TIME OF ORIGIN OF THE NEUROBLASTS IN THE NEURAL
TUBE OF THE CHICK EMBRYO DETERMINED BY
HISTOCHEMICAL OBSERVATION OF ACETYL—
CHOLINESTERASE ACTIVITY

AKINORI MIKI, YASURO ATOJI AND HUMIO MIZOGUTI

*Department of Anatomy, Kobe University School of Medicine,
Kusunoki-cho 7-5-1, Chuo-ku, Kobe 650*

Received for publication June 2, 1981

The time of origin of the neuroblasts in the neural tube of the chick embryo was determined by histochemical observation of acetylcholinesterase (AChE) activity.

In the central nervous system, the neuroblasts appeared first in the caudal half of the rhombencephalon of the 1.5-day chick embryo (13-somite, stage 11). They were round medium-sized cells and located in both the ventral and the dorsal halves of the wall of the neural tube. In the cervical, thoracic and lumbosacral neural tubes, the neuroblasts first appeared at stage 12, 14 and 17 respectively. At these levels, they were large and located in the ventral half of the wall of the neural tube. Occasionally they provided an axon which extended to the ventrolateral edge of the neural tube.

The time of origin of the neuroblasts in the neural tube of the chick embryo is more accurately determined by histochemical observation of AChE activity than by ^3H -thymidine autoradiography. The time difference between the actual neuroblastic differentiation and the appearance of AChE moderately positive cells is much smaller than that between the actual neuroblastic differentiation and the differentiation determined by ^3H -thymidine autoradiography.

So far the time of origin of the neuroblasts in the neural tube of the chick embryo has been determined exclusively by means of autoradiography using ^3H -thymidine. Some authors (Fujita, 1964; Martin and Langman, 1965; Langman and Haden, 1970; Hollyday and Hamburger, 1977) regarded the unlabeled cells on the autoradiogram as neuroblasts and others (LaVail and Cowan, 1971; Kanemitsu, 1977; McConnell and Sechrist, 1980) the heavily labeled ones as neuroblasts. The time when ^3H -thymidine was administered was considered as the time when these unlabeled or heavily labeled cells differentiated from the neuroepithelial cells to the neuroblasts.

According to Fujita (1963), the neuroblastic differentiation of a newly formed daughter cell is determined within the ependymal layer (matrix) while the cell is descending, and the neuroblastic differentiation becomes recognizable after the cell migrates out from the ependymal layer. Correctly speaking, at the time of administration of ^3H -thymidine the unlabeled cells should be regarded as the cells which had completed the final phase of DNA synthesis, and the heavily labeled ones should be regarded as the cells which started the final phase of DNA synthesis. Then, it

follows that neither unlabeled nor heavily labeled cells differentiated from the neuroepithelial cells to the neuroblasts when ^3H -thymidine was administered. As stated by Fujita (1964), the interval between the final DNA synthesis and the determination of differentiation is the possible difference between the actual date of the differentiation and that determined from the final DNA synthesis.

On the other hand, acetylcholinesterase (AChE) appears at the very early embryonic stage in the tissues of the nervous system and increases in activity and distribution with the progress of development. Thus, the relationship between the enzymatic activity and the neuronal differentiation has received attention for a long time, and several opinions have been proposed by many authors, i.e., (1) The appearance of AChE activity in the cells constituting the neural tube can be viewed as an indication of the neuroblastic differentiation of the neuroepithelial cells (Tennyson and Brzin, 1970; Pannese *et al.*, 1971). (2) The increase of AChE activity is a reflection of the differentiation and growth of the neuroblasts (Strumia and Baima-Bollone, 1964; Burt, 1968). (3) There is a close relationship between the morphological differentiation of synapses and the increase of AChE activity (Shen, 1958; Turbow and Burkhalter, 1968; Eränkö, 1972; Kim *et al.*, 1975; Kim, 1976).

Recently, using ^3H -thymidine autoradiography and histochemical observation of AChE activity on the same sections (Miki *et al.*, 1981a), we have investigated the relationship between the proliferating ability and the AChE activity of the neural tube cells in the early chick embryos and have reported that (1) as the cells showing AChE activity only along the nuclear membrane have the proliferating ability, they are regarded as undifferentiated neuroepithelial cells; (2) the cells showing AChE activity not only along the nuclear membrane but also in the cytoplasm no longer have the proliferating ability and they are regarded as neuroblasts; (3) especially, the elongated spindle-shaped cells located in the ependymal layer (matrix), which show AChE activity along the nuclear membrane as well as in the cytoplasm, are regarded as the cells descending from the ependymal layer after the last division, i.e., newly formed neuroblasts (Miki *et al.*, 1981b). These results suggest that the increase of AChE activity in the cells constituting the neural tube, particularly in the cytoplasm, is one of the signs which characterize the neuronal differentiation.

In the present study, the time of origin of the neuroblasts in the neural tube of the chick embryo was determined by the histochemical observation of AChE activity. The results of the histochemical observation were compared with those of the conventional autoradiographic study using ^3H -thymidine.

MATERIALS AND METHODS

Fertilized white Leghorn eggs were artificially incubated at 37°C in a humid atmosphere and chick embryos were staged carefully according to the stage series of Hamburger and Hamilton (1951). A total of 64 chick embryos at various stages were used in this study. They were fixed in 4% paraformaldehyde dissolved in a phosphate buffer (pH 7.2) of Millonig (1962) for 12 hr at 4°C and washed in the same buffer for 12 hr.

Unfrozen sections of $100\ \mu\text{m}$ were cut with a vibratome and incubated in the medium containing acetylthiocholine iodide as substrate and $10^{-5}\ \text{M}$ iso-OMPA

(tetra-isopropyl pyrophospholamide) as an inhibitor of non-specific cholinesterase for 2 hr at 37°C according to the method described by Karnovsky and Roots (1964). After washing two or three times in the phosphate buffer, without postfixation with osmium tetroxide solution, they were dehydrated and embedded in an epoxy resin mixture (Quetol 812) as usual.

Semithin sections, 1 to 2 μm thick, were prepared and examined with an ordinary light microscope.

Histochemical controls were made as follows: (1) incubation in a substrate-free medium; (2) incubation in a medium containing 10^{-5} M eserine (physostigmine), an inhibitor of cholinesterases; (3) incubation in a medium containing 10^{-4} M DFP (di-isopropyl phosphofluoridate); (4) incubation in a medium containing 10^{-4} M BW284-C51 (1,5-bis-(4-allyldimethyl-ammoniumphenyl)-penta-3-one dibromide), an inhibitor of acetylcholinesterase.

Definition of the levels of the neural tube.

The somites appear first at the level of the utmost upper end of the spinal cord and these somites are expressed as S_1 . The somites caudal to S_1 are referred to as S_2 , S_3 and so on successively. The levels of the neural tube are defined in this paper as follows: rhombencephalon, cervical tube (S_1 to S_7), thoracic tube (S_8 to S_{19}) and lumbosacral tube (caudal to S_{19}).

RESULTS

(1) AChE activity in the neural tube of the early chick embryo

Light microscopically, the reaction products showing AChE activity were observed as fine brown granules. In the early chick embryos, the reaction products were detected not only in the neural tube cells but in the cells of the somite, neural crest, notochord and surface ectoderm. But in the controls, no reaction products were found in any sections. Therefore, it can be concluded that these reaction products are due to AChE activity.

In the present study, AChE activity was found, though weakly, along the nuclear membrane of the cells constituting the neural tube of the 4-somite (stage 8), 9-somite (stage 9) and 10-somite (stage 10) chick embryos. In these early chick embryos (up to 1.5-day of incubation), the neural tube was exclusively composed of AChE weakly positive cells.

After day 2 of incubation, the cells constituting the neural tube are classified into three groups according to the intensity and distribution of AChE activity (Figs. 2, 3) as reported in our previous study (Miki *et al.*, 1981b). Cells in the first group show AChE activity only along the nuclear membrane. They are elongated fusiform and their nuclei are seen in the ependymal layer (matrix). Cells in the second group show AChE activity not only along the nuclear membrane but also in the cytoplasm. They are also fusiform and their nuclei are located in the ependymal layer. Cells in the third group show intense AChE activity along the nuclear membrane and in the cytoplasm. They are round and located near the basement membrane or in the mantle layer. In this paper, the cells of the first, second and third groups are referred to as AChE weakly positive, moderately positive and intensely positive cells respectively, as in our previous study (Miki *et al.*, 1981b).

TABLE 1. *Distribution of the AChE moderately positive and intensely positive cells in the neural tube of chick embryos.*

TABLE 1-1.

Number of Somite	H-H Stage	Day	Rh	C		Th		L-S
				Upper half	Lower half	Upper half	Lower half	
4	8	1.0	—	—	—	—	—	—
9	9		—	—	—	—	—	—
10	10		—	—	—	—	—	—
11	10		—	—	—	—	—	—
13	11	1.5	+	—	—	—	—	—
15	11	1.5	—	—	—	—	—	—
15	11	1.5	+	—	—	—	—	—
15	11	1.5	+	—	—	—	—	—
16	12		+	—	—	—	—	—
17	12		+	—	—	—	—	—
17	12		+	—	—	—	—	—
17	12		++	—	—	—	—	—
18	12		++	+	—	—	—	—
18	12		+	+	—	—	—	—
19	13	2.0	++	+	—	—	—	—
19	13	2.0	++	+	—	—	—	—
20	13	2.0	++	+	—	—	—	—
20	13	2.0	++	++	—	—	—	—
20	13	2.0	++	+	—	—	—	—
21	13	2.0	++	++	—	—	—	—
21	13	2.0	++	++	+	—	—	—
21	13	2.0	++	++	—	—	—	—
22	14		++	++	+	—	—	—
22	14		++	++	+	+	—	—
22	14		++	++	+	—	—	—
22	14		++	++	+	+	—	—
23	14		++	++	+	+	—	—
23	14		++	++	++	+	—	—
25	15	2.5	++	++	++	+	—	—
25	15	2.5	++	++	++	+	—	—
25	15	2.5	++	++	++	++	—	—
25	15	2.5	++	++	++	+	—	—

TABLE 1-2.

Number of Somite	H-H Stage	Day	Rh	C		Th		L-S
				Upper half	Lower half	Upper half	Lower half	
26	15	2.5	++	++	++	+	—	—
26	15	2.5	++	++	++	++	—	—
27	15	2.5	++	++	++	+	—	—
27	15	2.5	++	++	++	++	—	—
28	16		++	++	++	++	—	—
30	16		++	++	++	++	+	—
30	16		++	++	++	++	+	+
31	17		++	++	++	++	+	+
31	17		++	++	++	++	+	—
32	17		++	++	++	++	++	+
33	17		++	++	++	++	++	+
34	18	3.0	++	++	++	++	++	+
34	18	3.0	++	++	++	++	++	++
34	18	3.0	++	++	++	++	++	++
35	18	3.0	++	++	++	++	++	++
35	18	3.0	++	++	++	++	++	++
35	18	3.0	++	++	++	++	++	++
35	18	3.0	++	++	++	++	++	++
35	18	3.0	++	++	++	++	++	++
37	19		++	++	++	++	++	++
38	19		++	++	++	++	++	++
39	19		++	++	++	++	++	++
40	20	3.5	++	++	++	++	++	++
40	20	3.5	++	++	++	++	++	++
41	20	3.5	++	++	++	++	++	++
41	20	3.5	++	++	++	++	++	++
41	20	3.5	++	++	++	++	++	++
41	20	3.5	++	++	++	++	++	++
—	23	4.0	++	++	++	++	++	++
—	23	4.0	++	++	++	++	++	++
—	23	4.0	++	++	++	++	++	++
—	23	4.0	++	++	++	++	++	++

The number of AChE moderately positive and/or AChE intensely positive cells is rated on a three-point scale: —=none, +=1 to 5, ++=6 or more. H-H stage: stage series of Hamburger and Hamilton (1951), Day: incubation day, Rh: rhombencephalon, C: cervical neural tube, Th: thoracic neural tube, L-S: lumbosacral neural tube.

(2) Distribution of the AChE moderately positive and intensely positive cells in the neural tube of the chick embryo

The time of the appearance of the AChE moderately positive and intensely positive cells was examined at various levels of the neural tube. The results are presented in Table 1.

The AChE moderately positive and intensely positive cells were first detected

FIG. 1. AChE activity in the neural tube in the lower half of the rhombencephalon. 1.5-day chick embryo (13-somite, stage 11). $\times 320$ The wall of the neural tube is composed predominantly of AChE weakly positive cells. In the basal and alar plates, two AChE intensely positive cells (large arrows) are seen. They are round and smaller than the AChE intensely positive cells shown in Figs. 2 and 3.

FIG. 2. AChE activity in the neural tube at the upper half of the cervical level. 2-day chick embryo (20-somite, stage 13). $\times 200$ Almost all the cells in the neural tube are AChE weakly positive cells (small arrows). An AChE intensely positive cell (small double arrow) is seen near the basement membrane in the basal plate. It is large and provides an axon which extends to the ventrolateral edge of the neural tube. Many AChE intensely positive cells showing the enzymatic activity along the nuclear membrane and in the cytoplasm are also detected in the myotomes (M).

FIG. 3. AChE activity in the neural tube at the upper half of the cervical level. 2-day chick embryo (23-somite, stage 14). $\times 200$ In the neural tube, three kinds of cells, AChE weakly positive cells (small arrows), AChE moderately positive cell (large arrow) and AChE intensely positive cells (small double arrows) are seen.

TABLE 2. *Appearance time of the AChE moderately positive and intensely positive cells at the different levels of the neural tube of the chick embryo.*

Level of the neural tube		H-H Stage
Rh	Otic vesicle	12
	Lower half	11
C	Upper half	12 to 13
	Lower half	14
Th	Upper half	14 to 15
	Lower half	16 to 16
L-S		17 to 18

in the lower half of the rhombencephalon of the 1.5-day chick embryo (13-somite, stage 11) (Fig. 1). They were round medium-sized cells and located in the ventral and dorsal halves of the neural tube wall. In the 16-somite embryo (stage 12), they were also found at the level of the otic vesicle in the rhombencephalon. In the 2-day embryos (19- to 21-somite, stage 13), these cells were found not only in the rhombencephalon but also in the upper half of the cervical neural tube (Fig. 2). But at this stage, neither AChE moderately positive nor intensely positive cells were detected in the neural tube lower than the caudal half of the cervical level. The stages when the AChE moderately positive or intensely positive cells appeared first at different levels of the neural tube are shown in Table 2. At the lower half of the cervical neural tube, upper and lower halves of the thoracic neural tube and lumbosacral neural tube, these cells were first found at stages 14, 14 to 15, 15 to 16 and 17 to 18, respectively. In the spinal cord, the AChE moderately positive and intensely positive cells were first observed in the ventral half of the neural tube (basal plate) (Fig. 2). Generally, they were large and round, and occasionally provided an axon which extended to the ventrolateral edge of the neural tube. In the subsequent stages, these cells were detectable not only in the basal plate but in the alar plate or in the intermediate zone of the neural tube.

The distribution of AChE activity in the myotomes was also examined. At the early stages when neither AChE moderately positive nor intensely positive cells were detected in the neural tube, the myotomes were composed exclusively of the cells which showed low AChE activity only along the nuclear membrane. However, when a few AChE moderately positive or intensely positive cells appeared in the neural tube, the cells showing high AChE activity along the nuclear membrane and in the cytoplasm were found also in the myotomes at the corresponding levels (Fig. 2).

DISCUSSION

In our present study, AChE activity was detected light microscopically in the cells constituting the neural tube of the 4-somite (stage 8), 9-somite (stage 9) and

10-somite (stage 10) chick embryos. In these early embryos (up to 1.5-day of incubation), all the cells but a few in mitosis were spindle-shaped and exhibited AChE activity only along the nuclear membrane. At these stages, the wall of the neural tube was composed exclusively of the AChE weakly positive cells.

Recently, using a method of performing ^3H -thymidine autoradiography and histochemical observation of AChE activity on the same sections (Miki *et al.*, 1981a), we have investigated the relationship between the proliferating ability and AChE activity of the neural tube cells in the early chick embryos and reported that as the AChE weakly positive cells were labeled with ^3H -thymidine, they should be regarded as undifferentiated neuroepithelial cells (Miki *et al.*, 1981b). Previously, we also reported as a result of electron microscopic study that the cells showing AChE activity only in the cisterns of the nuclear envelope also should be regarded as undifferentiated neuroepithelial cells according to their morphological characteristics (Miki, 1981). Our findings were in accordance with the report of Zacks (1954) that AChE activity might appear before the morphological differentiation of the neuroblasts.

In the present study, AChE activity was detected in the neural tube cells as well as in the cells of the somite, neural crest, notochord and surface ectoderm as in our previous study (Miki *et al.*, 1981b). In the controls, however, no reaction products were detected in any sections. Therefore, it is evident that the reaction products indicate AChE activity. Previously, we demonstrated, by electron microscopic examination, AChE activity not only in the neural tube cells but also in the cells of the somite, neural crest, notochord and surface ectoderm (Miki, 1981). Other authors also reported the presence of AChE activity in these tissues in chick embryos (Zacks, 1954; Kussäther *et al.*, 1967; Usadel *et al.*, 1967; Drews, 1975) or in rabbit embryos (Tennyson *et al.*, 1968; Slotwiner and Tennyson, 1969; Hagopian *et al.*, 1970; Tennyson and Brzin, 1970; Tennyson *et al.*, 1971).

Based on the autoradiographic studies, Fujita (1963, 1964) schematically demonstrated the mitotic cycle of the matrix cell (neuroepithelial cell) and the differentiation of the neuroblast, and divided the generation time (T_g) into four phases: the DNA synthesis time (T_s), premitotic resting time (T_2), mitotic time (T_m) and postmitotic resting time (T_1). Fujita (1963) reported that the neuroblastic differentiation of a newly formed daughter cells is determined within the ependymal layer (matrix) while the cell is descending and the neuroblastic differentiation becomes recognizable after the cell migrates out from the ependymal layer. Therefore, if the unlabeled cells were regarded as neuroblasts and the time when ^3H -thymidine was administered as the time of the differentiation of the neuroepithelial cells to the neuroblasts, then, as Fujita (1964) stated, the interval between the end of the final DNA synthesis and the determination of the neuroblasts is possible discrepancy between the actual date of the differentiation and that determined from the final DNA synthesis. This discrepancy is between $(T_2 + T_m)$ and $(T_2 + T_m + T_1)$ and maximal range $(T_2 + T_m + T_1)$ is equal to the time $(T_g - T_s)$ (Fujita, 1964). When the heavily labeled cells were regarded as neuroblasts, the possible discrepancy may be between $(T_s + T_2 + T_m)$ and $(T_s + T_2 + T_m + T_1)$ and the maximal range $(T_s + T_2 + T_m + T_1)$ is equal to the generation time (T_g).

Previously, we reported that (a) since AChE weakly positive cells were labeled with ^3H -thymidine and the percentage of the labeled cell increased approximately

linearly with time, they should be regarded as undifferentiated neuroepithelial cells; (b) since AChE moderately positive cells were not labeled up to four hr and were first labeled at eight hr after the administration of ^3H -thymidine, they should be considered to have no longer the proliferating ability and therefore should be regarded as the cells which had passed through the final division and were descending the ependymal layer to form the mantle layer, i.e. newly formed neuroblasts; (c) AChE intensely positive cells were not labeled and were identical to the neuroblasts identified by ^3H -thymidine autoradiography (Miki *et al.*, 1981b).

According to our observations, the AChE moderately positive cells are the cells in the postmitotic resting time (T_1). Thus the difference in time between the actual differentiation to the neuroblasts and the appearance of the AChE moderately positive cells is the period of the postmitotic resting time (T_1) at the longest. In view of these results, the time of appearance of AChE moderately positive and/or intensely positive cells in the neural tube can be regarded as the time of origin of the neuroblasts. The time of origin of the neuroblasts can be determined more accurately by our histochemical method than by the autoradiographic study using ^3H -thymidine.

In the present study, the first neuroblasts in the central nervous system were detected in the lower half of the rhombencephalon of the 1.5-day chick embryo (13-somite, stage 11). They were medium-sized cells and located in both the ventral and the dorsal halves of the wall of the neural tube. Some authors carried out autoradiographic studies using ^3H -thymidine and reported that the first neurons in the central nervous system appeared in the lower half of the rhombencephalon (Sechrist, 1975; Fujita, 1977; McConnell and Sechrist, 1980). As to the region in which the first neurons appeared, our observations were in agreement with their reports. But as to the time when the first neurons appeared, Fujita (1977) stated that the first neurons appeared in the latter half of the second day of incubation. McConnell and Sechrist (1980) reported that some of the earliest neurons start their final DNA synthesis before twenty hr of incubation (Head process, stage 5 of Hamburger and Hamilton: 1951). As Fujita (1977) expressed the time of origin of neurons as days of incubation instead of the stage series of Hamburger and Hamilton (1951), his data cannot be strictly compared with ours. The disparity in time between the results of McConnell and Sechrist (1980) and ours is much larger than that due to differences in experimental methods.

The present study showed that the first neuroblasts appeared at the otic vesicle level, the upper and lower halves of the cervical level, the upper and lower halves of the thoracic level and the lumbosacral level at stage 12, stage 12 to 13, stage 14, stage 14 to 15, stage 15 to 16 and stage 17 to 18, respectively. These results evidently indicated the rostrocaudal gradients of the neuronal differentiation in the neural tube. This is in good agreement with the results obtained from the ^3H -thymidine autoradiographic studies (Nornes and Das, 1974; Hollyday and Hamburger, 1977).

Autoradiographic studies revealed that the number of neuroblasts formed in the cervical neural tube before the end of day 2 was very small (Martin and Langman, 1965; Langman and Haden, 1970). This is in close agreement with our results. Using ^3H -thymidine autoradiography, Hollyday and Hamburger (1977) reported that the first motor neurons of the brachial lateral motor column (LMC)

were born at stage 15; the earliest birthdates of the lumbar LMC neurons were at stage 17. Since the brachial LMC and lumbar LMC correspond to the lower half of the cervical neural tube and the lumbar neural tube respectively, our observations on the time of origin of the neuroblasts at these levels are in agreement with their reports. Generally speaking, the time of origin of the neuroblasts in the neural tube of the chick embryo determined by our histochemical observation of AChE activity is in close accord with that determined by ^3H -thymidine autoradiography.

So far as we investigated by the histochemical method, the neuroblasts first appeared in the ventrolateral portions of the wall of the neural tube caudal to and including the cervical level. They were large and occasionally provided an axon which extended to the ventrolateral edge of the neural tube. In subsequent stages, the neuroblasts were detected in the ventral as well as in the dorsal halves of the wall of the neural tube. McConnell and Sechrist (1980) reported that the neurons (heavily labeled cells) which first appeared in the spinal cord were medium-sized cells and located in the intermediate zone. As to the size of these neurons and their localization, our observations were inconsistent with their findings. Our observations supported the opinion that large-sized neurons generally arise earlier than do small sized ones (Altman, 1966; Hinds, 1968; Kanemitsu, 1977).

ACKNOWLEDGEMENT

The authors are grateful to Mr. Humihiko Hata and Miss Kumiko Tsujino for their valuable cooperation.

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