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HISTOCHEMICAL CORRELATION BETWEEN ACETYLCHOLINESTERASE ACTIVITY AND LIMB FUNCTION IN METAMORPHOSING TADPOLES

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ABSTRACT

C. Y. Chioü and H. M. Liang (1970) *Histochemical Correlation Between Acetylcholinesterase Activity and Limb Function in Metamorphosing Tadpoles*. Bull. Inst. Zool, Academia Sinica 9(1):7-14. A histochemical method was used for investigating the correlation between changes of acetylcholinesterase (AChE) distribution in brachial and lumbar enlargements and development of limb function in metamorphosing tadpoles. The results show that the enzyme activity was mild and diffused in the whole mantle layer before stage V; after that, AChE localization was gradually realized in the following order: ventro-lateral motor column, interneurons and dorsal roots. Enzyme differentiation was finally completed at stage XXV when froglets were formed. There was no appreciable difference in enzyme development between brachial and lumbar enlargements. This was because forelimbs and hindlimbs begin their functional locomotion simultaneously at stage XX. Enzyme differentiation in the spinal cord appears thus to be correlated temporarily with the functional development of the limbs.

Acetylcholinesterase (AChE) is an important enzyme for the maintainence of neuron function in both adults and developing systems. It was found that when the peripheral branch of a rabbit spinal nerve was severed, AChE activity in the sectioned fibers disappeared together with chromatolysis; however, immediate suture after the cut restored enzyme activity (5). Motor nerve fibers were demonstrated to maintain development and activity of AChE in muscle-tendon junctions of new born rats (12).

Limb ablation in Xenopus larvae was shown to stop differentiation of neuroblasts in the ventral horn; the already differentiated ones started to degenerate. All this coincided with the disappearance of AChE (6). In other amphibian larvae, demonstration of a close correlation between increase of AChE content and advance of behavior activity was reported (20, 21, 4, 26). In chick embryos, the enzyme was found during early developing stage (13, 27). In sheep fetuses, development of AChE was closely related to brain and spinal cord function (14). In vitamin-E-deficient rat fetuses, abnormal central nervous system was shown to be associated with a decrease of AChE activity (25). Thus it is eminent that in most vertebrate embryos, AChE is closely related to the developing central nervous system.

Before an organ functions, AChE must be present in strategic areas and the

neurons must be able to synthesize, store and transport the enzyme. This is because when nerve impulse starts, the chemical conductor, acetylcholine, must be hydrolysed and removed. In a developing system, different organs may function simultaneously or at different times, and development of AChE varies *pari passu*. The present study analysed histochemically the correlation between changes of the enzyme activity in brachial and lumbar enlargements and functional development of the forelimbs and hindlimbs in metamorphosing tadpoles.

MATERIALS AND METHODS

1. Preparation of spinal cord sections

Fertilized eggs of *Rana catesbeiana* were obtained from induced breeding by pituitary injection. Eight metamorphosing tadpoles each of Taylor and Kollros stages V, X, XV, XX and XXV (24) were used. Their spinal cords were dissected without anesthesia and fixed in formalin-sucrose-ammonia fixative (16) for 4 hours at 4°C, followed by washing with distilled water for 15 min. Two mm pieces of brachial and lumbar enlargements were incubated in the substrate medium for 6 hours at 22°C. The segments were washed twice again with distilled water and stained in block with dilute ammonium sulfide for 15 min. The segments were washed for a third time, then embedded in paraffin. Serial cross sections, 15 μ thick, were made for microscopic examination.

The substrate medium for the study of AChE was designed by Koelle and Friedenwald (9), later modified by Strumia and Bollone (22). However, the method is not satisfactory owing to the presence of precipitate and poor staining reaction. Thus it was further modified for this study as follows:

Acetylthiocholine iodide	5.6 mg
Buffered sodium acetate-acetic acid, pH. 6	2.5 ml
Glycocoll 3.75%	0.1 ml
Copper sulfate 0.1 M	0.4 ml
Distilled water	2.0 ml

The above ingredients were placed in a 10 ml micro-staining jar one by one in the order listed. Stirring was necessary after each addition. The resultant solution was clear and sky blue in color.

Control-sections were stained in this solution after adding 10^{-5} M of BW 284c51, a specific inhibitor of AChE (1), to the substrate medium. This is to prove that the dark brown deposit (copper sulfide) in the tissue results from a reaction between copper thiocholine, a product of hydrolysis of the iodide by AChE, and ammonium sulfide.

2. Measurement of neuron number and nuclear diameter

Cells in the ventro-lateral motor columns of both sides were counted in every other section under high power, totalling 50 sections each of the brachial and lumbar enlargements of stages XX and XXV. The diameter of motor neurons was measured also in every other section under high power with the aid of an occular micrometer. A total of 150 nuclei in stage XX and XXV, so measured, was finally calibrated by a known scale.

RESULTS

I. Development of AChE activity

The final product of dark brown deposit of CuS_2 in the tissue indicated the presence AChE. When the enzyme inhibitor, BW284c51, was added to the incubation medium, no such colored deposit resulted (Fig. 1). The validity of this localization of the enzyme was used as a basis for studying the development of the enzyme activity. The following progressive steps in this development were recognized:

1. Stage V—The distribution of AChE in brachial and lumbar enlargements was

similar: a mild and diffused deposition of the colored CuS_2 in the mantle layer only. However, the ventro-mesial motor neurons were distinguistable from the rest of the mantle layer by a more intensified color (Fig. 2).

2. Stage X—The pattern of enzyme distribution remained almost the same as that of the previous stage, except some colored motor neurons in the ventro-lateral area showed a tendency to protrude into the marginal layer, amply indicating an early concentration of the enzyme in this area.

3. Stage XV—AChE activity definitely appeared in the ventro-lateral motor neurons which protruded into the white matter (Figs. 3, 4). The cells of this column were large, with their nuclei and cytoplasm colored to the same extent but processes unstained (Fig. 4). The dorsal part of the white matter showed a diffused and light colored reaction (Fig. 3).

Stage XX—During this metamorphic climax AChE activity had greatly increased. The enzyme was clearly localized in ventro-lateral and ventro-mesial motor columns (Fig. 5). The lateral cells were definitely larger than the mesial ones. The differentiation of the motor neurons appeared approaching the final stage as their cytoplasm and processes were both fully loaded with the colored deposit while the nucleus was generally negative with only a dark dot in its center (Fig. 6). The dorsal roots also showed positive reactions (Fig. 5). Two branches showed AChE activity in both dorsal roots of the lumbar enlargement while only one in the brachial enlargement. Some positive reaction began to show up also in the white matter as dark radiating lines (Fig. 5).

5. Stage XXV—The development of enzyme activity was at its peak in this stage when froglets were formed (Fig. 7). Colored deposition in the motor neurons was darker than ever before; cell processes showed more details and the central positive dots in nuclei had disappeared (Fig. 8). Two branches in the dorsal root of the brachial enlargement were discernible, one along the mesial and the other along the lateral side of the root. The radial arrangement of enzyme distribution was more pronounced now.

II. Data on measurement

Table 1. Number of ventro-lateral motor neurons per section and their nuclear diameter

Stage	XX		XXV	
Enlargement	Brachial	Lumbar	Brachial	Lumbar
Number of neurons	13.51±3.33*	9.65±2.25	10.10±1.63	7.94±1.80
Diameter of nuclei (μ)	3.81±0.43	4.04±0.46	3.68±0.46	4.39±0.61

*denotes standard deviation

The development of AChE activity in the spinal cord of metamorphosing tadpoles observed by the present histochemical method was summarized as follows: The enzyme was at first distributed faintly and evenly in the mantle layer of both brachial and lumbar enlargements prior to stage V. AChE began to localize in ventro-lateral motor neurons beginning from stage XV. At stage XX differentiation of the enzyme distribution was about maximal. At that stage definite localization occurred not only

in the cell body but also in the processes of the ventro-lateral motor neurons. Positive enzyme activity was observed in the dorsal roots. At stage XXV, enzyme activity in the motor columns was completely differentiated, the positive reaction being strongest in the motor columns and entrance of dorsal roots, intermediate in other parts of the gray matter. No essential difference in enzyme development was observed between brachial and lumbar enlargements.

DISCUSSION

It is well known that cells in the mantle layer of early vertebrate embryo are the undifferentiated neuroblasts with a high mitotic activity. Therefore, at this stage, the distribution of AChE in the cord was confined to the mantle layer, and represented a diffuse and mild form.

During metamorphosis, cytological differentiation of the ventro-lateral motor neurons takes place (2). This differentiation probably depends on thyroxin concentration. The I^{131} uptake of tadpole thyroid gland was found to be weak before stage XII; the capacity gradually increased and reached its peak at stage XX (8). Thus, it appears that the present finding of the beginning of enzyme concentration in ventro-lateral motor neurons at stage XV and the almost complete enzyme differentiation at stage XX was in accord with this increasing concentration of thyroxin.

Experimentally, exogeneous thyroxin was found also capable of exerting an effect to increase the size of ventro-lateral motor neurons and to reduce the neuron number in tadpoles (3, 10, 17). The phenomenon is considered a normal process in motor neuron development; the result of this study histochemically substantiates it.

The results of limb ablation experiments in amphibians showed that ventro-lateral motor columns in brachial and lumbar enlargements controlled movements of forelimbs and hindlimbs respectively (2, 3, 19, 18). In this study, no appreciable difference in enzyme differentiation of ventro-lateral motor columns was observed between brachial and lumbar enlargements, although forelimbs emerge very much later than hindlimbs. This was because forelimbs and hindlimbs are functionally comparable. Forelimbs begin its growth under the skin at stage III and protrude from the body at stage XX while hindlimbs are outside the body during all that time. The latter, however, do not begin their effective movements before stage XVIII (23). Apparently, forelimbs and hindlimbs initiate their movements at about the same time to effect a well coordinated action. For this reason, enzyme differentiation of the ventro-lateral motor neurons in the brachial and lumbar enlargements was about the same.

On the other hand, enzyme localization in the dorsal root began earlier in lumbar than in brachial spinal cord. The difference is probably due to the fact that hindlimbs come into contact with the environment earlier. Since sensory activity in the hindlimbs is initiated earlier, so is the localization of AChE earlier in the lumbar than in the brachial enlargement.

That the enzyme localization of the mesial branch of the dorsal root appeared later (stage XXV) than the lateral branch (Stage XX) is a new and significant finding. It is known that the dorsal roots of amphibians enter the spinal cord by two branches: a major mesial one, passing into the dorsal white column, where it sends ascending and desending branches and also some collaterals into the gray matter; a small lateral one, passing into the dorsal lateral zone of the white matter and entering the tips of the dorsal horn (7, 15). The lateral branch in the gray matter sends connections to the ventro-lateral motor column of the same segment through the interneurons (11), while the mesial branch

CROSS SECTIONS OF THE SPINAL CORD OF *RANA CATESBEIANA* TADPOLES

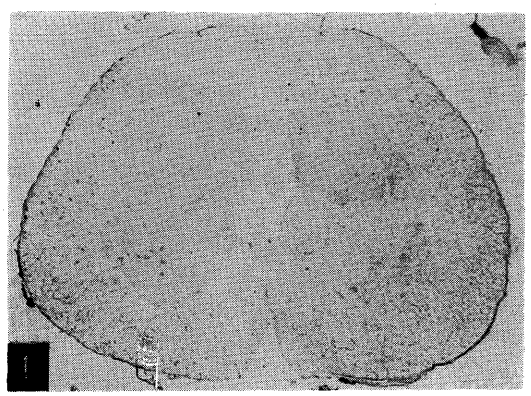

Fig. 1. Brachial enlargement, stage XXV, control section, ×260.

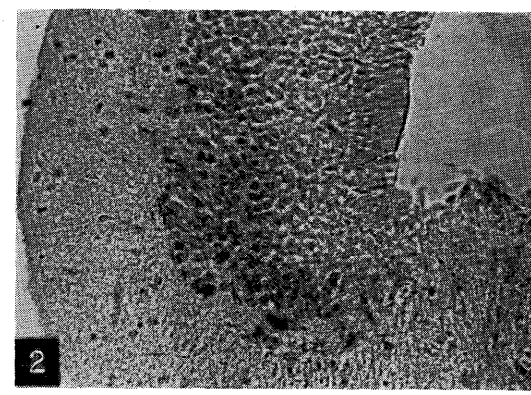

Fig. 2. Ventro-lateral motor column of lumbar enlargement, stage V, ×550.

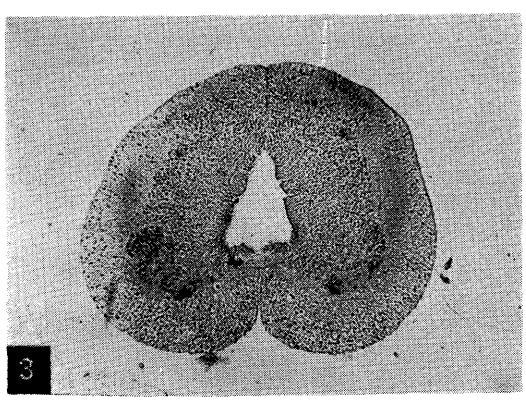

Fig. 3. Brachial enlargement, stage XV, ×260.

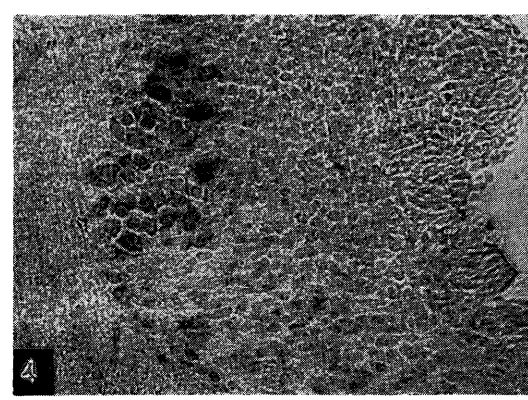

Fig. 4. Ventro-lateral motor column of lumbar enlargement, stage XV, ×550.

CROSS SECTIONS OF THE SPINAL CORD OF *RANA CATESBEIANA* TADPOLES

(Continued)

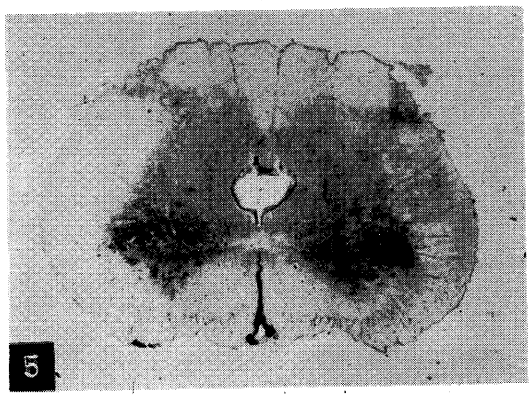

Fig. 5. Brachial enlargement, stage XX, ×260.

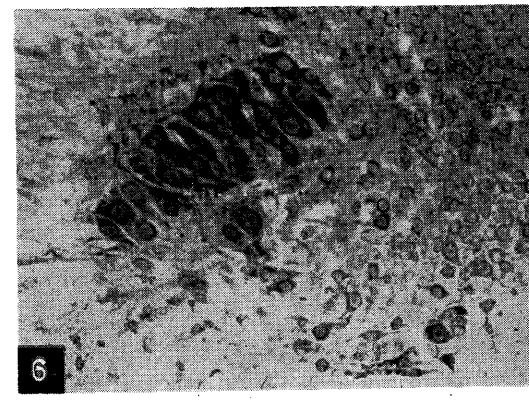

Fig. 6. Ventro-lateral motor column of brachial enlargement, stage XX, ×550.

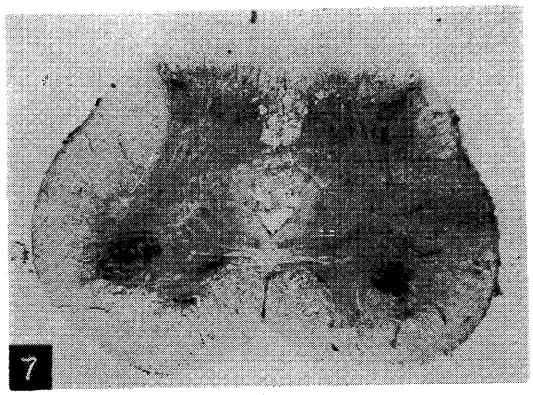

Fig. 7. Lumbar enlargement, stage XXV, ×260.

Fig. 8. Ventro-lateral motor column of lumbar enlargement, stage XXV, ×550.

conncets mainly intersegmentally in the white column (15, 11). The progressive development of enzyme localization correlated with morphological tracings in the two branches of the dorsal root as indicated by the present finding possibly means a temporal development of the function of intra-and intersegmental reflex arcs.

The ventro-lateral motor neurons in amphibians send out their dendrites to the white matter. This explains the presence of radiating lines of positive enzyme activity in this area.

The tendency of decrease of cell number in ventro-lateral motor columns in both brachial and lumbar enlargements of the spinal cord during metamorphic climax from stage XX to stage XXV observed in this study confirmed that of an earlier study (2).

The nuclear size of ventro-lateral motor neurons in the lumbar enlargement, according to our finding, was larger than that in brachial enlargement at both stage XX and stage XXV. The difference may be accounted by the fact that the axons in the hindlimbs are longer than those of forelimbs, for there is usually a high correlation between nuclear size and cell size.

CONCLUSION

1. Although there is a temporal difference in morphological development between forelimbs and hindlimbs, their functional development begins simultaneously at stage XX to coincide with an active movement in both of them. Correlating with this functional development, the differentiation of AChE in ventro-lateral motor columns in brachial and lumbar enlargements started at stage V and almost reached its peak at stage XX.

2. The enzyme development in central nervous system was found to occur sequentially in this order: ventro-lateral motor neurons, processes of sensory neurons entering the lateral branch of the dorsal root, dendrites of ventrolateral motor neurons, interneurons in the gray matter between dorsal and ventral horns and lastly the mesial branch of the dorsal root. Such an order may mean that, for limb movement, intrasegmental reflex arc developed earlier than intersegmental arc, which, in general, agrees with the principle of development, i.e., from simple to more complex.

3. The cytological differentiation of ventro-lateral motor neurons depends on thyroxin and so did development of AChE in the neurons. The enzyme began to show its activity in the cell body, both cytoplasm and nucleus exibiting the same color reaction. The activity then disappeared in the nucleus, leaving a single dot in its center to mark the position of the nucleolus. Finally, when cell processes were formed the enzyme followed their course while the central positive dot in the nucleus disappeared. The resulting pattern was clearly a positive enzyme activity in the cytoplasm and the processes, but a negative one in the entire nucleus.

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