inert α-aminoisobutyric acid. Also, no asymmetry was found for acid soluble proline, in contrast to the findings of Elam and Agranoff (1970) in a parallel study using the goldfish visual system. The most pronounced asymmetry occurred in the case of α-aminobutyric acid (GABA) where a significant differential was apparent within 30 min of injection. GABA is present in bovine optic nerve at a very low concentration (Florey, 1960) and has been shown to block impulses from the retina to the tectum in the chick optic system (Roberts and Kuriyama, 1969).

Table 3. *Transport of acid soluble material and protein to the optic tectum after monocular injection of ^{14}C amino acids*

I and C = as in Table 1

Specific Activities = (μ C/m-Mole), α-Aminoisobutyric 6.1, γ-Aminobutyric (GABA) 21.5, Glutamic 27, Lysine 4.45, Proline 254. 1.5 μC of each amino acid was injected.

0.5 h After Injection	Acid Soluble Counts/min/10 mg Tissue C	I	C/I	Protein Bound Counts /min/mg Protein C	I	C/I
(U-^{14}C)-Proline	39	36	1.1 ± 0.1	—	—	—
(1-^{14}C)-GABA	45	14	3.2 ± 0.5*	—	—	—
(1-^{14}C) α-Aminoisobutyric	69	88	0.8 ± 0.1	—	—	—
4h After Injection						
(U-^{14}C)-Proline	33	30	1.1 ± 0.1	184	88	2.1 ± 0.3*
(1-^{14}C)-GABA	25	3	8.1 ± 1.4*	120	8.4	14.3 ± 2.3*
(1-^{14}C) α-Aminoisobutyric	61	62	1.0 ± 0.1	2.0	2.3	0.9 ± 0.1
(U-^{14}C)-Lysine	21	14	1.7 ± 0.2*	1879	375	5.0 ± 0.8*
(U-^{14}C)-Glutamic	36	30	1.2 ± 0.2	137	31	4.4 ± 0.8*

* $P < 0.05$

Types of Proteins Migrating

Microtubule Protein

The specific activity of crude microtubule protein derived from the soluble fractions of optic lobes was determined 2 days after injection of 1.5 μC ^{14}C-proline into a single chick eye. Proline was chosen as a precursor as, following monocular injection in the goldfish, this amino acid has been shown to result in a large differential of radioactivity in protein from paired optic lobes (Elam and Agranoff, 1971). The difference of radioactivity of microtubular protein between ipsilateral and contralateral lobes was similar to that of soluble protein (Table 4). Thus,

Table 4. *Incorporation of radioactivity into protein fractions from paired optic lobes 2 days after monocular injection of 1.5 μC (U-^{14}C)-proline (254 μC/m-mole)*

I and C = as in Table I

Fraction	Counts/min/mg Protein		
	C	I	C/I
Total Protein	1282	46	27.9 ± 6.1
Soluble Protein	297	53	5.6 ± 0.8
Vinblastin Precipitable Soluble Protein	394	58	6.8 ± 1.4

microtubule protein was transported at a similar rate to a soluble protein and somewhat more slowly than total protein. Slower migration of vinblastin precipitable material relative to particulate protein has been described by Grafstein *et al.* (1970).

Glycoproteins

As several cerebral glycoproteins are very tissue specific (Warecka and Bauer, 1967), axonal glycoprotein transport was examined. Fucose was used as a precursor as it has been described as being exclusively utilized for the synthesis of glycoproteins within brain (Zatz and Barondes, 1970).

Table 5. *Incorporation of radioactivity into protein of optic lobes after monocular injection of 15 μC (l-^{3}H)-fucose (450 mC/m-mole)*

I and C = as in Table 1

Time After Injection	Acid Soluble			Protein Bound		
	Counts/min/10 mg Tissue			Counts/min/mg Protein		
	C	I	C/I	C	I	C/I
4h	117	59	2.0 ± 0.4*	2416	58	41.7 ± 11*
2d	—	—	—	1958	74	26.5 ± 7.7*

*$P < 0.05$

Monocular injection of 1.5 μC ^{3}H-fucose resulted in a very sharp asymmetry for radioactive protein of paired lobes after 4 hours and 2 days (Table 5). This indicated a considerable and very rapid movement of intact glycoprotein along the axon with the sugar residue being attached prior to transport. Elam *et al.* (1970)

have found a rapid migration of sulfated mucopolysaccharide proteins along the goldfish optic tract. In contrast, Barondes (1968) found incorporation of glucosamine into macromolecules at nerve endings. It may be that glycoprotein is completed within the nerve cell body but that reversible dissociation can take place at the nerve terminal.

Amino Acid Analysis

Two days after monocular injection of a different mixture of 15 ^{14}C-amino acids, the specific activity of residues from hydrolysed protein of optic lobes was determined. The ratio of radioactivity within protein, due to axon flow, to that not due to axon flow varied considerably from one amino acid to another (Table 6). In view of the differing amounts of each amino acid injected (Experimental Section) and their widely varying free cerebral pool sizes (Table 6), it is difficult to

Table 6. *Incorporation of radioactivity into protein of optic lobes 2 days after injection of 20 μC of a mixture of ^{14}C-amino acids*

I and C = as in Table 1

Chick optic lobe composition						
Residue	Protein (μ-moles/mg protein	Pool (μ-moles/100 mg tissue	Counts/min/μ-mole amino acid residue			C-I/I
			C	I	C—I	
Lys	0.66	0.013	1204	109	1095	10.0±1.6
His	0.21	0.006	550	97	457	4.7±0.7
Arg	0.49	0.024	853	90	763	8.5±0.7
Asp	0.90	0.342	128	45	83	1.8±0.3
Thr	0.52	0.042	679	73	606	8.3±0.4
Ser	0.56	0.096	530	74	456	6.2±0.4
Glu	1.13	0.778	312	24	288	12.0±1.2
Pro	0.43	0.012	1537	56	1481	24.4±4.1
Gly	0.68	0.115	607	30	577	19.2±2.7
Ala	0.78	0.036	470	62	408	6.6±0.9
Val	0.58	0.009	786	159	627	3.9±0.7
Ile	0.46	0.003	630	71	558	7.9±0.8
Leu	0.82	0.007	1373	188	1185	6.3±1.0
Phe	0.38	0.009	1539	289	1250	4.3±0.6
Hypro	—	0.008	—	—	—	—

correlate these data. However, by comparing paired lobes in this manner and making the assumption that free amino acid pools within the brain are similar in magnitude to those within the retina, it is possible that this ratio may give an indication of the composition of migrating proteins. As proline and glycine exhibit the highest ratios, such proteins may then be unusually rich in these amino acids. This is unlike the amino acid composition of protein from chick optic lobe (Table 5) or that of neuronal microtubule protein (Olmsted *et al.*, 1970), but may resemble collagen which is strikingly rich in glycine, proline and hydroxyproline (Leach, 1957).

Casola (1970) has shown a broad variety of proteins are transported along the optic nerve. It may be that a certain number of these proteins have collagen-like

features and these could play a role in the maintenance of axonal structure. The slow rate of appearance of the proline asymmetry (Table 3) suggests that proline-rich proteins may be relatively slow migrating. No hydroxylated proline was found in axon transported protein, although a trace was found in the acid-soluble pool (Table 6). Hydroxyproline-free collagen has been described by Gribble *et al.* (1969). It is of interest that a similarly high asymmetry of protein bound proline has been found after monocular precursor injection, in the goldfish visial system (Elam, Goldberg, Radin and Agranoff, 1970).

These results suggest that the phenomenon of axonal flow may not be readily divided into "slow" and "fast" components, but that a wide spectrum of transport velocities may exist.

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