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Biochemical Maturation of the Non-Innervated Chick Optic Lobe [1]

S.C. Bondy, K.C. Bonnet, M.E. Harrington, C.Z. Neurath, R. Nidess, A. Vernadakis, P.R. Vulliet and C. York

Departments of Neurology, Pharmacology and Psychiatry, University of Colorado Medical Center, Denver, Colo.; Department of Psychiatry, New York University Medical School, New York, N.Y., and Roche Institute of Molecular Biology, Nutley, N.J.

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Abstract. The development of the chick optic lobe was impaired following removal of the optic cup of the early embryo. Tectal cell number is reduced but cell size may be relatively normal. There was evidence of neuronal cell death and several neuron-associated proteins and enzymes (nerve-specific protein and acetylcholinesterase) showed selectively impaired maturation. However, other nerve-specific enzymes (choline acetyltransferase, tyrosine hydroxylase), develop normally on a per cell basis. The noninnervated optic lobe had a normal blood-brain barrier but a depressed ability to accumulate amino acids from plasma. Levels of 3′:5′-cyclic GMP were also reduced in the nonafferented lobe.

Introduction

Deafferentation of the optic tectum of the new hatched chick by eye removal leads to an arrest of its development. This operation results in the removal of approximately 90% of the afferent sensory input [12] and major degeneration of the synaptic terminals of the optic lobes becomes morphologically apparent [16]. The gross weight of the lobe increases very little after this procedure and the accretion of protein and RNA is severely reduced [3, 4]. The reduced growth rate is associated with a lower nutrient supply, caused by a depressed rate of blood flow to the affected lobe [5].

In spite of a reduced growth rate, considerable development of several indices of maturation occurs. Thus, the levels of nerve-related enzymes such as acetylcholinesterase continue to rise [4] and the high affinity neurotransmitter uptake mechanisms, associated with mature nerve tissue, also develop [6]. The maturation of blood-brain barrier is not completely prevented by deafferentation of the chick optic lobe [37]. However, other maturation processes such as the formation of myelin cerebrosides and the appearance of 2′:3′-cyclic nucleotide phosphohydrolase are selectively inhibited [2] by optic lobe deafferentation.

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If an optic cup of the chick is removed at 2–3 days after the start of incubation, the corresponding contralateral optic lobe does not receive its usual innervation from the optic nerve at 8–11 days of incubation. In this case the lobe is not denervated but rather it is non-innervated. Morphologically, such lobes develop normally until the 14th day of incubation after which time an increasingly severe hypoplasia is seen [14].

The outgrowing axons from retinal ganglion cells in the 3-day-old embryo are already committed to specific tectal destinations [15]. Few, if any, fibers grow to an ipsilateral destination after removal of the optic cup [14]. It is likely that any such aberrant fibers disappear before hatch [11].

We have examined a variety of biochemical indices of development of such noninnervated optic lobes in the 20-day-chick embryo, just prior to hatch. This time period was chosen in view of the high mortality associated with hatching. Monocularly enucleated embryos develop a characteristic 'cross bill' which makes pecking through the eggshell very difficult. Since the surgical removal of the optic cup was done at an early age, we have been able to determine the extent to which tectal maturation is intrinsic rather than dependent on its major afferent supply. We have found that although growth of the noninnervated optic tectum is severely retarded, the development of several processes associated with brain maturation still occurs.

Methods

Surgical Removal of the Optic Cup

Fertile chick eggs of a White Leghorn strain were maintained at 38 °C for 48 h in a rotating incubator, after which they were incubated in an upright position (with the blunt pole upward) for a further 24 h. A hole around 1 cm in diameter was then cut over the air space and the embryo was exposed by peeling back the allantoic membrane. The right eye, which invariably faced upward, was electrocoagulated using a sharpened bipolar forceps insulated except at the tip, attached to a Malis Bipolar Coagulator (Codman Inc., Randolph, Mass.). A Bausch and Lomb dissecting microscope was used to visualize the embryo. The egg was then resealed with a circle of paraffin wax (m.p. 56 °C) upon which a flame-heated coverslip was placed. The remainder of the incubation was carried out with the egg in a vertical position. The overall mortality up to the 20th day of incubation was 63 % of all operated eggs. On the 20th day of incubation, live embryos were removed and examined to ensure that the right eye had totally failed to develop and that the left eye was intact. Embryos with partial right eyes resulting from incomplete extirpation of the optic vesicle were rejected. For enzymic and chemical determinations, left and right optic lobes were rapidly dissected, weighed and immediately placed on dry ice prior to storage at −60 °C.

Measurement of Cerebral Blood Flow, Inulin Space, Proline Penetrance and Uptake of α-Aminoisobutyrate

Blood flow was measured by intracardiac injection of 0.1 ml n-methyl-^{14}antipyrine (10 µl 20.0 mCi/m*M*, New England Nuclear Corp., Boston, Mass.) into 20-day-old operated embryos. After 10 sec, optic lobes were dissected out, weighed and dissolved at 50 °C in 0.5 ml NCS tissue solubilizer (Nuclear Chicago, Inc., Chicago, Ill.). 10 ml of a compatible scintillation fluid were then mixed with this and radioactivity assayed in a Picker Liquimat scintillation counter at an efficiency of 86–88 %. Results were calculated as counts/min/mg wet tissue.

The inulin space was estimated after intracardiac injection of a mixture of ^{14}C-antipyrine and ^{3}H-inulin (875 mCi/m*M*) as previously described [32]. The blood-brain barrier toward proline was estimated by a similar injection of a mixture of ^{14}C-antipyrine and *L*-(5-^{3}H) proline (33 Ci/mmol) [32], a method first described by *Oldendorf* [30]. Briefly, a mixture of 8 µCi of the ^{3}H-compound and 0.17 µCi of ^{14}C-antipyrine in 0.14 *M* saline were injected into the exposed embryonic heart and optic lobes were dissected out 10 sec later. After solubilization radioactivity in ^{3}H

and ^{14}C was assayed. An aliquot of the injected mixture was also counted. Penetrance was calculated as

$$\frac{^{3}H/^{14}C \text{ in tissue}}{^{3}H/^{14}C \text{ in original mixture}}$$

The double label technique compares the penetration of the test compound into the brain with that of freely diffusible antipyrine.

The ability of tissue to accumulate amino acids was estimated using nonmetabolizable α-aminoisobutyric acid [29]. 10 μCi of 2-amino (1-^{14}C) isobutyric acid (60 mC/im*M*) were injected into the egg and after 24 h the relative radioactivity in optic lobes and serum was compared. Tissue was weighed and dissolved in tissue solubilizer before the determination of radioactivity.

Determination of RNA, DNA and Protein

Each weighed optic lobe was homogenized in 3 ml 0.3 *N* $HClO_4$ at 0°C. The homogenate was centrifuged (3,000 *g*, 10 min) and the precipitate resuspended in a further 3 ml 0.3 *N* $HClO_4$. Aliquots of this suspension were taken for determination of protein by the method of *Lowry et al.* [23] and the rest was recentrifuged. This precipitate was extracted twice with 3 ml ethanol and the final pellet was dissolved in 3 ml 0.3 *N* KOH and maintained at 37°C for 2 h. After cooling, the solution was made acid with 0.12 ml 70% $HClO_4$ and centrifuged. The pellet was washed twice with 1 ml cold 0.5 *N* $HClO_4$ and the absorbance of the combined supernatants at A_{260} (where there was a maximum) measured. Taking the absorbance of 1 mg hydrolyzed RNA/ml to be 30.0 [8], the RNA content of the lobe was calculated. The residual precipitate that had been washed with cold $HClO_4$ was then heated to 90°C for 15 min in 1 ml 0.5 *N* $HClO_4$ and the absorption maximum at A_{268} was measured. DNA content was calculated using a calf thymus DNA preparation as a standard. The absorbance at A_{258} of 1 mg hydrolyzed DNA/ml was 32.3.

Assay of Cyclic Nucleotides

Optic lobes were excised from brains of embryos whose heads had been frozen in liquid nitrogen for 2 min [9]. Frozen lobes were homogenized in 6% trichloroacetic acid at 0°C and centrifuged (3,000 *g*, 10 min). The supernatant was washed 4 or 5 times with diethyl ether to remove the trichloroacetic acid, then taken to dryness in a nitrogen stream. The residue was taken up in 0.6 ml 0.1 *N* sodium acetate buffer pH 4.2. Cyclic AMP was assayed by the method of *Gilman* [20] and cyclic GMP was determined by the radioimmunoassay technique of *Steiner et al.* [34].

Enzyme Determinations

Acetylcholinesterase was determined colorimetrically using the rate of hydrolysis of acetylthiocholine [17]. Nonspecific cholinesterase was similarly determined using butyrylthiocholine iodide as the enzyme substrate. 0.1% tissue homogenates in 0.07 *M* Na_2HPO_4 and 0.07 *M* KH_2PO_4, pH 8.0 were incubated at 37°C with 0.3 m*M* dithiobisnitrobenzoic acid and 0.5 m*M* acetylthiocholine or butyrylthiocholine.

Choline acetyltransferase was assayed by the radiochemical method of *Fonnum* [18]. Following a 25-min incubation at 38°C of a 20% homogenate, labeled acetylcholine was extracted with 0.5% tetraphenylboron in 3-heptanone. The reaction mixture contained 0.2% Triton X-100, 0.04% bovine serum albumin, 20 m*M* EDTA, 8 m*M* choline chloride, 0.1 m*M* eserine sulfate, 0.3 *M* NaCl, 0.2 m*M* ^{14}C-acetylcoenzyme A (New England Nuclear, Boston, Mass., 55 mCi/mmol) in 50 m*M* sodium phosphate buffer pH 7.4.

Tyrosine hydroxylase was assayed by the method of *Waymire et al.* [38]. This technique is based on the enzymic decarboxylation of ^{14}C-carboxyl-labeled *L*-dihydroxyphenylalanine formed from carboxyl labeled *L*-tyrosine. The evolved $^{14}CO_2$ is then trapped in base and assayed.

Determination of Nerve-Specific Protein (NSP)

NSP was determined by a radioimmunoassay method previously described [25]. Chick optic lobes were homogenized in 2.5 ml of 10 m*M* Tris-HCl, pH 7.6 and the suspension centrifuged (100,000 *g*, 60 min). Supernatants were then lyophilized and the powder taken up in 1 ml water for Lowry protein determination and radioimmunoassay. The method essentially consists of the tritiation of NSP by reductive alkylation and its subsequent immunological reaction with antibody-coated polystyrene beads [25]. After incubation, the amount of tritiated NSP that is bound to the antiserum complexed with beads is readily determined by centrifuging down the beads and washing them to remove noncomplexed radioactivity. Since chick NSP does not cross-react with antiserum to the same extent as does rat NSP, results have been expressed as rat-brain equivalents of NSP.

Table I. Weight and macromolecule content of optic lobes of 20-day-old chick embryos in which a single optic cup was removed on the 3rd day of incubation

	mg/lobe	
	E	C
Wet weight	49.3±1.1	67.2±1.3
Protein	3.91±0.09	5.38±0.21
RNA	0.105±0.005	0.143±0.006
DNA	0.037±0.001	0.050±0.002

E = Experimental lobe, contralateral to removed optic vesicle; C = control lobe contralateral to intact eye. Standard errors of the mean are given.

Results

Protein, RNA and DNA Content

The weight of the nonafferented optic olbes was 73% of that of innervated lobes from the same embryos (table I). The weights of innervated lobes were slightly lower than weights of lobes derived from unoperated embryos. This may be due to a minor developmental delay caused by the surgery. The deficit in weight of the nonafferented lobe was almost exactly paralleled by a reduced content of protein, RNA and DNA (table I). Thus, the concentration of macromolecules per milligram wet tissue was unchanged in experimental lobes.

Nutrient Supply of the Optic Lobes

The rates of blood flow through innervated and noninnervated optic lobes were compared by determining the uptake of ^{14}C-antipyrine from the blood stream. No significant difference in uptake per milligram tissue was found. Also, the inulin space of experimental lobes was similar to that of control lobes (table II).

The penetrance of blood-borne ^{3}H-proline into the optic lobe was roughly 10% of that of antipyrine (table II). This value is similar to that of the paired control lobe and also to the penetrance found in operated chick embryos of the same age [32]. Therefore, nonafferentation does not alter the development of the blood-brain barrier toward proline.

The ability of the noninnervated optic lobe to accumulate an inert amino acid from the plasma was seriously impaired (table II). The transport capacity of the nonafferented optic lobe was below that of the 13-day embryonic lobe. This represented a deterioration of the

Table II. Nutrient supply to optic lobes of 20-day-old chick embryos in which a single optic cup was removed on the 3rd day of incubation

	E	C	E/C
Inulin space (fraction of whole tissue)	0.16±0.04	0.17±0.05	0.98±0.07
Proline penetrance (relative to antipyrine)	0.10±0.03	0.11±0.04	0.97±0.12
Relative regional blood flow	–	–	0.96±0.06
Lobe concentration of α-aminoisobutyrate relative to plasma	2.90±0.62	3.89±0.52	0.77±0.06*

E, C as in table I. Standard errors of the mean are given. * = $p<0.05$ that value differs significantly from unity.

transport capacity of the optic tectum rather than merely an arrest of development.

Cyclic Nucleotides

The level of 3′:5′-cyclic GMP was lower in the nonafferented lobes than in control optic lobes, while 3′:5′-cyclic AMP values were not significantly different (table III). Cyclic nucleotide levels were also measured in 3-week-old chicks that had been unilaterally enucleated at hatch. In this case the denervated optic lobes also had an abnormally low concentration of cyclic GMP. Noninnervation of optic lobes caused a major fall in cyclic GMP levels while cyclic AMP values are changed to a much lesser extent. The ratio of cyclic AMP to GMP for individual operated embryos or 3-week-old chicks was elevated in optic lobes without afferentation from the retina.

NSP, Enzyme and Monoamine Levels

The activity of nonspecific cholinesterase was measured by the rate of hydrolysis of butyrylthiocholine. The concentration did not differ in experimental and control optic lobes (table IV). The activity of specific acetylcholinesterase was 15% lower in noninnervated lobes. Since cerebral acetylcholinesterase is a relatively specific index of neurons [24], this finding implied that surgery had affected nerve cells to a greater extent than glial cells. Since noninnervated lobes only weighed 73% as much as control lobes, the overall amount of both enzymes per lobe was depressed in experimental regions. Choline acetyltransferase activity per milligram wet tissue was identical in experimental and control lobes. This enzyme may be confined to cholinergic neurons and is concentrated at axonal endings [24]. Tyrosine hydroxylase levels were also not reduced in experimental

Table III. Levels of cyclic nucleotides in lobes of 20-day-old chick embryos in which a single optic cup was removed on the 3rd day of incubation and in 21-day-old birds unilaterally enucleated at hatch (pmol/mg wet weight)

	3′:5′-cyclic AMP	3′:5′-cyclic GMP	AMP/ GMP
Embryo			
E	0.71 ± 0.06	0.021 ± 0.02*	33.8*
C	0.71 ± 0.06	0.032 ± 0.03	22.2
21-day-old bird			
E	0.80 ± 0.06	0.022 ± 0.001*	36.4*
C	0.97 ± 0.09	0.035 ± 0.003	27.7

E, C as in table 1. Standard errors of the mean are given. * = Experimental value significantly differs from control ($p<0.05$).

Table IV. Enzyme and NSP levels in optic lobes of 20-day-old chick embryos in which a single optic cup was removed

	E	C
	µmol substrate hydrolyzed/min/g wet weight	
Acetylcholinesterase	23.9 ± 0.8*	28.3 ± 1.8
Butyrylcholinesterase	1.18 ± 0.03	1.20 ± 0.03
	nmol product synthesized/min/g wet weight	
Choline acetyltransferase	52 ± 2	50 ± 1
Tyrosine hydroxylase	3.87 ± 0.68	3.55 ± 0.22
	µg/g wet weight	
Nerve specific protein	21.6 ± 2.3*	28.0 ± 2.2

E, C as in table I. Standard errors of the mean are given. * = Experimental value significantly differs from control ($p<0.05$).

lobes. Thus, the maturation of enzymes involved in neurotransmitter synthesis in both cholinergic and catecholaminergic neurons was not impaired when assayed on a per cell basis.

The content of NSP was much lower in the nonafferented optic lobes than in the control lobes (table IV). The amount of NSP in the experimental lobes was thus only 56% of the control value, suggesting a large loss of differentiated neurons.

Discussion

Since DNA content can be taken as an index of cell number, the reduced DNA within the noninnervated (experimental) optic lobe relative to the control lobe implies a reduced cell number.

The absence of the developing eye has no detectable effect on cell proliferation in the optic tectum which proceeds normally up the 13th day of incubation [14]. The DNA content of the normal optic lobe increases very little between the 12th and hatch [26]. Thus, the reduced cell number in the experimental lobe is due to cell death as well as failure of division. The size and content of RNA and protein in surviving cells appears to be relatively normal as the amount of RNA or protein per unit DNA is identical in experimental and control lobes. These results are not analogous to the effects of monocular eye removal in the new hatched chick. In this latter case, while severe arrest of differentation takes place, tectal cell number remains constant [4] and protein content on a per cell basis is depressed.

The concentration of acetylcholinesterase in experimental lobes was significantly below that of control lobes while the butyrylcholinesterase concentration was unchanged. Acetylcholinesterase is relatively specific for neurons while nonspecific cholinesterase is associated with glia [19,36]. Thus, the cell population lost in the experimental lobes may have been predominantly neuronal rather than glial. This is substantiated by the reduced velocity of high affinity uptake of putative neurotransmitters or their precursors by the nonafferented experimental lobes [7]. Several uptake mechanisms are largely confined to the presynaptic area, especially those of choline, dopamine and serotonin [22] and so their reduced levels imply extensive failure of synaptogenesis. The reduced density of NSP in experimental lobes relative to tissue weight or protein content also suggests a selective loss of neurons.

Although the number of neurons in the experimental lobe is reduced, the levels of several nerve-specific enzymes and proteins and uptake mechanisms are much above levels found in the optic lobes of the 10-day-old embryo [7,10,35]. Therefore, the biochemical maturation of many surviving neurons may be relatively normal.

The lack of detectable changes in the blood-brain barrier and in the inulin space of the experimental optic lobe could be attributed to relatively unimpaired glial maturation. Thus, the overall evidence indicates a large neuronal loss in the nonafferented optic lobe and a rather normal development of glial cells. The conclusion is supported by morphological evidence suggesting neuronal cell death [14].

Cyclic AMP levels have been related to the state of tissue differentiation while proliferating tissue is frequently characterized by a relatively high concentration of cyclic GMP [33]. Cyclic AMP analogues have been shown to induce neurite formation and inhibit cell

division in tissue cultures of neuroblastoma [31]. The ratio cyclic AMP/cyclic GMP has been used as an index of differentiation rather than proliferation activity [33] although this relationship is not totally accepted [28]. The ratio is greater in experimental than in control lobes of operated chick embryos and this may be associated with the failure of normal tectal differentiation by removal of the major potential source of afferentation. We have found the major changes in cyclic nucleotide levels in the nonafferented embryonic lobe to be cyclic GMP rather than AMP. Cyclic GMP levels seem capable of greater fluctuation than those of cyclic AMP [21]. There is evidence that cyclic nucleotides promote transsynaptic enzyme induction during development [13] and thus a reduced cyclic GMP level may be the cause of failure of development of neuronal enzymes, especially those of cholinergic neurons.

In the functioning chick brain, after hatch, eye removal causes a rapid and prolonged fall in blood flow through the deafferented optic lobe [5]. A significant drop in tectal blood flow occurs after merely suturing an eye shut [1]. Thus, after hatch, factors regulating tectal blood flow may include the intensity of visual input and perhaps also trophic influences of the optic nerve. In the nonafferented embryonic optic lobe, no significant reduction in blood flow occurs relative to the control lobe. Thus, the embryonic optic nerve does not regulate tectal blood flow or capillary density. At these early developmental stages, cerebral blood flow may be solely intrinsically regulated perhaps by carbon dioxide concentrations.

The proper migration of many tectal cells and their processes depends on intact afferentation from the retina [14]. This work shows that the absence of these key axons, which form only a small fraction of all tectal synapses, severely affect the biochemical maturation of the optic lobe.

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S.C. Bondy, Departments of Neurology and Pharmacology, University of Colorado Medical Center, Denver, CO 80262 (USA)