

XI. NEUROPHYSIOLOGY

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A. SYNTHESIS OF ACETYLCHOLINE AND GAMMA-AMINOBUTYRIC ACID IN THE TECTUM OF THE TIGER SALAMANDER, AMBYSTOMA TIGRINUM

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The tectum of the salamander has a significant but restricted distribution of acetylcholinesterase activity. In previous work¹ we showed histochemically that the activity was coincident with optic-nerve fibers and terminals. The tectum can be divided into two concentric parts: an inner area where the cell bodies are concentrated, and an outer plexiform area free of cell bodies. The optic fibers and terminals and the accompanying AChE activity are restricted to the external half of the outer plexiform area. In other than a few scattered cells of the mesencephalic V nucleus and the walls of blood vessels, the tectum has very low AChE activity. Most investigators have tentatively accepted the fact that the demonstration of AChE activity implies the presence of cholinergic neurons.² Another step, however, toward proving that a set of neurons employs a particular neurotransmitter is to show that they can synthesize the neurotransmitter.

We have used a radiochemical procedure recently detailed by Hildebrand et al.³ that can demonstrate the synthesis of a variety of neurotransmitters using labeled neurotransmitter precursors (choline for acetylcholine, glutamate for GABA, tyrosine for catecholamines and tyramines, tryptophan for serotonin). Given the restricted distribution of AChE, we have looked for additional candidates for tectal neurotransmitters.

Adult tiger salamanders were beheaded, and their brains were quickly removed under aseptic conditions and immersed in an operating medium of L-15 Leibovitz (Grand Island Biological) diluted 20% by 1000 units/ml penicillin-streptomycin. Under a dissecting microscope, the midbrain was excised from the diencephalon and the medulla. The dura was teased away, and the midbrain was cut to separate the dorsal half (tectum) from the ventral half (tegmentum). The midbrain is a cylindrical structure, but dorsal

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was easily distinguished from ventral because pigment granules are present exclusively in the tegmental region.

The tectal slabs were transferred to an incubating medium of 8 parts L-15 deficient in choline, L-glutamine, L-tyrosine, and L-tryptophan; 2 parts water, 1 part fetal calf serum; and 50 $\mu\text{C}/\text{ml}$ of choline-methyl- ^3H chloride alone or paired with L-glutamic- $^3\text{H}(\text{G})$ acid or L-tyrosine-3,5- ^3H or L-tryptophan- $^3\text{H}(\text{G})$ (all radiochemicals from New England Nuclear Corporation). The tissue was incubated 20 min, 1 h, 4 h, and overnight (approximately 16 h) at 21°C , washed several times in unlabeled incubating medium, homogenized in 50 ml of pH 1.9 formic acid/acetic acid buffer and prepared for electrophoresis as described by Hildebrand et al. Samples were variously coelectrophoresed with unlabeled markers of choline chloride, acetyl-choline chloride, GABA, tryptophan, serotonin and noradrenaline. The runs were made at 6 kV for 1.5 h with the origin near the anode. The electrophoresed paper was dried for 1 h at 60°C . The markers were stained as follows: tryptophan and serotonin with Ehrlich's reagent stain for indole derivatives (1/2 gm p-dimethylaminobenzaldehyde in 100 ml 95% ethanol plus 2 ml concentrated HCl). The tryptophan stain was yellowish, the serotonin stain was bright purple.⁴ The noradrenaline, ACh and Ch were stained with iodine spray (1% in acetone); tyrosine, GABA and glutamine with ninhydrin dip (0.2 ninhydrin in acetone, then warmed over a hot plate).

The paper was divided longitudinally into 1-cm strips and cut sequentially. Each strip was placed in an individual scintillation vial containing a toluene-solvent scintillation fluid with 15 gm/gallon 2,5-diphenyloxazole (PPO) and 0.19 gm/gallon 1,4-bis-[2-(5-phenyloxazole)] (POPOP). Counts were made in an LS 250 Beckman Liquid Scintillation System. All labeled precursors were routinely checked separately for purity by electrophoresis.

Tectal tissue incubated for 20 min, or for 1 h, or with the dura intact, showed little or no ACh synthesis. Figure XI-1 shows the results of an overnight incubation in labeled choline. An additional peak for ACh in the incubated tissue clearly demonstrates its synthesis by the tectum.

Having shown ACh synthesis, we screened other potential neurotransmitters by pairing each of the other labeled precursors with labeled choline. Synthesis of ACh would crudely indicate viability of the tectal tissue sample. In this way we found positive results only for GABA. Figure XI-2 shows a subsequent run made after tectal tissue was incubated only in labeled glutamate. A clear peak coincident with GABA is shown.

The negative results for the other potential neurotransmitters do not absolutely preclude them as candidates. It is possible that their synthesis could have a different time course, or depend more closely on the integrity of the tectum because either they are synthesized elsewhere than in the tectum or synthesis is more sensitive to the trauma caused by the operative techniques, or the levels of synthesis are below the

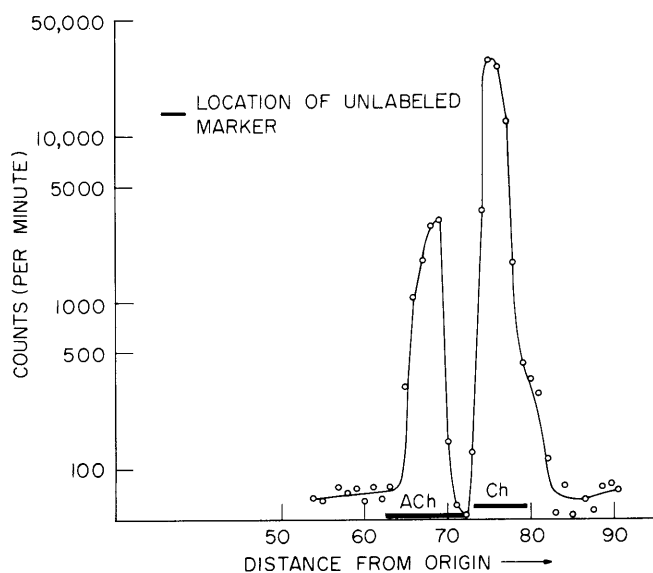


Fig. XI-1. Tectum incubated overnight in ^3H -choline. Radiochemical analysis of electrophoretogram with scintillation counts of paper cut in 1-cm strips. Peaks were found for both Ch and ACh.

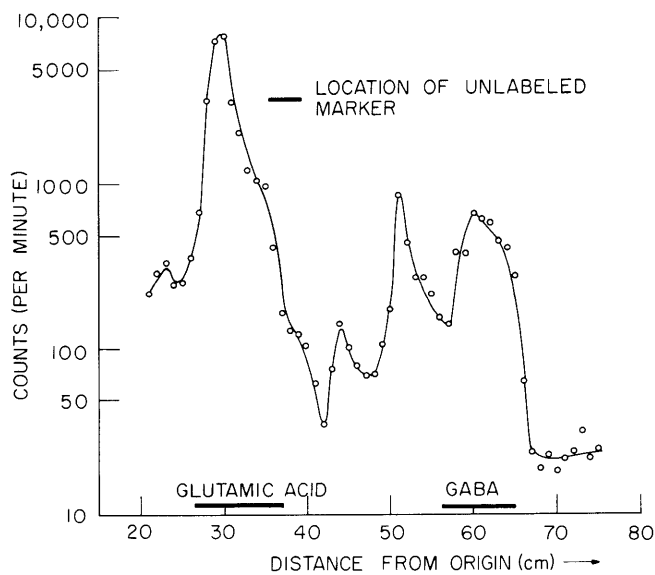


Fig. XI-2. Tectum incubated overnight in ^3H -glutamic acid. Radiochemical analysis as in Fig. XI-1. Peaks were found for glutamic acid and GABA.

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resolution of the technique that we employed.

The positive results do not of themselves prove that ACh and GABA are neurotransmitters. Ultimately, it must be shown that these substances are released by nerve-fiber terminals in response to their electrical activity and have an electrical effect on the postsynaptic membrane.

References

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