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PUTATIVE NEUROTRANSMITTERS OF THE AVIAN VISUAL PATHWAY

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SUMMARY

The ability of homogenates of the chick optic lobe to accumulate a series of possible neurotransmitters has been studied. High affinity uptake of several possible neurotransmitters was examined in optic lobes of 21-day-old embryos that had a single eye removed on the third day of incubation and in 23-day-old chicks that had an eye removed at hatch. Embryonic enucleation resulted in severe reduction of development of the ability of the contralateral optic lobe to take up tritiated GABA, dopamine, choline, serotonin and glutamate from solutions around 10^{-8} M. Unilateral eye removal of new-hatched chicks caused failure of the denervated optic lobe to grow, but only the uptake capacity for glutamate was significantly reduced. This deficit was apparent as early as 4 days after enucleation. The transport of other compounds was unimpaired. The uptake of glutamate by homogenates of the optic tract was 43 % of that of the optic lobe. This was a much greater fraction than the corresponding value for other postulated neurotransmitters. These data suggest that glutamate may be the primary neurotransmitter of the fibers of the optic tract originating in the retinal ganglion cells.

INTRODUCTION

In the preceding paper⁷ we present evidence suggesting a close correlation between the level of a possible neurotransmitter in a brain region and the ability of homogenates of that region to take up the same compound from very low concentrations in the medium. This process is energy requiring, sodium dependent and is predominantly localized in the nerve ending. Uptake is relatively specific for a given possible neurotransmitter²¹.

The avian visual pathway is completely crossed over¹² and receives most of its afferentation from the retina of the contralateral eye¹⁰. We have examined the ability of chick optic lobes to take up putative neurotransmitters either after prevention of lobe innervation (by early ablation of the early embryonic eye) or after lobe

denervation (by removal of an eye of the new-hatched chick). The first of these procedures caused widespread secondary failure of lobe development, while eye removal in the chick initially caused only degeneration of the distal termini of the retinal ganglion cell axons.

Deficits in the high affinity uptake of suspected neurotransmitters in deafferented optic lobes may give an indication of the nature of the specific transmitter in the chick optic pathway.

MATERIALS AND METHODS

Chick and chick embryos of a White Leghorn strain were used. Eyes were removed or sutured shut under light chloroform anesthesia. Animals rapidly recovered from these procedures. After decapitation, tissues were rapidly dissected out and accurately weighed. Unilateral enucleation of 3-day-old embryos was carried out by bipolar electrocoagulation. After surgery, eggs were sealed with a coverslip held in place with paraffin wax.

The standard incubation medium consisted of Krebs-Ringer buffer consisting of 121 mM NaCl, 4.0 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1 mM ascorbic acid, 0.015 mM EDTA, 20 mM glucose and 40 mM Tris · HCl, pH 7.6. An inhibitor of monoamine oxidase (nialamide) was also present (7.5×10^{-5} M) as was aminoxyacetic acid (1×10^{-5} M), an inhibitor of γ -aminobutyric acid transaminase. A single radioactive compound (New England Nuclear, Boston, Mass.) was then added to this medium for each incubation. Final μ M concentrations of each compound were: DL-[3-³H]glutamic acid, 0.01 (8.2 Ci/mmol); [2,3-³H]GABA, 0.01 (10 Ci/mmol); [1,2-³H]hydroxytryptamine (serotonin), 0.024 (4.25 Ci/mmol); DL-[7-³H]-norepinephrine, 0.013 (7.5 Ci/mmol); [methyl-³H]choline chloride, 0.017 (2.34 Ci/mmol); [ethyl-1-³H]3,4 dihydroxyphenylethylamine (dopamine), 0.0125 (8.0 Ci/mmol); and [1,2-¹⁴C]taurine, 1.08 (4.61 mCi/mmol).

This medium was gassed with 95% O₂-5% CO₂ and then 0.9 ml of this was mixed with 0.1 ml of either a 5% (v/v) tissue homogenate or of a synaptosomal suspension in 0.32 M sucrose. The GABA uptake mechanism was intensely active. Thus, in order to maintain linear kinetics, 0.1 ml of a 0.5% homogenate was used in these studies. In this case, 0.1 ml of 5% liver homogenate was added as a carrier at the end of the incubation. Incubation was at 37 °C for 5 min with continuous shaking. To allow for non-energy dependent neurotransmitter-binding, identical mixtures were held at 0 °C, and these served as controls.

All samples were then centrifuged at 0 °C and $28,000 \times g$ for 10 min. Supernatants were drawn off for determination of radioactivity remaining unbound to particulate matter. Pellets were resuspended in 4 ml isotonic (0.14 M) NaCl and recentrifuged ($28,000 \times g$, 10 min). The washed pellets were then dissolved in 0.5 ml tissue solubilizer (NCS, Amersham Searle, Arlington Heights, Ill.) at 45 °C. Radioactivity in these samples was determined. It was thus possible to calculate what percentage of the total radioactive compound in each incubation tube was actively taken up by the particulate fraction.

Fig. 1. High affinity uptake of compounds by homogenates of normal or non-afferented optic lobes of 21-day-old chick embryos, 18 days after removal of a single optic cup. Tubes contained 0.1 ml of a 5% tissue homogenate except for the GABA study where a 0.5% homogenate was used. Incubation was for 5 min in oxygenated Krebs-Ringer buffer. For detailed procedure see text. Results are expressed as the ratio of uptake capacity of the non-innervated experimental lobe to that of the normal control lobe (open columns). The ratio of total uptake capacity of experimental to control lobes is also shown (shaded columns). Standard errors of the mean are given. *, experimental significantly different from control ($P < 0.05$).

Determination of GABA, catecholamines and serotonin

The concentrations of all suspected neurotransmitters were determined in tissue that had been frozen at -10°C immediately after weighing. Alderman and Shellenberger² have found GABA levels to rise rapidly after death.

GABA was estimated by the method of Cozzani¹⁴. Tissue extracts were incubated together with ketoglutarate and a crude mixture of GABA transaminase and succinic semialdehyde dehydrogenase derived from *P. fluorescens* ('Gabase', Sigma Chemical Co., St. Louis, Mo.), and the conversion of NADP to NADPH was followed spectrophotometrically at 340 nm. In the absence of 'Gabase', the amount of NADPH formed was reduced by 96%. GABA concentrations were calculated using a standard curve.

Norepinephrine and dopamine were estimated by a method based on that of Coyle and Henry¹³. Extraction was with toluene-isoamyl alcohol (3:2 v/v) rather than ethyl acetate. Overall recovery was checked by the addition of [^{14}C]S-adenosylmethionine after incubation with [^3H]S-adenosylmethionine. Serotonin was assayed fluorimetrically²⁵.

Amino acid analysis

Tissues were homogenized in 20 vols of cold 5% trichloroacetic acid (TCA). After centrifugal preparation of the supernatant ($10,000 \times g$ 10 min), TCA was

Fig. 2. High affinity uptake of compounds by homogenates of normal or denervated optic lobes of chicks 23 days after unilateral eye removal. Tubes contained 0.1 ml of a 5% tissue homogenate except for the GABA study where a 0.5% homogenate was used. Incubation was for 5 min in oxygenated Krebs-Ringer buffer. For detailed procedure see text. Results are expressed as the ratio of uptake capacity of the deafferented (experimental) lobe to that of the intact (control) lobe (open columns). The ratio of total uptake capacity of experimental to control lobes (shaded columns) is also shown. Standard errors of the mean are given. *, experimental significantly different from control ($P < 0.05$).

removed by extraction with ether three times. The final neutral aqueous solution was lyophilized and taken up in pH 2.2 citrate buffer. Portions (2 ml) were analyzed for amino acid content by column chromatography with a Beckman Spinco Model 120 C Amino Acid Analyzer²⁶.

RESULTS

(a) Removal of a single eye in the 3 day embryo

The optic lobe contralateral to an eye that has been extirpated prior to outgrowth of optic nerve fibers to the lobe develops normally until around the 13th day after which it undergoes an increasingly severe hypoplasia¹¹. We found the non-innervated optic lobe just prior to hatch to weigh 69% of the corresponding value for the innervated lobe of the same eye. A similar deficit in protein content was also found.

The transport capacity of homogenates of these lobes was determined and expressed as total uptake per lobe and also uptake per unit wet weight (Fig. 1). There was a reduction in the overall ability of the non-innervated lobes to accumulate all compounds tested. This deficit was approximately equal for all such putative neurotransmitters and the precursor of acetylcholine. When data were calculated per

mg tissue, experimental and control lobes had similar uptake abilities. This suggested that the density of surviving synapses in the non-innervated lobe was the same as in a normal lobe.

(b) *Enucleation of the new-hatched chick*

Removal of an eye at hatch arrests the subsequent growth and accretion of protein of the denervated optic lobe⁴. Twenty-three days after enucleation the ability of homogenates of experimental and control lobes to take up a variety of compounds was tested (Fig. 2). The total uptake capacity per lobe for GABA, dopamine, choline and serotonin was not significantly reduced in the deafferented lobe. Since the lobes were smaller than control lobe, the density of uptake capacity per mg lobe for these 4 compounds was in fact significantly higher in the experimental lobe than in the normal lobe.

In the case of glutamate transport, a different profile was observed. Both the total uptake and the uptake per unit weight of glutamic acid were significantly depressed in homogenates of the denervated optic lobe. Thus, deafferentation caused a selective loss of one high affinity uptake mechanism while leaving others intact. A kinetic analysis of the high affinity uptake of glutamate by homogenates of normal and denervated optic lobes was performed. The $V_{\max}$ for normal lobes was 23.4 ± 1.6 nmoles/g tissue/min while that for denervated lobes was 19.6 ± 1.0 nmoles/g tissue/min. Since this difference was significant ($P < 0.05$, one-tailed t -test), the experimental lobe appeared to have a lower density of sites for glutamate transport. Control and experimental lobes had very similar K_m values (8.30×10^{-6} M and 8.12×10^{-6} M respectively). This suggested that surviving uptake sites in the deafferented lobe had essentially the same affinity for glutamate as sites in the control lobe. These data are consistent with the idea that the deficit of the high affinity uptake system in deafferented lobes is due to a selective loss of primary synapses that are able to take up glutamate.

A low affinity uptake system for glutamate was also found in optic lobe homogenates. This system was very similar in control and denervated lobes. The K_m values

TABLE I

Concentration of compounds in optic lobes of day-old chicks and of chicks 23 days after removal of the left eye

	nmoles/g tissue			
	1-day-old chick	23-day-old chick		
		Left lobe (control)		Right lobe (deafferented)
Dopamine	0.027	0.08 $\pm$	0.01	0.08 $\pm$ 0.02
Norepinephrine	0.11	0.34 $\pm$	0.01	0.39 $\pm$ 0.01
Serotonin	2.48	2.6 $\pm$	0.3	2.9 $\pm$ 0.1
GABA	2270	2980 $\pm$	105	2870 $\pm$ 73
Glutamate	—	7110 $\pm$	1010	6600 $\pm$ 30
Aspartate	—	3870 $\pm$	450	3710 $\pm$ 580
Glutamine	—	6890 $\pm$	960	5250 $\pm$ 660

were $4.4 \times 10^{-4} M$ (control lobes) and $4.5 \times 10^{-4} M$ (experimental lobes) while the corresponding $V_{\max}$ was 260 nmoles/g tissue/min for control lobes and 267 nmoles/g tissue/min for denervated lobes. These kinetic parameters are similar to those reported for the crude mitochondrial fraction of the deafferented pigeon tectum²⁰.

The levels of putative neurotransmitters in optic lobes were measured 23 days after unilateral eye removal (Table I). There was no significant difference in values of dopamine, serotonin, GABA or norepinephrine in experimental and control lobes. In fact, norepinephrine and serotonin concentration was somewhat increased in the experimental lobe. This may be a reflection of the increased density of surviving synapses in the denervated lobe whose growth is impaired. The GABA value for the new-hatched chick was similar to that reported previously by Siskin et al.²⁷. The excess of NE relative to dopamine suggested that NE synapses constituted most of the catecholamine uptake sites of the optic lobe. No significant difference could be found in levels of glutamate, aspartate or glutamine although they all tended to be lower in the denervated optic lobe.

(c) Modification of the transport capacity of intact neurons

The cerebral hemispheres of the chick receive no direct innervation from the optic nerve. However, significant biochemical and physiological changes can rapidly occur in the indirectly associated hemisphere after eye removal^{3,5}. In order to see whether changes observed following enucleation were primary or indirect, the uptake capacity of homogenates from hemispheres was studied after unilateral enucleation. No differences were found between left and right hemispheres in their uptake capacity for GABA, dopamine, glutamic acid, norepinephrine or choline 23 days after removal of the left eye. Unilateral eyelid suture does not cause death of retinal ganglion cells but rapidly changes the rate of blood flow through the corresponding optic lobe^{6,8}. These changes are almost as large as those following eye removal⁶. We were interested to see whether this could also be reflected in alterations of transport ability. However, after 23 days of continuous monocular suture, no alteration in the capacity for neurotransmitter uptake was found in the lobe innervated by the sutured eye. These negative results, together with those from the cerebral hemispheres described above, were obtained from regions where no primary neuronal degeneration is taking place. Thus, the changes observed after enucleation of the new-hatched chick are largely related to loss of the primary synapses of the visual pathway.

(d) Uptake by the optic nerve

Homogenates of the optic nerve of the 3-day-old chick took up significant amounts of labeled compounds (Table II). This transport was generally low compared to the uptake capacity of the optic lobe. However, glutamate was taken up by the optic nerve at 43% of the corresponding value for the optic lobe.

DISCUSSION

Since the uptake capacity of optic lobe tissue is very low at 11 days of incubation⁷, much uptake capacity must have developed in the non-innervated optic lobe after eye

TABLE II

High affinity uptake by homogenates of the optic lobe and optic tract of 3-day-old chicks

The concentration of compounds was between 1.0×10^{-8} M and 2.3×10^{-8} M. Tubes contained 0.1 ml of a 5% tissue homogenate except for the GABA study, where a 0.5% homogenate was used. Incubation was for 5 min at 37 °C in oxygenated Krebs-Ringer buffer. For detailed procedure see text. Standard errors of the mean are given.

³ H-compound	pmoles labeled compound accumulated/min/g tissue	
	Optic tract	Optic lobe
DL-Glutamate	6.3 ± 0.2	14.8 ± 0.2
GABA	119 ± 5	840 ± 80
Dopamine	2.1 ± 0.1	12.4 ± 1.2
Choline	2.4 ± 0.1	12.0 ± 1.9
Serotonin	10.5 ± 0.9	80.9 ± 4.6

removal of the 3-day-old embryo. Thus, the development of the bulk of the synapses in the optic tectum may be genetically predetermined and independent of afferent innervation. Since the preceding paper⁷ presents data suggesting that the uptake mechanisms being studied are largely synaptosomal, the observed reduced uptake may be due to a reduction in the total number of synapses. This depression is not selective for any specific neurotransmitter. Complete synaptic induction in the optic lobe requires innervation from the retinal ganglion cells. While early removal of the optic cup causes extensive cell death and hypoplasia⁴, surviving neurons may develop synapses with normal properties of neurotransmitter uptake.

Results of enucleation of the young chick also show failure of normal development and cessation of growth. The effect of denervation is to retard further increase in cell size in the optic lobe while the total cell number remains constant⁴. However, the uptake capacity per lobe develops almost normally for most compounds studied. This results in a greater density of uptake mechanisms in the denervated lobe. After enucleation the usual posthatch increases in dopamine, choline, serotonin uptake take place while the uptake for GABA remains high in the denervated lobe. While the distance between neurons does not increase in the deafferented lobe, most neurons may develop a roughly normal uptake capacity which implies a normal complement of synapses.

Acetylcholine may be the major neurotransmitter in the amphibian optic system¹⁸, but the avian optic nerve is devoid of choline acetyltransferase¹⁹. Enucleation of the chick does not affect the rate of choline uptake by the denervated optic lobe to a greater extent than other compounds examined. Choline acetyltransferase levels in the outer layers of the denervated optic lobe which are directly innervated by the retinal ganglion cells are not reduced 20 days after eye removal²³. Enucleation has a very minor effect on the acetylcholinesterase level of the chick optic lobe²⁴. Thus acetylcholine is not likely to be the major neurotransmitter of the chick optic nerve.

The transport of glutamate was different than that of other compounds in the

denervated optic lobe of the 23-day-old chick. The total uptake of glutamate was only 68 % of that of the control lobe and the density of uptake capacity was also significantly reduced. This anomalous behavior of glutamate could be accounted for if this chemical were the neurotransmitter of the direct afferent fibers from the retinal ganglion cells to the optic tectum. Glutamate has also been suggested as the excitatory neurotransmitter of the pigeon optic nerve²⁰. In the pigeon, 4 weeks after enucleation, these primary axons and synapses have disappeared¹⁵, but transneuronal degeneration may not be well established at this time. The absence of primary terminals within the deafferented tectum may be reflected by the reduced uptake of glutamate by this region.

The role of glutamate is further suggested by the finding that glutamate level is depressed in the denervated optic lobe. Other possible neurotransmitters had very similar levels in experimental and control lobes. However, aspartate is another putative neurotransmitter that is taken up by the same high affinity system that transports glutamate²². Thus, it is not possible to determine the natural substrate of the uptake mechanism by this means.

There is no evidence of glial cell loss or proliferation in the chick optic lobe, 3 weeks after enucleation⁵. Therefore, the reduced glutamate uptake by the denervated optic lobe is unlikely to result from changes in glial number. Since glutamate uptake is relatively high in the optic lobes of the young chick embryo where neuronal but not glial differentiation has occurred⁷, there is probably a considerable neuronal contribution to glutamate uptake.

The data from the amino acid analyzer showed that several compounds, including both glutamic and aspartic acids, were somewhat depressed in the denervated optic lobe (Table I). Since this depression was widespread and did not reach significance, we could not use this approach to distinguish between aspartic and glutamic acids as possible neurotransmitters in the visual path. De Belleruche¹⁶ found endogenous glutamate to be much more concentrated than aspartate in mammalian synaptic vesicles. This suggests glutamate as the more likely preponderant neurotransmitter at least in the mammalian cortex. After eye removal in the frog, the level of glutamate but not of aspartate is considerably reduced in the contralateral optic tectum²⁸. This is also suggestive of the possible role of glutamate as the neurotransmitter of the visual system.

The ability of optic nerve to take up most compounds studied was under 20 % of the corresponding value for optic lobes. However, the optic nerve took up glutamate at 43 % of the rate of the optic lobe. This uptake may be due to axonal or glial elements. Several putative neurotransmitters can apparently be taken up from very dilute solutions by non-synaptic neuronal areas^{1,9,17}. The emphasis on glutamate uptake by the optic nerve is consistent with the concept of glutamate as the major neurotransmitter of the optic pathway in the chick.

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