STUDIES ON THE TIME OF ORIGIN OF MAUTHNER'S NEURON IN *XENOPUS LAEVIS*

by

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ABSTRACT.

The time of the last DNA replication of the Mauthner's neuron precursor cell has been investigated using radioautography. Embryos of Xenopus laevis were labeled at different stages of early development by single microinjections of tritiated thymidine. Labeling times were designed to cover the entire period of development between gastrula and hatching stages. The embryos were fixed at later stages (41 to 44, according to Nieuwkoop and Faber, 1967), when the Mauthner neuron can be readily distinguished by its characteristically large size and large nucleolus.

Mauthner neurons of embryos which received tritiated thymidine from stage 10 (beginning of gastrulation) to stage 12 (advanced gastrula, medium yolk plug) were always labeled. Those embryos which received the isotope at or after stage 12-1/2 (advanced gastrula, small yolk plug) were never found labeled. These results imply that the last DNA replication of the cell destined to give rise to the Mauthner neuron occurs during the last gastrula stages. This last DNA replication immediately precedes the time of the so-called "histogenetic determination" of the Mauthner neuron proposed to correspond to stage 13 (slit blastopore) by Stefanelli (1951).

Therefore it appears that the developmental program of the Mauthner neuron involves a remarkably early cessation of DNA replication closely followed by histogenetic determination. This is the earliest known event of this type for a specific, well characterized neuron in the amphibian embryo.

Bromodeoxyuridine, a thymidine analog, was injected into embryos at stages in which the Mauthner precursor cell was still synthesizing

DNA. Even though the bromodeoxyuridine was incorporated into the Mauthner cell during its last DNA synthetic period, it failed to affect its differentiation. The cell attained normal size, with large dendrites and a large axon which decussated at the neural tube midline. Implications of the results of the bromodeoxyuridine experiments are discussed.

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INTRODUCTION.

A. History of Mauthner's neuron: structure and function.

The Mauthner neurons are a single pair of contralaterally placed, exceptionally large neurons found in the medulla oblongata of teleost fishes and many amphibians (for review see Stefanelli, 1951). They are situated between the gray and white matter at the level of emergence of the vestibular nerve. Each neuron has four primary dendrites, three medial ones and a large lateral one. The axon emerges from the dorsomedial dendrite, courses ventromedially to decussate in the midline, and then posteriorly in the medial longitudinal fasciculus (Tagliani,1905; Beccari, 1907).

The Mauthner cells are considered part of an integrative motor associative center in the medulla. They receive and convey impulses from the head sensory organs (eyes, ears, tactile receptors) and lateral line organs; their dendrites branch among the afferent VII, VIII and IX nerves (Szepsenwol, 1936; Leghissa, 1941; Stefanelli, 1946). The Mauthner cells' axons course down the spinal cord and collaterals synapse with motor neurons which innervate segmental musculature throughout trunk and tail. The Mauthner neurons are thought to mediate quick avoidance responses such as the startle reflex seen in fish and aquatic phase of amphibians (Tagliani, 1905; Detwiler, 1947; Rodgers and Melzack, 1963; Furukawa, 1966).

Even though these cells are unique because of their size and characteristic morphology, their differentiation follows the general pattern of developing nerve cells in other systems (Chezar, 1972; Billings, 1972). The appearance of filopodia precede the emergence of dendrites and general growth of the body. Accumulation of Golgi bodies

correlate with the tremendous growth that the cell must undergo. There is an increase in numbers of ribosomes and endoplasmic reticulum membranes at the time of nucleolar enlargement, but they do not appear in discrete bodies. Instead they are found scattered in the cytoplasm, especially around the nucleus. The Mauthner's nucleus is striking because of its large size as well as the large size of its nucleolus. The nuclear to nucleolar size is approximately 3.5:1. In spite of the large nuclear volume, the cell contains a diploid amount of DNA (Billings and Swartz, 1969). Because of its striking, unique morphology and easy localization in the medulla, the Mauthner cell system offers many advantages for the detailed analysis of the differentiation process of a single nerve cell. To understand the developmental sequence of the Mauthner neuron, it is essential to determine its "time of origin," that is, the time at which the last DNA replication of the Mauthner's precursor cell occurs (Sidman, 1960).

B. Studies on the time of origin of cells of the nervous system.

Previous work (for review see Angevine, 1970) has shown that the differentiation of neurons, growth of neurites, can be detected only after the last mitosis of nerve cell precursors. This time marks the transition from a germinal, proliferating cell to a non-proliferative, immature neuron. The "just born" neuron then continues the process of differentiation and migrates from its place of origin to a specific site in the nervous system (Sidman et al., 1959).

On the basis of radioautographic data (Fujita, 1965; Angevine, 1965, 1970; Hinds, 1968; and Taber Pierce, 1966, 1967) have constructed elaborate calendars of neuron birth dates for various regions of the brain. Day to day follow-through of migrations of populations of

neurons has been done after tritiated thymidine injections (for a comprehensive review see Angevine, 1970).

Radioautography has been essential in elucidating cell origin, cell cycle and cell migration in the central nervous system (Boulder Committee, 1970; Fujita, 1963, 1966; Watterson, 1965).

In determining the birthday of a nerve cell, two things are taken into consideration: 1) Thymidine is a specific precursor of DNA, thus radioactive thymidine is almost exclusively incorporated into DNA at the time of DNA replication (Friedkin et al., 1956); 2) Once a neuroblast has left the proliferative ventricular or subventricular regions of the developing nervous system, its capacity to divide is arrested (Fujita, 1963). Thus a label on a neuroblast is permanent. Choosing appropriately the stages at which radioactive thymidine is injected into an embryo, one can label a neuron precursor cell in its last DNA synthesis. The nucleus of the fully differentiated cell arising from this labeled neuroblast will be labeled if the isotope was administered during a significant portion of the S phase of the cell cycle prior to the last mitosis. The cell will also be labeled to a lesser extent (depending on the time of availability of the precursor and on the duration of the cell cycle) if the labeling period coincides with the next-to-last mitosis. The administration of the radiochemical after completion of the last DNA synthetic period will result in an unlabeled nerve cell.

It is well known that nervous tissue is already determined as such by the completion of gastrula stages (12, 12-1/2), but this does not necessarily mean that at these early stages any given embryonic cell is "determined" to give rise to a specific nerve cell or group of nerve cells. This histogenetic determination is a gradual process.

Stefanelli, studying the development of the nervous system in frog larvae, has shown that the Mauthner neurons are already determined as such at the late gastrula stages (Stefanelli, 1951). This study is interesting because it dissects the determination of a single, highly specific nerve cell at a remarkably early stage of development. It implies that a Mauthner precursor cell or field of cells is present in the early neurula stage, even though the characteristic morphology of the cell cannot be recognized until hatching stages. Thus one must ask what happens to the precursor cell during this prolonged period of time. At the time of its histogenetic determination, is the Mauthner cell precursor a cell that has ceased cell division, hence DNA replication, or is it a cell that will proceed to divide and give rise to a progeny, any one of which may become a Mauthner cell? These are important questions to answer if we are to understand the processes that precede the cytologically visible differentiation of a nerve cell. The virtue of the Mauthner cell system is that it allows one to approach these questions at the level of a single, unique nerve cell.

Of particular interest is the question of whether or not the time of the last mitosis of the Mauthner precursor cell coincides with its time of determination. In order to investigate this question, the time of the final DNA replication which precedes the last mitosis of the Mauthner's precursor cell in developing Xenopus laevis embryos has been studied. The experimental approach was based on the injection of tritiated thymidine into embryos at specific stages of early development followed by radioautographic examination of the Mauthner's cell nucleus at later stages, when the neuron can be identified unequivocally.

Using these methods, the time of the last DNA replication in the

cell line which gives rise to the Mauthner neuron has been determined.

C. Studies on the effects of 5-bromodeoxyuridine on developmental systems.

5-bromodeoxyuridine (BrdU), a thymidine analog, has been used extensively to suppress the terminal differentiation of many cell types without altering cell growth or cell division (Wilt and Anderson, 1972; Holtzer et al., 1972a; Younkin and Silberberg, 1973; for review see Holtzer et al., 1972b). BrdU's mode of action seems to be dependent on its incorporation into DNA strands and so it effectively affects differentiation only when administered to replicating precursor cells (Stockdale et al., 1964; Bischoff and Holtzer, 1970; Ostertag et al., 1973; Mayne et al., 1973). In several cell lines some inhibition of expression of a differentiated product can result from the presence of BrdU during a single round of DNA synthesis (Turkington et al., 1971; Mayne et al., 1973; Scher et al., 1973; O'Neill and Stockdale, 1974). However, more inhibition is obtained by exposing cells to BrdU one or more cycles earlier (Weintraub et al., 1973).

Studies have been done showing that BrdU interferes with transcription of hemoglobin mRNA (Preister et al., 1973; Ostertag et al., 1973). It has also been shown that the repressor of the lac operon binds more tightly to BrdU substituted DNA than to unsubstituted, normal DNA (Lin and Riggs, 1972). Lapeyre and Bekhor (1974) propose that "if the primary effect of BrdU is to modify protein-DNA interactions,. . .it is possible to explain the differential inactivation of genes relative to the number of hits characteristically produced with low BrdU substitution in DNA." They have found that BrdU affects the properties and structure of chromatin, even with low levels of incorporation. For example, there is 1) an increase in thermostability

of the A-T base pairs where thymidine is substituted by BrdU, 2) modification of non-histone protein interactions with DNA by increasing the negative charges in chromatin, thereby effecting a possible increase in supercoiling. Studies by Stellwagen and Tomkins (1971) further indicate that transcription of only certain genes is inhibited although BrdU is uniformly incorporated into DNA.

BrdU has been found to inhibit the synthesis of so-called "luxury proteins" (cell and tissue specific proteins as opposed to "house-keeping" enzymes). Examples of these are hemoglobin production in red blood cells, myosin, actin and tropomyosin in replicating myogenic cells; tyrosine aminotransferase in hepatoma cells (for review see Holtzer et al., 1972b). It has been suggested that BrdU inhibits the initiation of a new synthetic activity in a lineage (Weintraub et al., 1971; Holtzer et al., 1972b). Only if it is added at a particular time during the mitotic history of a cell lineage will it affect the differentiation of the progeny in that cell line. It apparently inhibits some function necessary for the commitment of that cell line to make its specific product(s).

Several studies have been made on the effects of BrdU on the differentiation of the nervous system. Concentrations of BrdU similar to those used to inhibit tissue-specific products in other systems inhibit myelination in explants of newborn rat cerebellum (Younkin and Silberberg, 1973). BrdU was used to find the time of determination of oligodendrocytes as myelin-producing cells. It is not known what the neurons might contribute to this process of myelination in the cerebellum by virtue of their interaction with glial cells.

Degenerating neural cells can be seen in explants of amphibian gastrulae (stages 10-1/2, 10-3/4) after they have been exposed to

BrdU (Tencer and Brachet, 1973). However, the doses used (10^{-3}M) are cytotoxic (Ostertag et al., 1973; Hunt et al., 1974).

Studies on explants of chick retinas have shown that BrdU inhibits radial orientation of peripheral cells. These cells have centripetally extending nerve fibers when cultured without BrdU. In the presence of BrdU even though nerve processes formed, they were not arranged in layers. The cells were able to form aggregates but in a random fashion (Morris, 1973). The concentration of BrdU used in this study was 5×10^{-5}M (15 µg/ml).

Recently, in vivo studies on the effects of BrdU on amphibian retinas have been done (Bergey et al., 1973; Hunt et al., 1974). BrdU treatment of retinas at stage 24±1 (tail bud) completely inhibited neuronal cytodifferentiation of central retina ganglion cells. The cells were devoid of neurites and morphologically similar to undifferentiated neuroepithelial cells. Absence of cell alignment and layering was noted. BrdU treatment at later stages affected the differentiation of other neuron types of the retina while permitting ganglion cell differentiation to occur.

In studies done with developing mouse neocortex, Webster et al. (1973) have used high doses of BrdU for different periods of time. They found that abnormal mitotic figures and grossly abnormal nuclei appeared in the neuroepithelial cells. It was not possible to determine whether or not these cells differentiated normally because of the impossibility of identifying them from the many other cells in the cortex.

The Mauthner cell system can provide many insights into the problem of BrdU effects on developing systems. On the basis of information concerning the mitotic history of the cell obtained in the first part of this study, BrdU was administered to embryos before and during the

last DNA synthetic period, and its effect on Mauthner cell differentiation, as well as on differentiation of the rest of the brain, was studied.

MATERIALS AND METHODS.

A. Breeding and maintenance of embryos.

Adult Xenopus laevis (Daudin) were obtained from the Lemberger Co., Oshkosh, Wisconsin. Males and females were kept in separate tanks which contained de-chlorinated tap water or spring water (Crystal Spring Water Co.) at room temperature (22-24°C). They were fed beef liver cut in 1 cm. cubes twice a week. Animals of the same sex were distinguished from each other by clipping the claws on their hind limbs and noting their number and position.

Embryos were obtained by mating adult males and females after injection of gonadotropic hormones (Nieuwkoop and Faber, 1967). The adult male was injected with 1 cc. of gonadotropic hormone (Antuitrin S, Parke Davis) containing 280 I.U. dissolved with saline (NaCl) solution. The female was injected with 580 I.U. of the same hormone. A pair of animals, or two males and one female, were placed in a container with de-chlorinated water containing 0.010 g/l of Penicillin-Streptomycin (Gibco). A wire mesh was placed two inches from the bottom of the tank to prevent damage to the embryos by the adult frogs. The tank was then placed in a dark, reduced-noise level area. The injections were administered late in the afternoon and eggs were obtained early the next morning. Larger doses of gonadotropin (1-1/2 - 2 times larger) were needed during the summer months.

The fertilized eggs were collected and reared in de-chlorinated water in finger bowls at 22-24°C. Unfertilized, damaged or otherwise abnormal looking eggs were eliminated immediately.

Following the injection of isotope, the embryos were transferred to individual containers in 1/10 strength Holtfreter's solution

containing Penicillin-Streptomycin (Gibco, 0.01 g/l).

Staging of the embryos was done according to Nieuwkoop and Faber (1967).

B. Method of labeling embryos.

1. Injection apparatus. Jelly coats surrounding the embryos were removed manually with watchmaker forceps. Removal of vitelline membrane was difficult and often resulted in fragile or dead embryos. Exposure of embryos to a solution of 2% cysteine hydrochloride and 0.01% papain at pH 7.9 (Graham and Morgan, 1966) was also tried. The quicker and less detrimental method to the development of the embryo was manual removal with forceps. The embryos were injected using a chemically cleaned glass micropipette drawn to a 15 - 20 micron tip diameter (O.D.) using a micropipette puller (David Kopf Instruments). The micropipette was attached to a 2 microliter syringe (Precision Sampling Co.) by polyethylene tubing (0.015 in. internal diameter) mounted on a micromanipulator (Pfeiffer PBL-2) (modification of Elsdale et al., 1960). The tubing was filled with Squibb mineral oil through a side arm in the syringe so as to eliminate all air bubbles from the system. The micropipette was filled with oil separately and the tubing was inserted into it, again avoiding formation of air bubbles in the closed system. This was very important at the time of drawing the isotope or other solutions into the micropipette as creation of even the most minuscule bubble decreased the effective pressure needed for injecting and for refilling the pipette. An interphase was created between the oil and the aqueous solutions making it possible to check the delivery of the liquid into the embryos and leakage from the embryos. As an additional precaution the mineral oil was evacuated prior to use.

2. Labeling of embryos: methyl tritiated thymidine. For the injection of tritiated thymidine the embryo was positioned into a depression carved in a piece of paraffin wax, and rotated so that the ventral side was facing the injection apparatus. The micropipette was slowly lowered till the tip entered the embryo and the solution was delivered. By slowly withdrawing the pipette, the formation of a "plug" consisting of yolk released from broken cells and membranes was facilitated.

a. Dosage injected: Methyl tritiated thymidine (aqueous TdR-methyl-^{3}H, specific activity 10 Curies/mmole, 5m Curies/ml, International Chemical and Nuclear Corp.) was administered in single 0.05 to 0.15 microliter injections of approximately 0.50 to 0.75 microCuries. The embryo was then allowed to develop to a later stage at which time it was fixed. At least ten embryos of each stage were injected in this manner. In addition, to ensure the availability of isotope for long periods of time (more than 6 hours), multiple injections of 0.05 microliter were given (one every 4 hours) to ten embryos. Injections in these cases were given at different stages: 3 embryos received 3 injections at 4 hour intervals starting at gastrulation (stage 11); the other 7 embryos received 3 injections at 4 hour intervals beginning at neurula stages (14-23).

Approximately 500 embryos were injected. The stages injected ranged from stage 9-1/2 (2 hours before the beginning of gastrulation) up to and past late tailbud stages 29-30. A few embryos (about 3-4 embryos at each particular stage) were injected at stages 31, 37-38. Less than 5% of embryos injected before the onset of gastrulation survived long enough to be fixed at later stages. Mortality in general was about 30% for older embryos, 70% for embryos at gastrula stages.

Survival was improved by addition of penicillin-streptomycin to the medium in which the embryos were grown and by reduction of the volume of liquid injected (from 0.15 µl to 0.05). Of approximately 300 embryos injected successfully, only about a third, those showing excellent development and high radioactivity incorporation (see "tail test") were chosen for radioautographic examination.

b. Tail test: In order to monitor the level of radioactivity of each embryo, a tail test was developed. Each embryo (stage 37-38+) used for radioautography was cut into two parts prior to fixation so that one part contained head structures and whole gut and the remaining part consisted only of tail structures. The head portion was fixed in Carnoy's or Bouin's solution. The tail portion was digested with 15% hydrogen peroxide. The digest was placed in Brays' scintillation fluid and counted in a Packard scintillation counter.

In preliminary experiments the radioactivity of the tail portion and of the remainder of the embryo was determined for a series of twelve embryos injected at stages 18-25. These embryos were processed in this manner after they reached stage 37-38. The ratio of counts found in the tail to counts in the rest of the embryo was approximately 1:3.9. A conservative ratio of 1:3.5 was used for calculations (see Table I, RESULTS). This provided a suitable index for assessing whether enough counts for radioautography were incorporated in each embryo and for approximate determination of exposure times.

Control embryos were injected with 1/3 strength Barth's solution X (Barth and Barth, 1959) and processed in the same manner as experimental embryos.

c. ^{3}H-thymidine incorporation: Incorporation of methyl-tritiated-thymidine into acid-insoluble material was assayed by a modification of

the Schmidt-Thannhauser procedure (Schmidt and Thannhauser, 1945). Labeled embryos were homogenized in 10 mM Tris pH 7.4, and subsequently precipitated with 10% w/v Trichloroacetic acid (TCA). An aliquot of the acid-soluble supernatant was taken for counting and after two additional TCA washes (showing negligible cpm) the pellet was treated with 1 N KOH for one hour at 37°C. The digest was chilled and TCA was added to 10% w/v. After centrifugation an aliquot was taken to assay for alkali-sensitive radioactivity. The final pellet was washed, treated with 0.1 N KOH and NCS (tissue solubilizer, Nuclear Chicago Corp.), incubated overnight at 36°C, and counted in toluene scintillator in a Packard scintillation counter. See Table II.

d. Radioautography: Embryos were fixed overnight in Bouin's or Carnoy's fixatives, dehydrated in a graded series of ethanol and embedded in paraffin (Paraplast) (Guyer, 1953). Serial sections 3-5 microns thick were cut with a rotary microtome (Spencer "820", American Optical Co.) and mounted on appropriately labeled slides coated with albumen adhesive. After sections were deparaffinized, re-hydrated, then air-dried, the slides were coated with Kodak NTB-2 photographic emulsion according to a slightly modified dipping method of Kopriwa and Leblond (1962). Coating was uniform when the emulsion was diluted in a 1:2 or 1:3 ratio with distilled water. The slides were air dried, then placed in light-tight boxes in a pre-determined order. Two slides per embryo were coated and put in separate boxes: One slide had sections in which Mauthner's cells could be found; the other had sections which had other parts of embryo's brain. This was done as an added precaution in order to check exactly the time of exposure needed for each embryo.

The light-tight boxes were placed in a larger box containing small bags full of drierite and exposed for four to ten weeks at 4°C.

Radioautographs were developed in Kodak D-19 developer (diluted 1:2 H_2O) for 3-1/2 minutes, fixed in Kodak Rapid Fixer for 8-10 minutes and stained through the emulsion. Sections were stained for 10-15 minutes in Harris' Hematoxylin, rinsed in tap water, blued in cold saturated $LiCO_3$, dehydrated in a graded series of ethanol and counter-stained for 2-3 minutes in Eosin (Davenport, 1960). Dehydrated sections were cleared in xylene and mounted with Permount. All solutions were cooler than room temperature (18-20°C) as warm solutions tend to strip the emulsion off.

e. Enzymatic extraction of nucleic acids: In order to ascertain that label was incorporated preferentially into DNA, specific removal of DNA or RNA from tissue sections was effected by deoxyribonuclease (DNA'ase; ribonuclease-free, crystallized, Worthington) or ribonuclease (RNA'ase, Worthington) (Amano, 1962).

DNA'ase treatment: Sections of labeled embryos, which were fixed in Carnoy's fixative, were mounted on slides, deparaffinized and hydrated. Half of the sections on the slide were incubated for approximately 24 hours at 37°C in a solution containing 0.05 mg DNA'ase/ml of Gomori's tris buffer with 0.10 M $MgSO_4 \cdot 7H_2O$ pH 5.7. The other half of the sections were not exposed to the DNA'ase solution, serving as a control. Sections of un-injected embryos were also exposed to the DNA'ase solution. Additional controls were provided by sections of labeled embryos incubated in the buffer alone. The slides were then rinsed in several changes of distilled water, and processed for radioautography as previously described.

RNA'ase treatment: Sections of labeled embryos were incubated in a solution containing 1mg of RNA'ase/ml of distilled water. The enzyme was heated to 90°C for ten minutes prior to use in order to inactivate

any contaminating DNA'ase. The sections were incubated at 40°C for 4 hours with gentle shaking every hour. Control slides were incubated in distilled water.

After incubation, both enzyme-treated and control slides were washed thoroughly in distilled water and coated with Kodak NTB-2 emulsion. Exposure time was for 30 days. Staining through the emulsion with Hematoxylin-Eosin was done as specified above.

3. Labeling of embryos: 5-bromodeoxyuridine experimental treatment. Five series of embryos derived from different matings, each series from one clutch of eggs, were injected with 5-bromo-2'deoxyuridine at stage 10 through stage 25. They were prepared for observation by light or electron microscopy when embryos had reached stage 37-38 or later. Three different stock solutions of BrdU were used for injections in order to obtain different experimental conditions. Thus, some embryos were injected with "cold" BrdU to obtain an estimation of dosage effects; some were injected with high specific activity labeled BrdU for radioautographic experiments; yet others were injected with a mixture of "cold" and "hot" BrdU for calculation of internal dosage and observation of dosage effects.

Solution A: Solution A consisted of varying concentrations of non-radioactive BrdU ranging from 1 to 10 mg/ml (BrdU Sigma; made in 1/3 dil. Barth's solution X).

Solution B: Stock solution B consisted of high specific activity radioactive BrdU (New England Nuclear Co., 5-Bromo 2'deoxyuridine-6^{3}H; ethanol: water 7:3; 25 C/mmole, 5mC/ml).

Solution C: Stock solution C consisted of 150 µl of "cold"

BrdU (Solution A) 1-5 mg/ml mixed with 50 µl of radioactive BrdU (Schwartz-Mann; -5'bromo-2-deoxyuridine 6^{3}H; 15.3C/mmole; 0.5 mC/ml).

For each set of embryos injected with BrdU, control embryos were injected with 1/3 diluted Barth's solution X and the same amount of radioactive BrdU tracer as other embryos. They were processed in identical manner as experimental embryos.

a. Dosage effects: Series 1. Fifty four embryos ranging from stage 11 to stage 23 were injected with 0.05 to 0.10 µl of stock solution A which would theoretically give internal concentrations of 50-500 µg/ml (assuming the volume of an embryo to be 2 µl). Actual concentrations inside each embryo are not known because of uncertainty about the actual internal concentration of BrdU (the tail test was not used). Due to the known light sensitivity of BrdU, embryos were exposed to light only for the brief duration of injection, after which they were kept in the dark. They were allowed to reach stage 40+ and fixed in Carnoy's solution; serial sections were deparaffinized, dehydrated and stained with H and E. See Table I.

b. Radioautography: Series 2. Twelve embryos (stages 10, 10-1/4, 10-1/2, 11, 13-1/2) were injected with a modified stock solution B. A drop of 20 µl of high specific activity BrdU (61.4 µg/ml) was evaporated in a vacuum dessicator in order to evaporate the ethanol. An equal amount of 1/3 diluted Barth's solution X was added to the evaporated spot. Embryos were injected with 0.05 to 0.10 µl of this solution, allowed to develop to stages 40+ and fixed in Carnoy's fixative. Processing for radioautography was identical to that used for the thymidine injected embryos. Before fixing the embryos, the tails were removed for the tail test.

c. Light and electron Microscopy: Series 3 through 5.

Series 3. Effect of BrdU on development - Light microscopy, H & E. Each of sixty nine embryos (stages 10, 10-1/4, 10-1/2, 11-1/2) was injected with 0.05 to 0.10 µl of stock solution C (1.0 - 2.0 mg/ml). The embryos were allowed to develop in the dark until they reached stage 37-38+ and fixed in Bouin's fixative and stained with H & E. See Table IV.

Series 4. Light microscopy - silver stained. Thirty one embryos ranging from stage 10 to stage 25 were injected with 0.05 to 0.10 µl of stock solution C (2.7 mg/ml). The embryos were allowed to develop in the dark until they reached stage 37-38+, fixed in Lawdosky's fixative and processed for silver-staining (Bodian, 1936). After deparaffinization and hydration, sections were incubated for 24-48 hours at 37°C in a solution of 1% Protargol-S (Winthrop Laboratories) with 4-6% copper, reduced in 1% hydroquinone and toned in 1% gold chloride (Merck). Residual silver salts were removed with 5% sodium thiosulfate. Sections were thoroughly washed in distilled water, dehydrated and mounted in Permount (see Table V).

Series 5. Light microscopy - silver stain and epon embedded material. Fifty embryos (stage 10-1/4 to stage 25; 25 embryos were injected before stage 10) were injected with 0.05 to 0.10 µl of stock solution C (2.0 - 5.0 mg/ml). Some embryos were fixed in Lawdosky's fixative for routine processing for light microscopy and silver stained as described above. Embryos to be embedded in epon were fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer at 4°C, and post-fixed in 1% osmium tetroxide. Tissues were dehydrated in a graded series of ethanol and passed through propylene oxide before embedding in Epon 812 (R. P. Cargille Labs). See Table VI. One micron thick sections

were cut with glass knives in a Sorvall Porter Blum MT-2 microtome and stained with 1% toluidine blue.

d. Tail test. The "tail test" (see results) was performed for Series 2, 3, 4, 5 and the BrdU concentration inside of embryos was calculated according to the formula shown below. The ratio of counts in the tail to counts in the head was found to be 1:2.7. A longer piece of the tail (compared with tail test for thymidine ^3H) was cut for improved statistical analysis of cpm. The tail was cut exactly at the posterior end of the gut.

1. ["cold" BrdU inside whole embryo] = $\frac{\text{amt of BrdU in embryo}}{\text{volume of embryo}}$ = $\frac{1}{V}$ (amount of BrdU in embryo)

2. amt. of BrdU in embryo:
   (cpm in tail) (3.7 whole embryo) $\frac{[\text{BrdU mg/ml (stock soln)}]}{\text{cpm (cpm/ml}}$

3. [BrdU] = $\underline{\text{X mg/ml (or } \mu\text{g/ml)}}$; $\underline{\text{cpm in soln}}$ = 6.25×10^2 cpm/ml
   6.25×10^7 cpm = (o.125 mC/ml) $(0.5 \times 10^9$ cpm)
   50 µl of 0.5 mC/ml ^3H-BrdU is diluted 4 times by 150 µl of "cold" BrdU
   1 mC=2.2×10^9 dpm -- 0.5×10^9 cpm (assuming a 22-23% efficiency of counting.)

4. $\underline{\text{BrdU whole embryo}}$ = 3.7 (cpm in tail) $\frac{(\text{X } \mu\text{g/ml}}{6.25 \times 10^7 \text{ cpm/ml})}$

5. [cold BrdU inside whole embryo] = $\frac{1}{2 \times 10\text{-}^3\text{ml}}$ $(3.7)(\text{cpm in tail}) \frac{(\text{X } \mu\text{g/ml}}{6.25 \times 10^7 \text{ cpm/ml})}$
   volume of embryo 2×10^{-3}ml
   $= \frac{0.296 \text{ (X } \mu\text{g/ml) (cpm in tail)}}{10^4}$

This formula is to be used with solutions containing 0.5 mC/ml. The number 3.7 in calculation no. 2 refers to the ratio of counts of BrdU found in the whole embryo to those found in the tail. Original

estimates of the volume of an embryo were done by weighing embryos and calculating the volume by assuming the density of an embryo to be approximately 1.26 mg/ml. These values were later verified by direct measurement of the displacement of liquid by 50 embryos in a 300 µl calibrated tube.

RESULTS.

A. Tritiated thymidine studies.

The time of the last DNA synthesis in Mauthner's cell can be found by checking the last time that labeled thymidine is incorporated into the nucleus. Amphibian embryos have a very low permeability to the labeled precursors needed for analysis of macromolecular synthesis (Landesman and Gross, 1968). It is thus necessary to microinject the precursors directly into the embryo.

Methyl-tritiated thymidine was injected into embryos, each at a specific stage of early development. The embryos were then allowed to develop to later stages, and fixed at this time, when the Mauthner cell has attained a large size. This was followed by radioautographic examination of the Mauthner's nucleus.

Approximately 500 embryos were injected in this manner. Of these about 300 survived to reach later stages (37-38 to 41-42). The remaining 200 or so embryos developed abnormally or died. Improvement of the survival rate was achieved by adding antibiotics to the medium in which embryos developed and by keeping the volume of fluid injected to a minimum. Rate of growth of experimental embryos (injected with tritiated thymidine) was the same as control embryos (injected with saline).

The micropipette used for injections was manipulated in such a way as to aid the formation of a plug of cytoplasm and membranes. This was very helpful in preventing too much leakage of injected fluid. In most instances fluid leaked out of the embryo, especially in the case of embryos injected after neurula stages, when motor reactions to external stimulation commence. Embryos were observed for some time

after delivery of fluid. On many occasions leakage was seen at this time when none was observed right after injection. In quantitative experiments where the concentration of isotopes or other chemicals inside of the embryo must be known, it cannot be assumed that the concentration injected reflects the actual internal concentration.

Tail Test. Preliminary experiments were done in which the amount of radioactivity in different parts of the embryo was determined several days after injection (at late embryonic stages 41 or 42). The tail of each embryo was cut, digested with a tissue solubilizer and counted in a scintillation counter separately from the rest of the embryo. The results are shown in Table I. In 10 out of 12 embryos the radioactivity in the body was 4 times that found in the tail. As a conservative estimate, a ratio of 1:3.5 of counts in the tail to counts in the rest of the embryo, was used for determining the relative exposure times of radioautographs.

B. Determination of the effective labeling period.

Using a modified Schmidt-Thannhauser assay (see Materials and Methods) preliminary experiments were done to investigate the effective length of the labeling period following a single injection of tritiated thymidine. The time course of incorporation of the label into acid-insoluble alkali resistant material, mostly DNA, was followed for 8 hours following injection (Figs. 1a and 1b). In early embryos (stages 12-12-1/2, late gastrulation) incorporation of the labeled precursor into DNA takes place for at least 8 hours. Conversely, most of the counts found in the acid-soluble pool (unincorporated thymidine) decrease accordingly. As expected, incorporation of label into alkali-labile material (mostly RNA) is insignificant and remains at about 2%

of the recovered counts throughout the experiments. The incorporation of label into DNA in embryos injected at tailbud stages (21-24) increases rapidly for at least six hours; incorporation continues at a reduced rate. Approximately 75% of the total recovered counts are incorporated into alkali-resistant material in eight hours for early embryos and in approximately six hours for later ones. The rate of incorporation of label into DNA is greater in older embryos than in younger ones. There is the possibility that the thymidine pool of late gastrula stage cells is larger than that of tailbud stage cells. Rates of nucleoside transport could be altered by various factors thus affecting the kinetic behavior of intracellular pools; also, many enzymatic activities (for example, thymidylate kinase) could be different at different developmental stages (for review see Hauschka, 1973). In older embryos approximately 45% of total cpm available are incorporated into DNA in the first two hours after injection. In contrast, approximately the same percentage is incorporated in four hours in earlier embryos. The actual counts incorporated are shown in Table II. On the basis of these experiments, it was concluded that the effective labeling period is at least six hours. Radioactive thymidine is being incorporated into DNA for more than six hours so the label is available for at least this length of time.

C. Radioautographic experiments.

Having determined the length of the labeling period, the following experimental plan was adopted. Each embryo was injected only once at a particular stage, then was allowed to develop with no further injections up to stage 40 or 42, when the Mauthner neuron had attained a large size and could be unequivocally recognized. The idea was to cover with each embryo a single six-hour long developmental period

during which the isotope was known to be available for incorporation. The large number of different stages injected and the numbers of embryos injected at each stage insured that the labeling intervals overlapped extensively, thus covering all the possible times at which a DNA synthesis could occur.

Stages 10 through 13:

Embryos injected with ^{3}H-thymidine at stage 10-10-1/2 consistently had heavily labeled Mauthner cells in radioautographs (Figs. 2-4). Figs. 1a and 1b show the two Mauthner cells of one embryo, with heavy label in both. On many occasions the nucleus looks "empty" with seeming chromatic condensations in a cogwheel shape; the following serial sections through the nucleus would show more label over the seemingly empty parts. The nucleolus was always heavily labeled (Figs. 2-4).

Embryos injected at stage 11 have Mauthner cells that are slightly less labeled in comparison with surrounding cells and with Mauthner cells from embryos injected at stage 10-1/2 (Figs. 5a and 5b). The overall incorporation of tritiated thymidine was lower due to less availability of the injected precursor. Cpm from the tail tests done on these embryos indicate that less isotope was injected or more isotope leaked out of the embryos. Very seldom did the Mauthner cell's nucleus appear in only one section as the sections were seldom more than 5 μ thick. Figs. 6a and 6b show the pattern of labeling in two serial sections of the same cell. Empty-looking spaces are prominent in Fig. 6a. This is probably due to clumping of chromatin. Fig. 5b on the other hand shows no spaces and a uniformly labeled nucleus. Much more of the Mauthner neuron's cytoplasm can be appreciated. The empty looking spaces between different cells in the rest of the neural tube could be due to washing out of yolk depositions during

fixation. Early amphibian embryonic tissues have some remaining yolk platelets.

The labeling pattern of the Mauthner cell in embryos injected at stages 12 and 12-1/2 was variable (Figs. 7-11). Some embryos exhibited labeled Mauthner cells and yet others had two Mauthner cells which were not labeled above background. This was not unexpected, considering that there may be some small variability in the timing of developmental events from one embryo to another. Of particular interest is the case of one embryo injected at stage 12 which showed a single labeled Mauthner cell (Figs. 7a and 7b). The poorly defined nuclear area is covered with silver grains (arrow, 6a). The nucleolus can be made out in Fig. 7b, again, covered with grains. The contralateral Mauthner cell, however, showed no appreciable label over its nucleus or nucleolus (Fig. 7c). Serial sections throughout the whole cell showed an unlabeled Mauthner nucleus (Fig. 7d). It therefore appears that in the same embryo a Mauthner cell precursor on one side of the medulla incorporated tritiated thymidine in its nucleus (therefore was still replicating its DNA) while the contralateral precursor cell did not.

Radioautographs of another embryo injected at stage 12 are seen in Figs. 8a and 8b. These slides were exposed for a longer time because counts in the embryo were low. The cytology is very clear and both the nucleus and nucleolus are labeled to the same extent in both cells.

Fig. 9a is a radioautograph of a Mauthner cell from an embryo injected at stage 12-1/2. Label can only be seen on the nucleolus and in its immediate vicinity. The following serial section shows a section through the nucleus in which label is not above background (Fig. 9b).

Figs. 10 and 11 show Mauthner cells of two different embryos injected at stages 12-1/2. The cells are not labeled above background. The same is true for two different embryos injected at stage 13 (Figs. 12 and 13). No label was found in any part of the cell in subsequent sections.

Stages 14 through 37-38:

All the embryos injected at stages 14 through stage 37-38 (the time at which a Mauthner cell can be distinguished unequivocally) also exhibited unlabeled Mauthner cells (Figs. 14-24). In all these cases the nucleolus of the cell was also unlabeled. The cells around the Mauthner cell showed different labeling patterns depending on the stage injected and the stage at which the embryo was sacrificed.

Some radioautographs permit the observation of cytological detail while others appear "fuzzy" or blurry. This is probably due to unevenness of emulsion coating so that some slides exhibit a thinner film over the sections than others.

Among the embryos examined there were two or three cases of double Mauthner formation, i.e., two Mauthner cells on the same side of the medulla (Fig. 20). The cytology of this pair of Mauthner neurons was normal at the light microscope level, though possibly some abnormalities (of the axon, for example) could have escaped detection in the absence of silver staining. On the contralateral side, it was found that one, and only one, Mauthner cell was present.

Fig. 24 shows the radioautograph of an embryo injected at stage 37-38. This is a stage by which a Mauthner cell in Xenopus laevis can be seen to be quite differentiated. It has abundant cytoplasm at that stage and an axon (see Chezar, 1972 for review), therefore it is highly unlikely that it would be engaged in DNA synthesis.

As can be seen from Figs. 23 and 24, only a few cells in the hind brain are labeled in embryos injected at stages 33-38, although other regions of the brain show a somewhat larger number of labeled cells. Fig. 21 also shows a few cells labeled, but in this case the amount of radioactivity in the embryo was low. As the embryo grows older, fewer and fewer cells are labeled (less and/or slower mitotic activity in cells giving rise to neurons). Glial cells continue to proliferate and thus are labeled if tritiated thymidine is available.

Results of enzymatic extraction:

The specificity of incorporation of the ^{3}H-thymidine label into DNA was confirmed by DNA'ase treatment. DNA'ase treated sections showed no appreciable label, while untreated sections of the same brain region of the same embryo are heavily labeled (Fig. 25). Fig. 25a is a radioautograph of a section through the beginning of the telencephalon. It is heavily labeled with silver grains. The following section (Fig. 25b), which was exposed to deoxyribonuclease treatment, shows no appreciably labeled nuclei in any tissue. Fig. 25c is a section through the foramen of Monro in the same embryo. The cells of the neural tube are heavily labeled. Fig. 25d shows the following section, which is unlabeled after treatment with DNA'ase.

The experimental design and the results are summarized in Fig. 26. The filled circles pinpoint the stage at which the embryo was injected (stage 12 or later); the lines that extend from the circles indicate the stages covered in the six hours during which the label is known to be available. In all of these embryos the Mauthner cells were unlabeled. The bars represent embryos injected prior to stage 12; in all these embryos, the Mauthner cells' nuclei were found to be labeled to varying degrees. The label is known to be available for incorporation

for at least six hours (see also Hunt & Jacobson, 1972). However, the isotope could not have been incorporated during the hours covered by the clear portion of the bars, as shown by the embryos represented by the filled circles and lines. Embryos injected singly from stage 10 to stage 29-30 are represented in the graph.

A couple of embryos (not shown in Fig. 26) injected at stages earlier than stage 10 exhibited labeled Mauthner nuclei. Six embryos were injected at stages later than 29-30 (not shown in graph). The later stages injected ranged from 31-32 to 37-38. All these embryos had unlabeled Mauthner cells.

D. Effects of 5-bromodeoxyuridine on the development of Mauthner's cell.

1. Dosage effects. Preliminary experiments were done to determine which dosage of BrdU would have an effect on development of the embryo while permitting some percentage of survival (see Table III). Dosages used for in vitro studies in other systems varied from 10^{-3} to 10^{-6} M BrdU (0.3 to 300 µg/ml) (Stockdale et al., 1964; Schubert and Jacob, 1970; for review see Wilt and Anderson, 1972). There was no attempt to measure the actual concentration of BrdU inside the embryo, only non-radioactive BrdU was used. The overall percentage of survival was 43%, the earliest injected stages resulting in the highest mortality percentage. For embryos injected at stages 11-11-1/2 and stages 14-15, survival was 31-32%, for those injected at later stages (stages 18-19, 22-23) 62-80%. The trend was for survival to increase with lower dosages and by injection of embryos at late stages.

Embryos injected at stage 22-23 with high concentrations of BrdU showed very low mortality. Even though development was not perfect (eyes did not develop normally, had transparant centers) they showed

good pigmentation and good organ development (i.e. heart and gut).

Embryos which were injected at earlier stages all showed varying degrees of the same type of abnormalities, i.e. very low pigmentation, body deformities, incomplete development of the eyes.

In embryos where Mauthner cells were found, the cells exhibited large nucleoli and darkly staining cytoplasm. In some embryos, the marginal layer did not seem to be as well developed as in normal or thymidine injected embryos.

The results obtained through these experiments indicated that only concentrations of BrdU ranging from 1-5 mg/ml should be used as stock solutions for injection of embryos. Due to leakage from the embryo it must not be assumed that the concentration of BrdU or any other chemical injected into an embryo reflects the actual internal concentration. Only by use of tracers such as isotopes can a relatively accurate estimate of internal concentrations be obtained.

2. Results of radioautographic experiments: Series 2. The thymidine analog BrdU has been shown to be preferentially taken up by replicating cells in different biological systems (for review see Holtzer et al., 1972b). An experiment was designed to determine through radioautography the intracellular site of incorporation of BrdU in the amphibian embryo.

Radioactive BrdU of high specific activity was injected into nine embryos at the developmental stage of the last DNA synthesis of the Mauthner precursor cell (stage 10-1/2) and three embryos at a later developmental stage (stage 13-1/2) when no DNA replication occurs in that cell. In these experiments only BrdU of high specific activity dissolved in saline was used. The effects of BrdU on development were not studied in this series, therefore no additional non-radioactive

BrdU in concentrations used for series 1 and 3 embryos was used. The molarity of the BrdU injected was of the order of 2×10^{-5} M (6 µg/ml), approximately 100 times less than the molarity injected to obtain a BrdU effect.

The embryos used in this experiment came from a summer mating that gave a relatively smaller proportion of viable embryos (refer to page 9 of Materials and Methods) and development was not always good.

Mauthner cells could be unequivocally identified in three out of nine embryos which were injected at stages 10-1/4 to 11. All three embryos exhibited heavily labeled Mauthner nuclei. Cells from one such embryo are shown in Fig. 27.

The cytoplasm of the Mauthner cell is clearly visible and the nucleus and nucleolus are heavily labeled (Fig. 27a). The contralateral cell is also heavily labeled (Figs. 27b and 27c). All of the other cells of the brain are also labeled. Cells near the neurocoel are not as heavily labeled as cells near the marginal layer. Radioautography of three embryos injected at stage 13-1/2 showed Mauthner cells with unlabeled nuclei (see Fig. 28). Cells in the vicinity of the Mauthner cell are labeled heavily.

The results of these radioautographs show that bromodeoxyuridine is indeed incorporated into the Mauthner cell's nucleus. Furthermore, they confirm the finding that the Mauthner cell's precursor is synthesizing DNA at stages 10-1/2-11 and synthesis of DNA has terminated by stage 13-1/2.

3. Light Microscopy: H & E, silver stained material: Series 3 through 5. In these experiments radioactive BrdU was used as a tracer in order to calculate the approximate concentration of non-radioactive BrdU inside the embryos. The results are shown in Table II. There was

42% survival. BrdU concentrations used were calculated to give internal concentrations of 50 - 100 µg/ml. Actual internal concentrations obtained were 2 - 13 µg/ml.

Five embryos that were injected at stage 10 were examined histologically. Relatively well differentiated Mauthner cells were found in these embryos; large nucleus and nucleolus, abundant cytoplasm compared to other cells. Varying degrees of cellular disarray, abnormalities in the morphology and cytology of the neural tube were found to correlate with increasing internal concentrations of BrdU. An embryo with an internal concentration of 11 µg/ml had an abnormally shaped, distorted neural tube and eyes which were not well developed.

Similar results were found in embryos that had been injected at stages 10-1/4 through 10-1/2. The neural tube was found to be rounded up in some embryos; there was not a clear distinction, for example, between the characteristic shape of the medulla and the rest of the nervous system. In this case the embryo had a concentration of BrdU of 9 µg/ml.

An embryo injected at stage 11-1/2 with the same internal concentration of 9 µg/ml BrdU did not show the abnormalities mentioned above. The general cytology of the embryo, in particular the nervous system, was quite good. However, there seemed to be many more cells in the neural tube when compared with controls; the marginal layer seemed somewhat decreased in these embryos. This was only a qualitative observation, no quantitation was attempted. The Mauthner cells of these embryos differentiated well. The cell's axon could not be distinguished in sections stained with Hematoxylin and Eosin.

4. Effects of BrdU on Development: Light microscopy, silver stain: Series 4. In this set of experiments the internal concentration of

BrdU in the embryos was increased to double the previous concentrations obtained. Eleven embryos out of thirty-one injected managed to survive until stages 37-38+. Normal development was retarded compared to controls and greatly impaired. They were edematous, had skeletal deformities, eyes developed very incompletely and, in some cases, not at all. The gut development was incomplete and the embryos were characteristically devoid of body pigment.

In four of these embryos, Mauthner cells were found to be quite well differentiated. These embryos were silver stained so that observation of more cytological detail was possible. The Mauthner cells in two embryos injected at stages 10-10-1/4 and with internal concentrations of BrdU of 16 and 67 μg/ml differentiated quite well.

The embryo with 16 μg/ml BrdU appeared to have a neural tube which had a small marginal layer in proportion to the number of cells compared with non-BrdU embryos. The axon and its decussation at the midline of the neural tube could be distinguished in several contiguous sections.

The embryo that had an internal concentration of BrdU of 67 μg/ml was very badly developed both at the gross and microscopic level (see Figs. 29a and b). The neurocoel was almost non-existent. The cells in the vicinity of the Mauthner neuron were few and in no specific order; there was a small amount of marginal layer. The cells seemed to be undergoing necrosis due to cytotoxic levels of BrdU used. However, the Mauthner cell still had a very large normal-looking nucleus and nucleolus and abundant, well-stained cytoplasm, and some of its processes could be seen including a long axon coursing ventro-medially. Fig. 31 shows the structure that developed in place of a normal eye in this embryo. There is total disarray of cells, which are undergoing

necrosis; some pigment from pigmented cells of the retina remains.

These embryos surely incorporated BrdU in their Mauthner precursor cells for at least one round of DNA synthesis.

Two embryos injected at stage 14-15, which had internal concentrations of BrdU of 22 µg/ml and 50 µg/ml, showed very poor development. There was very little pigmentation; the eyes and gut were small and distorted. Histological examination revealed very poor general cytology, and very incomplete development of the nervous system. The neural tube was abnormal in size, shape and cytostructure. The marginal layer was reduced in area and did not have the characteristic configuration in transverse sections (compare Fig. 33 showing the embryo with an internal BrdU concentration of 50 µg/ml, with sections of normal embryos Fig. 27). The neurites did not form the typical peripheral layer, but formed large and small fascicles with no apparent regular disposition. Mauthner cells with abundant cytoplasm and large nuclei could be identified in these embryos. Figs. 33 a-d show the Mauthner cells of this embryo.

The Mauthner cell of an embryo injected before stage 10 and having an internal BrdU concentration of 33 µg/ml is shown in Fig. 30a and b. The nucleus was large and had the characteristically large nucleolus. The axon was very well stained and its ventro-medial course could be easily followed. The cell was well differentiated, judging from its size, shape and large processes. The nuclei in the vicinity of the Mauthner are probably of neurons and glial cells. The marginal layer is not well developed; it is scant. This can be better appreciated in Fig. 30d. Many cells can be seen in the neural tube but their processes have not contributed much to the marginal layer. However, there is still some preservation of the general, normal pattern and shape of

the neural tube of this embryo in this region (medulla). Other parts of the nervous system were highly abnormal. Fig. 30c, for example, shows a region of the neural tube (diencephalon) which had almost enveloped the notochord. The cells were not organized in any normal, recognizable pattern. The cells of the somites seemed to be undergoing necrosis (see Fig. 32).

5. Epon embedded material. (Figs. 35a and b show light micrographs of an embryo injected with saline at stage 15). Fig. 36a shows an overall view section of the neural tube of an embryo injected at stage 10-1/4 and with an internal concentration of BrdU of 28 µg/ml. The Mauthner cell (Fig. 36b) has differentiated and has a well-developed abundant cytoplasm and processes. The rest of the cells in the neural tube look very abnormal (compare with control, Fig. 35a and b). The nuclei are highly irregular, cells are in disarray; there are some cells that appear to have been extruded into the lumen (Fig. 36a). Fig. 36c shows the contralateral Mauthner cell with a long cellular projection, probably a dendrite.

Some embryos that were injected with high concentrations of BrdU developed in perfect conditions. None of the observed abnormalities in other BrdU treated embryos were present. After analysis of the tail test, the internal concentration of these embryos were found to be of the order of 2 - 4 µg/ml. Lack of incorporation of BrdU into the embryos was due in all probability to leakage of injected chemical from the embryo.

The rate of development of embryos injected with BrdU was slower by a couple of hours than that of control embryos.

Figs. 37a, b, c are 1 micron sections stained with toluidine blue. Serial ultrathin sections were cut thereafter. This embryo was

injected at stage 24 and had an internal BrdU concentration of 105 μg/ml. It was found, at the microscopic level, that the neural tube had developed in a more or less normal fashion with respect to size and shape of the various encephalic regions. However, there was an abnormal amount of intercellular space, and the shapes of the nuclei were more irregular than in the neural tube of control embryos, especially the ventricular cells (compare Figs. 37a and 35a).

Another finding which was characteristic of embryos injected with BrdU was the growth or extension of cells into the lumen of the neural tube (see Fig. 36a and 37a). This was never observed in control embryos nor in embryos injected with ^{3}H-thymidine for radioautography.

The amount of marginal layer in this embryo was somewhat less than control embryos. Figs. 37a and 37b show part of a Mauthner cell. Fig. 37c shows the contralateral cell. Even though much of the nucleus had already been cut away, the size of the cell's cytoplasm can be compared with that of other cells around it. What is presumed to be a lipid or yolk inclusion at the upper end of the cell's cytoplasm was present in both cells, but is more prominent in the cell shown in Fig. 37c.

At the gross morphological level this embryo exhibited good pigmentation and good intestinal development. No skeletal deformities were found but eye development was not entirely normal. A micrograph through the eye of this embryo is shown in Figs. 37d and 37e. The retina is highly abnormal when compared to an embryo injected with aqueous ^{3}H-thymidine (no BrdU). There is no formation (in the experimental embryo) of plexiform layers or of a pattern distinguishing a receptor cell layer from a ganglion cell layer as in a normal embryo

(Fig. 38). There is no indication of lens fiber formation. Only cells can be distinguished. Cells from the pigmented retina have formed some pigment.

DISCUSSION.

A. Time of origin of Mauthner's neuron.

It is difficult to determine precisely the time of the last mitosis of the Mauthner precursor cell. However, it was possible to determine the time of its last DNA synthesis. The time of the last DNA replication in that cell line was found to occur between stages 11 and 12-1/2. Injections of isotope after stage 12-1/2 fail to show any incorporation of label into the Mauthner cell's nucleus. Any embryo injected before stage 10 and up to and including stage 12 always incorporated tritiated thymidine in its Mauthner cell.

The last mitosis was expected to take place at some point between gastrulation and stage 29 - 30, since neural induction occurs only after gastrulation has begun and stage 29 - 30 is the earliest stage at which the Mauthner neurons can be distinguished from other cells around them (Chezar, 1972).

The labeling protocol of the experiments was thorough enough to exclude the possibility of an additional DNA replication at later developmental stages. All possible times and stages of larval development during which a DNA replication could have occurred were covered by the injection of several embryos at each particular stage. There was extensive overlapping of the individual time periods, covering the interval from stage 10 up to and past stage 29 - 30. Embryos injected as late as stage 37 - 38 were found to have unlabeled Mauthner cells. As mentioned above, by stage 29 - 30, the Mauthner neuron can be seen to have an axon, and in general, a nerve cell does not divide further once its axon starts developing (Lyser, 1964; Jacobson, 1970; Angevine, 1970).

Billings and Swartz (1969) have shown that the Mauthner neuron is a diploid cell. This implies that a mitotic event must take place after the last replication of DNA has occurred. According to the studies of Flickinger and co-workers (1967) in Rana pipiens embryos, the S period of the cell cycle (during which DNA replication occurs) of ectoderm cells in gastrula stages lasts approximately 1.5 - 1.75 hours and the G_2 period of interphase lasts about the same length of time. The M phase lasts approximately 30 minutes and remains constant from stage 9 to stage 19 (Graham and Morgan, 1966).

As stated above, the last S phase of the Mauthner precursor cell ends at about stage 11 - 12-1/2 (12 - 14 hours of development), thus it is probable that the last mitosis of that cell occurs at about 15 hours of development, stage 13. It is also possible that the Mauthner precursor cell, in contrast to other ectodermal cells of the gastrula stages, goes through a disproportionately long G_2 period, but this would seem unlikely. However, Graham and Morgan (1966) found that variations in the length of cell cycles of endoderm cells from gastrula to neurula stages were due to increasing length of the G_2 phase. At stage 13, the G_2 phase in endoderm cells is about six hours, S and G_1 phase being 3.5 and 2.5 hours, respectively. In contrast, Flickinger et al. (1967) found for stages 10 - 13 (gastrulation stages in Rana pipiens) that S and G_2 remained at 1.5 hour each and G_1 phase lasted approximately 16 hours. The latter measurements were done on ectoderm-mesoderm cells. Taking 3.5 hours as a rough average of the time spent by neuroectoderm cells on G_2 phase, if the Mauthner precursor cell stops DNA synthesis at stage 11 (in the majority of cases) then mitosis would be completed by stage 13 or 13-1/2.

Another implication of this study is that the Mauthner cells are

not mitotic sisters. The last S phase and subsequent cell division apparently take place at a time when the precursor fields are known to be physically separated (Piatt, 1950; Stefanelli, 1951). Further evidence of this is the fact that one embryo injected at stage 12 was found to have a labeled Mauthner cell on one side of the medulla and an unlabeled cell on the other. Other cells in the medulla were labeled. It appears that there are two precursor cells, one for each Mauthner cell that finally differentiates. The time of their last DNA synthesis is probably at the same time but some variability from embryo to embryo is evident.

It is important to note that the probable time of the last mitosis of the Mauthner precursor cell coincides in time with the so-called stage of histogenetic determination of this neuron, proposed to occur at early neural plate (stage 13) by Stefanelli (1951). Based on the results of experiments involving selective unilateral extirpation of the Mauthner cell region in the medulla of anurans at different time points in development, Stefanelli established that the time of determination of the Mauthner cell occurred during the advanced gastrula to early neural plate stages. Before stage 13, the remaining portions of the medulla restore the missing region including a Mauthner cell. After stage 13, again there is restoration, but a Mauthner cell is lacking so that there is not full histological restitution. Were there other neurons which one could distinguish from other nerve cells, it is possible that one could observe the same phenomenon. Stefanelli concluded from these experiments that at early gastrula stages the Mauthner cells are not yet determined. They become determined progressively during late gastrulation; after these stages the determination becomes irrevocable. According to Stefanelli, this determination

occurs in two steps. First the neuroblasts attain their general differentiation as nerve cells (prolongations, Nissl bodies, neurofibrils); then other secondary characters specific to the Mauthner cell such as shape, form, size of cell body, of axon, begin to develop.

In these studies the term determination has been used in referring to the acquisition of information by a cell or field of cells which will predispose it (them) to give rise to a Mauthner neuron. The precise nature by which the cell acquires this information is not known. In general, two ways in which the Mauthner cell may be determined can be considered: 1) there could appear in the late gastrula stage a single neuroblast committed to differentiate as a Mauthner cell, or 2) there could be a group of cells in the presumptive Mauthner histogenetic field, any one of which could eventually differentiate as a Mauthner cell. Perhaps at later stages, when one of these cells starts to exhibit Mauthner neuron characteristics, the rest of the cells in that field are inhibited from doing the same. At this time there is no way of distinguishing between these two possibilities. It can only be said that, at these late gastrula stages, the cell which will eventually become a Mauthner neuron has stopped DNA replication. In instances in which there is ipsilateral double formation of Mauthner cells, the two cells could be mitotic sisters, or they could be two cells from this histogenetic field mentioned above. Double formation of Mauthner cells has been found in about 2% of embryos examined (also see Chezar, 1972). We have never observed more than two Mauthner cells on the same side of the medulla.

B. Comparison of Mauthner's cell's mitotic history with that of other nerve cells.

It is of interest to compare our findings on the developmental

program of the Mauthner neuron to the development of other nerve cells. Extensive studies have been made on the generation of different types of cells in the nervous system of vertebrates. During neurogenesis four main events occur which shape the future adult brain: cell proliferation, cell migration, cell differentiation, and cell death. As to the first of these, there is a timetable of neuron origin specific for the different regions of the brain studied (for reviews see Angevine, 1970; Jacobson, 1970). A spatially and temporally ordered program of origin for different neuron types has been found in studies done in mammals (Sidman et al., 1959; Angevine, 1965; Altman, 1967), birds (Fujita, 1966), and amphibians (Jacobson, 1970; Straznicky and Gaze, 1971). It has been suggested that large neurons with long axons generally arise early in development and are highly specified at that time, while smaller neurons arise and are specified later in development (Hinds, 1968; Jacobson, 1970). The ganglion cells of the retina have been shown to belong to the former type, i.e., they arise early in development and the pattern of retinal connections seems to be at least generally and perhaps provisionally, specified at the time of their last mitosis. Jacobson (1968) has shown a correlation between the cessation of DNA synthesis in ganglion cells of the retina and some events in the development of specification of their future central connections with the tectum in Xenopus laevis larvae (within a period of ten hours; less than one cell generation cycle). Thus, though the process is certainly more complicated (Gaze, Chung, and Keating, 1972; Hunt and Jacobson, 1973) than earlier experiments suggested (Jacobson, 1968) some important events in the process of neuronal specification are closely related to the final mitosis.

The Mauthner cell system is an outstanding example of a large, highly specified neuron determined very early in development. These studies indicate a very early cessation of DNA replication followed within a couple of hours (the probable length of the G_2 period) by histogenetic determination. It is interesting to note that in the case of the retinal cells, "determination" or "cellular specification," as the case may be, seems to take place during or shortly after the final cell cycle. Approximately 22 hours elapse between the time of the last DNA replication of the Mauthner cell's precursor and the stage at which the neuron can be recognized as a Mauthner cell for the first time. It is not known precisely what biochemical events are taking place during this interval. It is not known, for example, if the cell has grown appreciably during this period of time. The size of neuroectodermal cells in Xenopus laevis range from 15 to 30 microns at stage 13 (Karfunkel, 1971). The size of the Mauthner neuron at stage 29-30 falls within this range, measuring approximately 26 microns in length. The nucleus measures 9 microns and is not appreciably larger than nuclei of other neighboring cells at this time (Chezar, 1972). If the Mauthner precursor cell belongs to the larger neuroectodermal cells, it is possible then, that there is little or no cellular growth during the interval between stage 13, the apparent time of origin of the neuron, and stage 29-30. However, if the precursor cell measures 15 microns, an increase in diameter to 26 microns represents a large increase in volume. In both cases, of course, there would be tremendous increase in axonal volume. Because the neuron already has a relatively long axon in the earliest stages examined, the neuron must have achieved a certain polarity which determined the site and direction of axon emergence. Biochemical, synthetic activities

occurring at this time are, of course, not discernible by microscopy.

C. 5-Bromodeoxyuridine effects on Xenopus laevis embryos.

The differentiation of several organ systems in Xenopus laevis embryos is interfered with when animals are exposed to the thymidine analog -- 5-Bromodeoxyuridine. Embryos injected with different concentrations of the analog showed abnormal development of body pigmentation, skeletal deformities and eye organs. With progressively higher concentrations of BrdU these abnormalities were accentuated. Cytologically, one can observe larger intercellular spaces and abnormal looking cells in the neural tube of experimental embryos as compared with controls. There is also disorganization of cells and a dearth of axons in the neural retina.

Mauthner cells developed in all these embryos with nervous system abnormalities. Even in embryos with very high cytotoxic internal concentrations of BrdU there were cells that by their location, shape and size could be identified as Mauthner cells.

Even though BrdU inhibited the organization and neurite development in retinal cells, it did not affect the differentiation of the characteristic shape and axon of the Mauthner neuron when injected at stages 10-1/4 - 11. BrdU was incorporated into the nucleus of the Mauthner cell as ascertained by radioautography. When the chemical was given at stage 13-1/2, the nucleus of the Mauthner cell was unlabeled. This confirms the results obtained with ^{3}H-thymidine, i.e., that the last DNA replication of the Mauthner precursor cell occurs before stage 13 - 13-1/2.

As far as could be observed at the light microscope level, the Mauthner cell developed without any obvious abnormalities. In pre-

liminary studies at electron microscope level, what seemed like normal looking organelles were observed; endoplasmic reticulum studded with ribosomes was seen, the mitochondria were a bit swollen and the cristae somewhat distended. But this could be due to inadequate fixation. A more detailed study of electron micrographs taken of embryos injected at early stages would have to be done in order to determine the effects of BrdU at the ultrastructural level.

Even though BrdU substitution experiments, which would measure the amount of substitution of thymidine in the DNA by BrdU, were not done, it is likely that at least 50-80% of the new strands of DNA were replaced by BrdU. Fabian and Wilt (1973) studying erythrogenesis in chick embryos, have obtained 80% replacement of thymidine by BrdU in each new strand of DNA at a concentration of 50 μg/ml. The range of exposure to BrdU reported in several systems (Bergey, et al., 1972; Weintraub, et al., 1973; O'Neill and Stockdale, 1974) has been one to two generations; generally, the differentiation of a majority of cells is inhibited only after one round of S synthesis.

There may be several explanations as to why the Mauthner cell's differentiation was not affected by exposure to BrdU: 1) the cell could be "resistant" to BrdU by virtue of the fact that its particular program of differentiation had already been started before stage 10 and therefore BrdU could not inhibit it. This same phenomenon has been observed in other systems (see Holtzer, et al., 1972a). Apparently BrdU does not simply block terminal expression of molecules of a specific product but can inhibit the initiation of new synthetic products in a cell lineage. After this program has been started, the system becomes resistant to BrdU. 2) BrdU has to be present for more

than one S phase of the cell cycle. It is interesting to note that other nerve cells (such as the ganglion cells of the retina) only need to be exposed to BrdU for one S phase (see section on results of BrdU treatment). While this work was in progress, similar experiments on the effects of BrdU on retinal ganglion cell differentiation came to our attention (Bergey, et al., 1972; Hunt, et al., in press). Not only was the terminal differentiation of central retina ganglion cells suppressed by BrdU (suppression of neurite growth, morphology similar to neuroepithelial cells) if added at a critical time in their mitotic history, but also the time of axial specification of these cells was blocked. The actual, internal concentration of these embryos is not known, but they were injected with amounts of BrdU that would presumably give concentrations of BrdU in the embryo of up to 250 µg/ml (0.5 µg injections per embryo; assuming a 2 µl volume for an embryo). Hunt, et al. (1974) used 0.5± .2 µl of a 1 mg/ml BrdU stock solution in studies on the retinal development of Xenopus laevis. 3) It is possible that exposure of BrdU for one S phase of the Mauthner precursor cell is enough, but that due to factors such as a large size of thymidine pool, the cell is not able to incorporate BrdU in its DNA. If this is the case, a DNA-BrdU substitution experiment (which could only give substitution in an average of cells) will not tell us what particular substitution by BrdU there is in the cell. It is likely that the thymidine pool is large in the Mauthner cell, as compared to other cells, because of its early cessation of DNA replication. However, radioautography shows that there is a substantial incorporation of BrdU. Whether this could be below a certain threshold is open to question. There are several suggestions that can be explored to solve this problem: 1) to use larger doses of BrdU; 2) to inject much

earlier in development so that more than one S cycle is covered.

It does not seem feasible to inject higher doses of BrdU in Xenopus laevis embryos without deleterious effects on overall development. Very high concentrations of BrdU were injected at stages which preceded stage 10 by at least one or two hours. If injections of BrdU in Xenopus laevis were done extremely early (before stage 8) and a Mauthner cell was found to be either missing (a statistical analysis would be imperative) or poorly differentiated, there is always the possibility that this effect is not due to BrdU. Hydrostatic pressure on such a delicate stage embryo could damage it in such a way that development would be totally abnormal. A large number of control animals would have to be used to circumvent this problem.

It is also possible that the microenvironment of the Mauthner cell is disrupted at a stage when the correct "influence" and support from neighboring cells is essential for the neuron's development.

In both these cases it can be argued that the lack of differentiation of the Mauthner cell was not due to a direct effect of BrdU on its terminal differentiation.

It should be possible to inject early enough in the development of the embryo so that several S phases of the Mauthner precursor cell lineage are exposed to BrdU in such a way that survival and overall development is not severely impaired. If a Mauthner cell still develops, then its response to BrdU can be compared to that of other nerve cells.

Tissue cultures of early gastrula embryos could be prepared and incubated with very high concentrations of BrdU. Differentiation of the Mauthner cell could be easily followed in the explants.

Even though in the set of experiments described the differentiation of the Mauthner cell did not seem to be affected by exposure to BrdU,

the thymidine analog can be a useful tool in studying differentiation. In these studies retinal ganglion cells and retinal organization in general were disrupted by BrdU. Only certain cellular characteristics were affected (formation and organization of axons was deficient); no other abnormalities were observed at the light microscope level.

In conclusion, this work has shown that the time of origin of the Mauthner cell in Xenopus laevis occurs very early in development (late gastrula stages) and that this event coincides with the histogenetic determination of the cell. It is possibly one of the earliest (if not the earliest) neurons to be determined in the amphibian brain; this correlates with the finding that large neurons with long axons in general arise before smaller neurons.

Elucidation of the mitotic history of the Mauthner cells further extend our knowledge of its developmental program. It is now possible to alter various biochemical and environmental factors at this specific critical period (its "time of origin") and evaluate their effect on the Mauthner cell differentiation. One such agent used in these studies is BrdU.

In the present study exposure of retinal ganglion cells to BrdU during their last DNA synthetic period affected severely their differentiation. These results confirm similar studies done in amphibian and chick retinas.

It was found that in contrast to these nerve cells, the Mauthner cell's differentiation (axonal growth, for example) was not affected by exposure of BrdU during the last DNA synthetic period of its precursor cell.

Furthermore, radioautographic analysis of embryos exposed to BrdU at different stages of the Mauthner cell's differentiation confirm that the time of origin of the cell occurs at late gastrula stages.

Table I

Injection stage	Tail cpm A	Rest of embryo cpm B	Ratio B/A
20	1,450	5,800	4.0
23	3,296	13,398	4.1
25	4,851	19,000	3.9
24	5,266	21,000	4.0
24	5,840	23,000	3.9
18-1/2	12,002	48,008	4.0
22	15,702	62,800	4.0
25	17,816	71,000	4.0
20	32,920	132,000	4.0
19	44,349	177,000	4.0
21-1/2	9,073	23,615	2.6
22	33,305	145,929	4.4

Mean: 3.91 Standard Deviation: ±0.432

Determination of radioactivity counts in different parts of the embryo after injection with ^{3}H-thymidine.

Table II

Schmidt-Thannhauser assay on embryos injected at stages 12 and 12-1/2.

% of cpm injected	Total Incorp. cpm	Hrs. Post Injection	Soluble cpm	Acid Labile cpm	Acid Resistant cpm
56	560,195	0	548,991 (98.8%)	2,801 (0.5%)	8,403 (1.5%)
34	341,645	2	241,789 (70.8%)	3,416 (1.0%)	96,441 (28.2%)
45	446,570	4	241,578 (54.1%)	13,323 (2.9%)	191,670 (42.9%)
39	393,683	6	142,780 (36.3%)	9,680 (2.5%)	241,227 (61.3%)
35	352,666	8	85,342 (24.2%)	6,915 (2.0%)	260,409 (73.8%)

Schmidt-Thannhauser assay on embryos injected at stages 21-24.

78	780,362	0	767,876 (98.4%)	4,214 (0.6%)	8,584 (1.1%)
51	510,722	2	274,000 (53.6%)	9,922 (1.9%)	227,271 (44.8%)
80	795,026	5	177,380 (22.3%)	16,170 (2.0%)	601,476 (75.7%)
68	679,771	8	115,500 (17.0%)	19,990 (2.9%)	544,281 (80.1%)

Table III

Effects of varying doses of BrdU on mortality of embryos.

Stage Injected	Calculated Approximate [BrdU] Injected µg/ml	Number Embryos Injected	Dead
11-11-1/2	500	8	8
11-11-1/2	250	8	5
11-11-1/2	125	4	2
11-11-1/2	50	5	2
14-15	500	5	4
14-15	250	5	3
14-15	125	6	3
18-19	500	4	3
18-19	50	4	0
22-23	500	5	1

Survival 43%

10 control embryos were injected with Barth's saline. 10 survived and developed well. They were injected at the same stages as experimental embryos.

Table IV

Embryos injected for light microscopy - Hematoxylin - Eosin Stain.

Stage Injected	Approx. [BrdU] Inside Embryo µg/ml	Number Embryos Injected	Number Embryos Dead	Number Embryos Moribund	Number Embryos Fixed
10	5-13	23	13	5	5
10-1/4	7	18	11	4	3
10-1/2	3-9	18	12	3	3
11-1/2	2-9	10	4	2	4

42% Survival

Table V

Embryos injected for light microscopy: silver stained.

Stage Injected	Internal [BrdU] µg/ml	Number Embryos Injected	Number Embryos Died	(Moribund) Number Embryos Fixed
10	16 and 24	10	7	3
10-1/4	67	11	7	4
14-15	22	10	6	4

35% Survival

Table VI

Embryos injected for light and electron microscopy (Series 4 and 5).

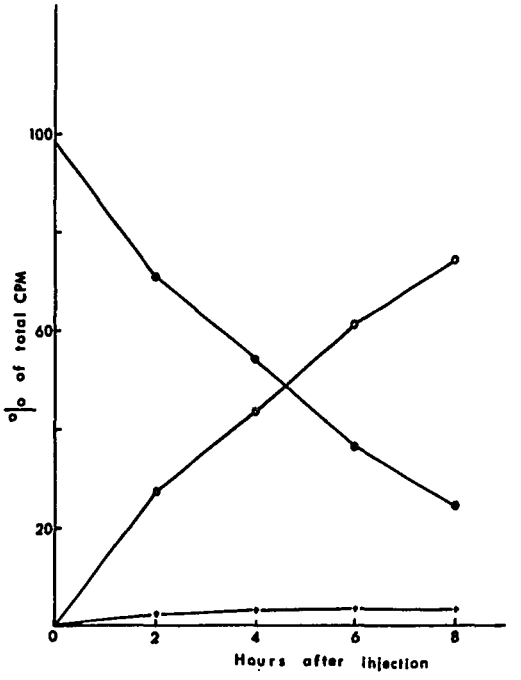
Stage Injected	Internal [BrdU] µg/ml	Numbers Embryos Injected	Numbers Embryos Dead	Microscopy
Before 10	3 and 33	24	22	Light
10-1/4-10-1/2	19 and 28	6	4	Light & Electron
15	40 and 50	5	2	Light
15	34			Light & Electron
20-21	13 and 29	5	1	Light
24	24	4	2	Light
24	105			Light & Electron
25	116	6	1	Light & Electron
25	2,7,15,74			Light

36% Survival

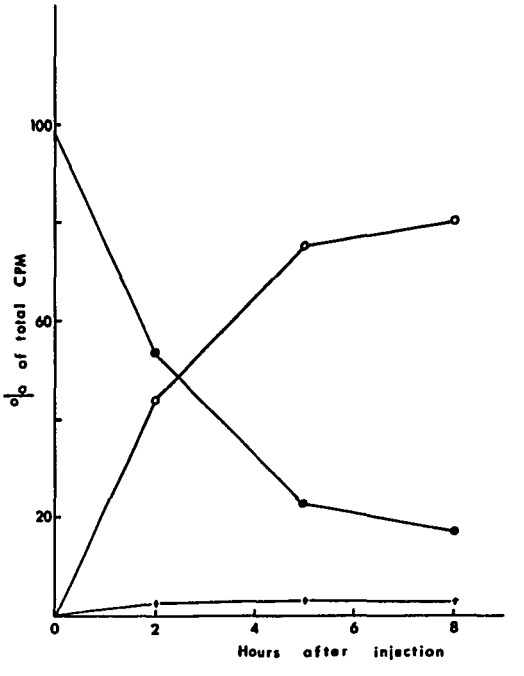
Fig. 1 Time course of incorporation of ^{3}H-methyl thymidine into TCA insoluble material in injected embryos. ●—● , TCA soluble; O—O, TCA insoluble; + —+ alkali labile. TCA = Trichloroacetic acid. Incorporation assays were done as indicated in Materials and Methods, page 12.

a. Groups of 4 embryos were injected with 0.5 μC each at late gastrula stages (12 or 12-1/2) and extracted at 0, 2, 4, 6, and 8 hours after injection.

b. Groups of 4 embryos injected at stages 21-24 (before tailbud) and extracted after 0, 2, 5, and 8 hours.


100
60
20
% of total CPM
0
2
4
6
8
Hours after injection

a


100
60
20
% of total CPM
0
2
4
6
8
Hours after injection

b

Figs. 2 - 25. Light micrographs of radioautographs of transverse sections through the medulla. This set of embryos were injected with ^{3}H-thymidine. Embryos were fixed at stages 37-38+ in Bouin's or Carnoy's fixatives and stained through the photographic emulsion with H & E.

Fig. 2. Embryo injected at a stage just before stage 10.

Fig. 2a. The Mauthner cell's nucleus (N) is heavily labeled the density of grains is equal to that of other cells in its vicinity. The cell's cytoplasm is abundant and clearly seen in this micrograph (m). Magnification x 980

Fig. 2b. Contralateral Mauthner cell (M). The nucleolus can be made out in the dorsal aspect of the nucleus (N) which is also heavily labeled. Magnification x 810

Figs. 3-4. Embryos were injected at stage 10-1/2.

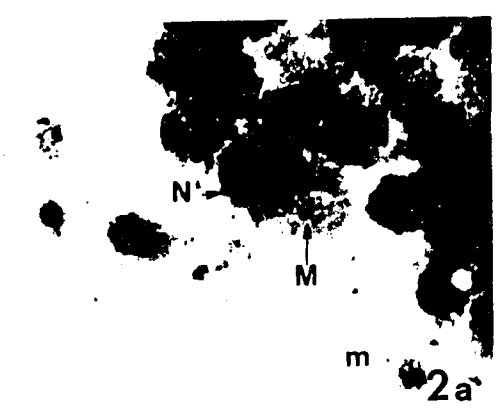
Fig. 3a. The Mauthner cell (M) is heavily labeled.

Fig. 3b. Serial section following that shown in a. More grains are seen in this section. Magnification x 600

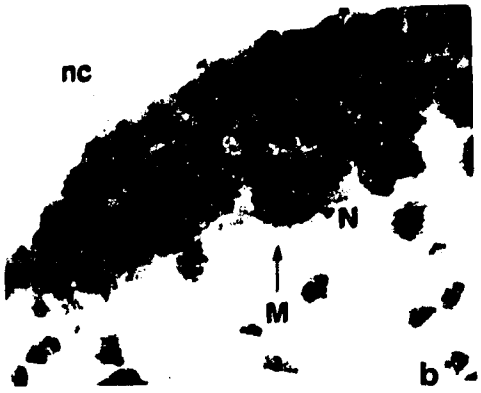
Fig. 4a. Mauthner cell with labeled nucleus and a clearly visible nucleolus (n). Magnification x 710

Fig. 4b. Contralateral Mauthner cell (M) showing labeled nucleus (N). Magnification x 600

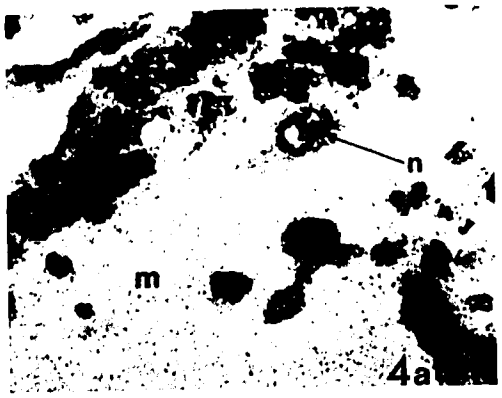
m = marginal layer of neural tube nc = neurocoel



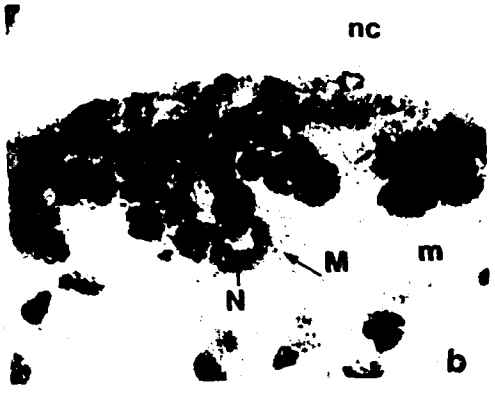
2a



3a



4a



Figs. 5-6. Embryos injected at stage 11.

Fig. 5a. The Mauthner cell (M) in this section is more lightly labeled. The nucleolus (n) is very clearly stained in this section. Magnification x 720

Fig. 5b. The contralateral Mauthner cell. The Mauthner cell in this and following sections was less heavily labeled than in embryos injected before stage 11. Magnification x 730

Fig. 6a. Mauthner cell with "empty" looking nucleus. The nucleolus (n) is seen surrounded by strands of nucleoplasm (probably clumped chromatin). Magnification x 1240

Fig. 6b. Following section showing silver grains over the nucleus (N). No empty spaces are seen in this section. Magnification x 1240

m = marginal layer of neural tube nc = neurocoel

cy = cytoplasm

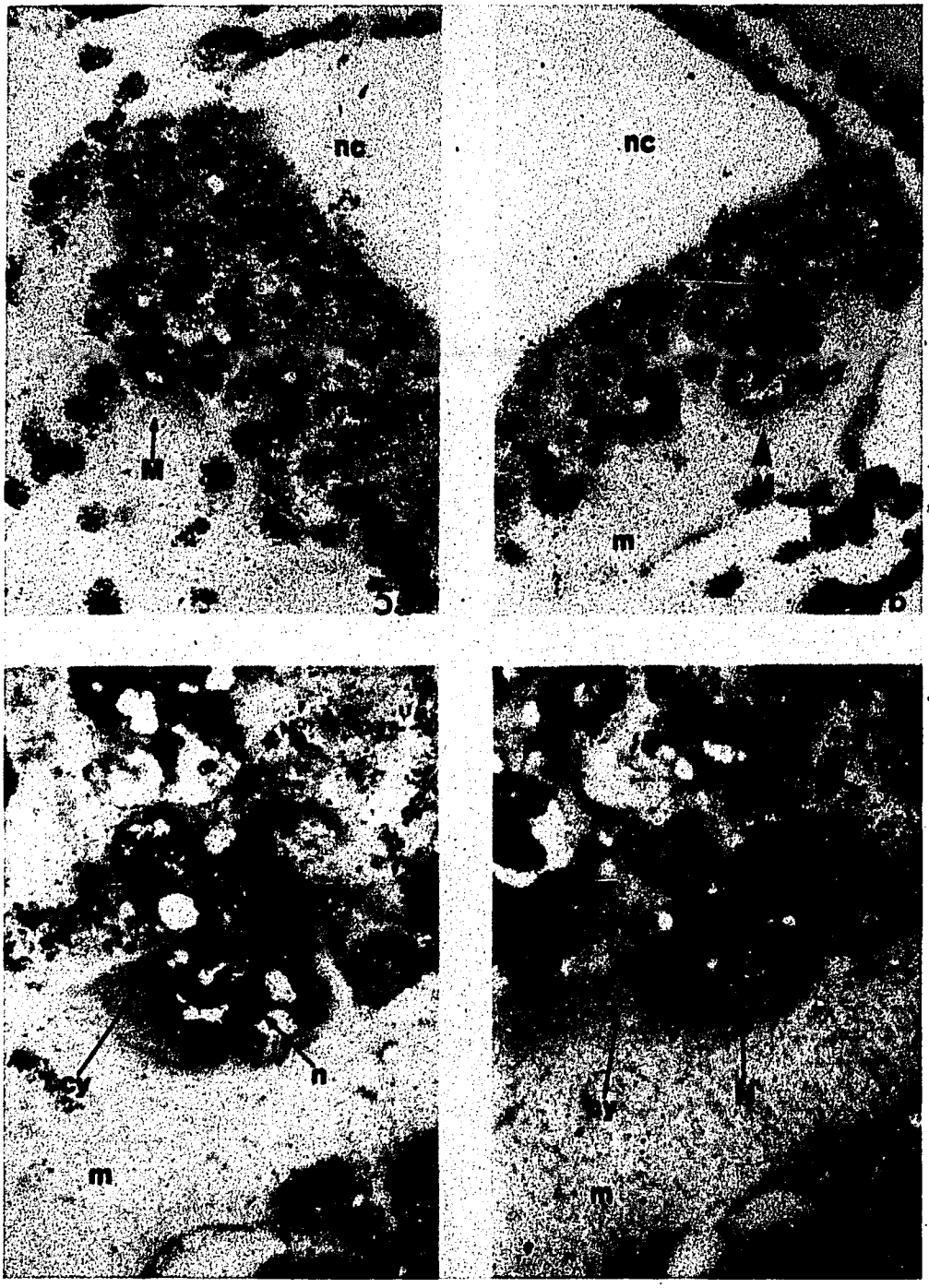

nc
n
5
nc
m
6
cy
n
m
cy
n
m

Fig. 7. Embryo injected at stage 12.

Fig. 7a. Mauthner cell showing poorly defined nuclear area (N) covered lightly with silver grains. Magnification x 1310

Fig. 7b. Following serial section showing the rest of the Mauthner (M) cell's nucleus, this time through the level of the nucleolus (n), and heavier label over the nuclear area. Magnification x 1310

Fig. 7c. Contralateral Mauthner cell showing no label above background. Magnification x 1500

Fig. 7d. Following serial section showing nucleus again unlabeled and large stream of cytoplasm. Magnification x 410

m = marginal layer of neural tube

nc = neurocoel

S = somite

cy = cytoplasm

e = ear

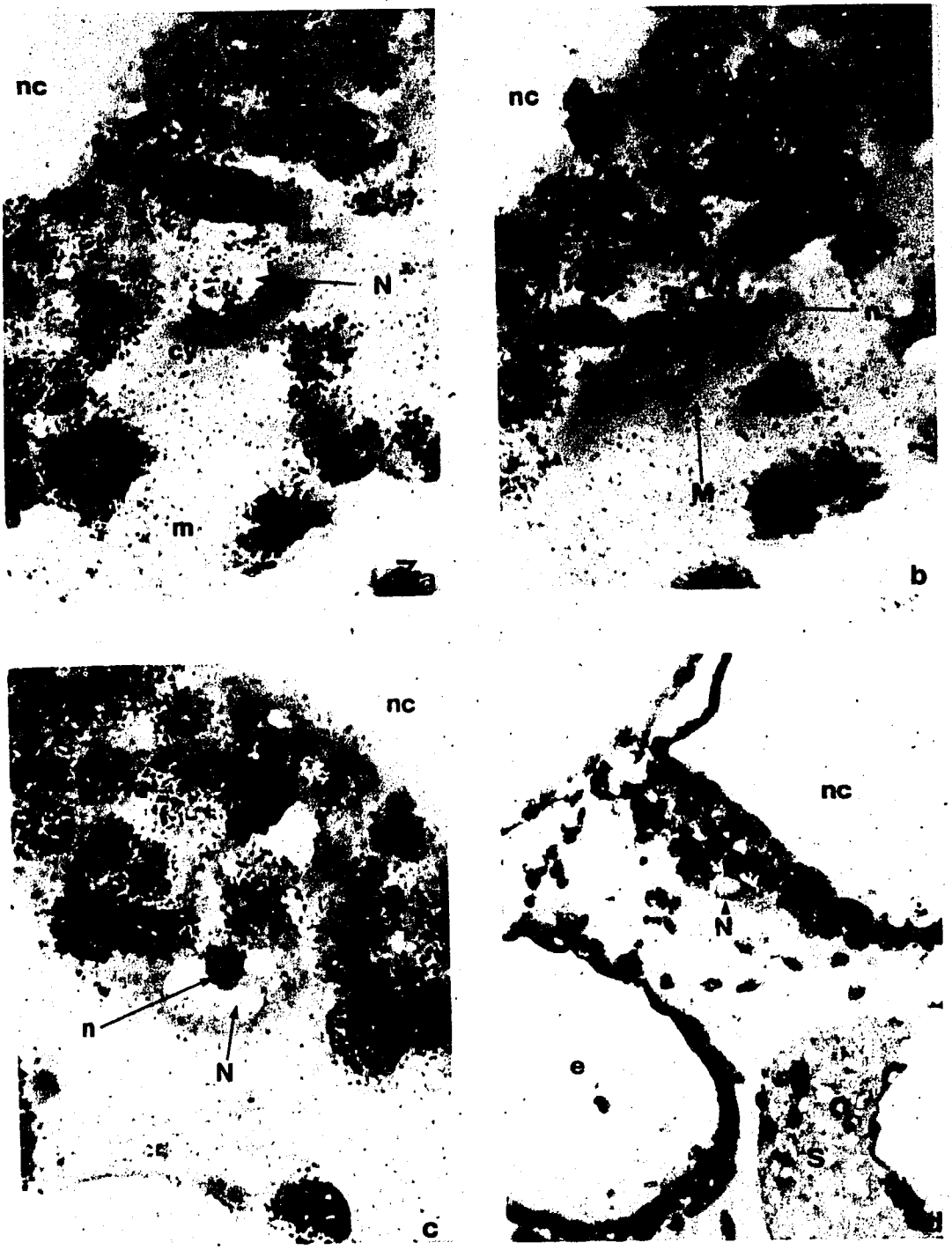

nc
N
m
nc
n
M
b
nc
n
N
c
nc
N
e
S

Fig. 8. Embryo injected at stage 12.

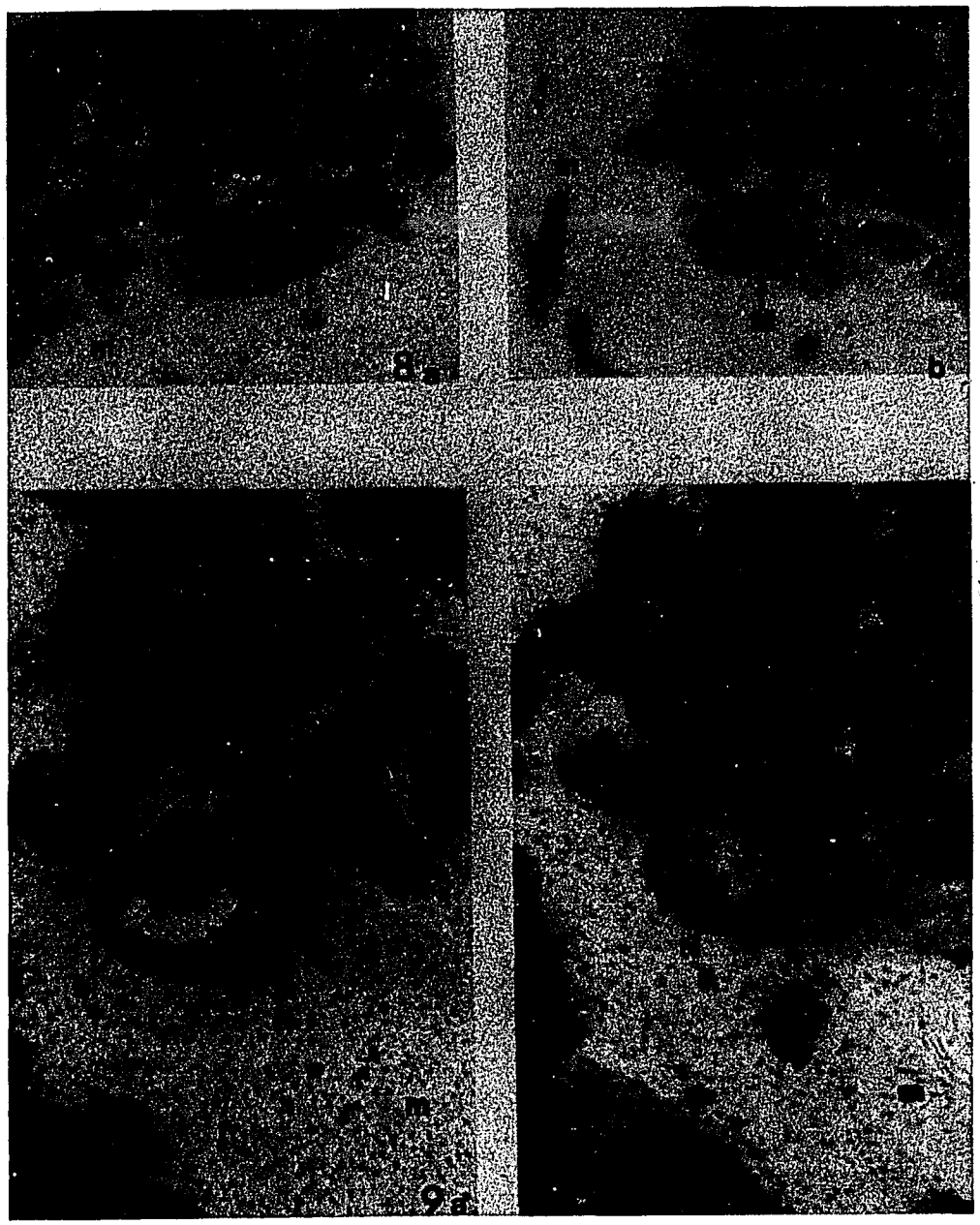
Fig. 8a. Mauthner cell (M) with labeled nucleus and nucleolus. The abundant cytoplasm contrasts with that of surrounding cells. Magnification x 730

Fig. 8b. The contralateral Mauthner cell. Magnification x 730

Fig. 9. Embryo injected at stage 12-1/2.

Fig. 9a. The nucleus (N) of the Mauthner cell is not labeled above background. Some grains are visible over part of the nucleolus. Magnification x 1240

Fig. 9b. Following serial section through the cell showing rest of unlabeled nucleus and cytoplasm. The rest of the cells in the neural tube are heavily labeled. Magnification x 1240


8a
b
9a

Figs. 10-11. Embryos injected at stage 12-1/2.

Fig. 10. Unlabeled Mauthner neuron (M) surrounded by labeled neurons and glial cells. Magnification x 510

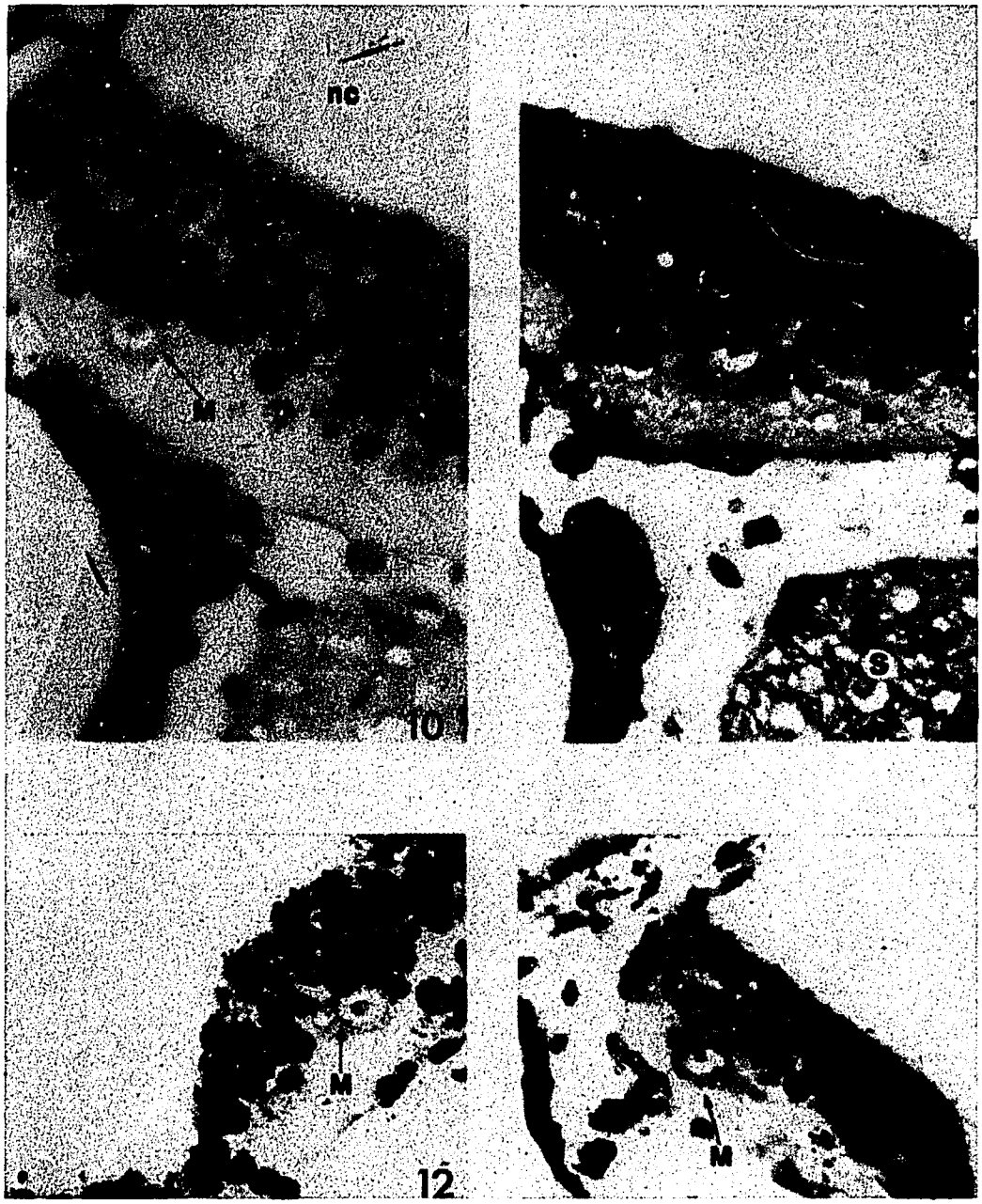
Fig. 11. Mauthner's neuron in another embryo injected at same stage. Neither the nucleus nor nucleolus is labeled. Magnification x 530

Figs. 12-13. Embryos injected at stage 13.

Mauthner cell's (M) nucleus unlabeled. The cells can be readily distinguished from surrounding cells in the neural tube. Magnification x 510, 430

nc = neurocoel

S = somites


nc
M
10
S
M
12
M

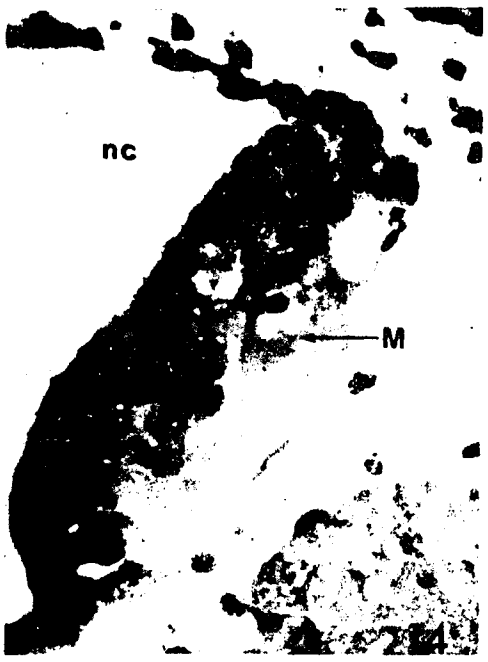
Figs. 14-17. Mauthner cells (M) whose nuclei were unlabeled.

Fig. 14. Embryo injected at stage 16. Magnification x 610

Fig. 15. Embryo injected at stage 17. This embryo was fixed at stage 44. The marginal layer is well developed. Compare with Figs. 14 and 16. Magnification x 560

Fig. 16. Embryo injected at stage 18. Magnification x 410

Fig. 17. Embryo injected at stage 20. Magnification x 620



15

16

Fig. 18. Embryo injected from stage 22. Two serial sections through the unlabeled nucleus (N) of Mauthner's neuron (M) are shown. Multiply injected. Magnification x 540

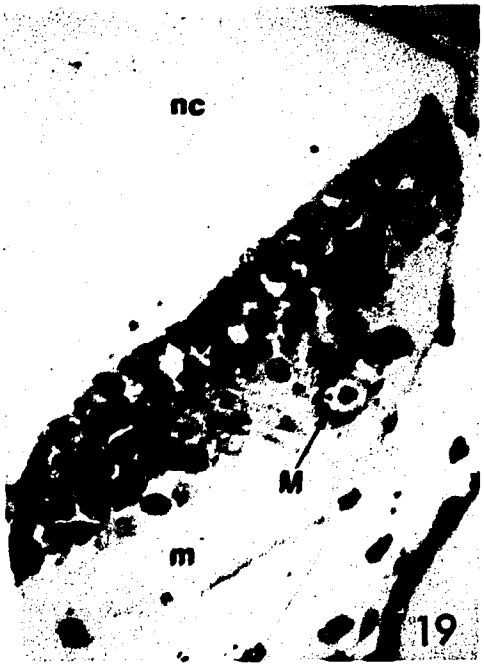
Fig. 19. Embryo injected at stage 24 (tailbud stage). Magnification x 390

Fig. 20. Embryo injected at stage 26. Two Mauthner's neurons (arrow heads) clearly stand out from the rest of the cells. Both have large nuclei and very large nucleoli. The cytoplasm is seen more clearly on following serial sections (not shown). On the contralateral side of the medulla only one Mauthner cell was found. Magnification x 470

m = marginal layer of neural tube nc = neurocoel

e = ear

18



19

20

Fig. 21. Micrograph of embryo injected at stage 27. This embryo did not incorporate much radioactivity but some nuclei are clearly labeled above background. The nucleolus is especially clear in this section. Magnification x 520

Fig. 22. Embryo injected at stage 29-30. By this stage the Mauthner cell can be recognized from other surrounding cells. This embryo was fixed at stage 41. The shape of Mauthner cell's (M) processes is somewhat clearer than in other sections. Magnification x 470

Fig. 23 and 24. Mauthner cells (M) injected at stages 33 and 37-38 respectively. The embryos were fixed at stage 44. Magnification x 520, 540

m = marginal layer of neural tube

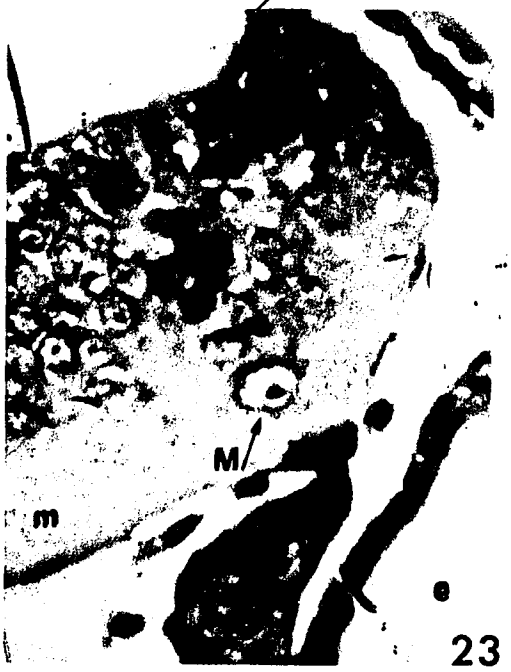
g = ganglion

d = dorso lateral dendrite

nc = neurocoel

e = ear

22



23

24

Fig. 25. Radioautographs of sections of embryos injected with ^{3}H-thymidine and digested with deoxyribonucleose (DNA'ase).

Fig. 25a. Section through telencephalon showing heavily labeled cells in the nervous system. The cavities are the telencephalic ventricles. Magnification x 330

Fig. 25b. Third section following section a was exposed to a solution of DNA'ase. More than 90 per cent of the counts have been removed by the enzyme from cell nuclei. Magnification x 330

Fig. 25c. Section approaching the foramen of Monroe of same embryo as sections a and b above. Neural tube cells are heavily labeled. Magnification x 330

Fig. 25d. Section through same region of the brain as c, but digested with DNA'ase. Magnification x 330

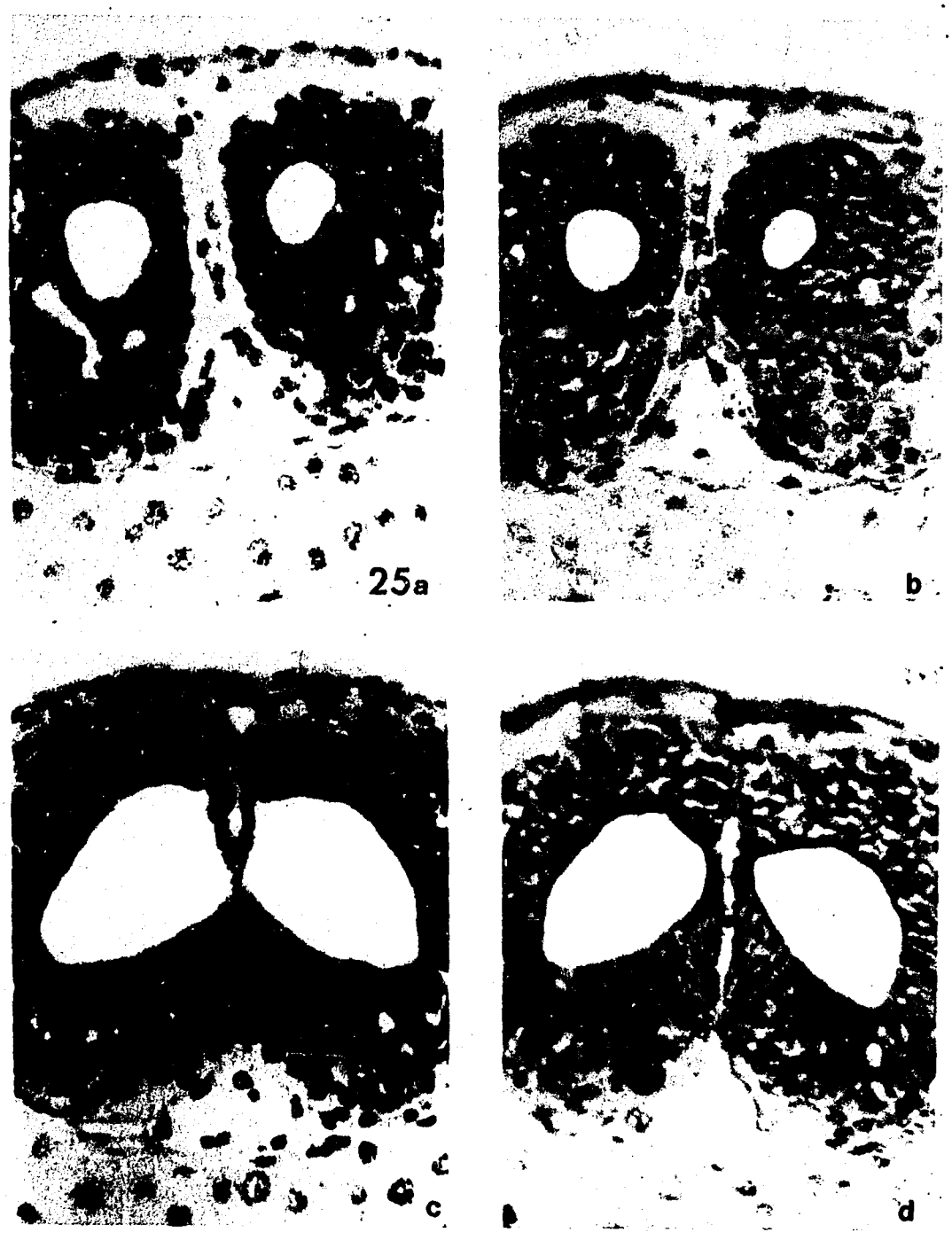
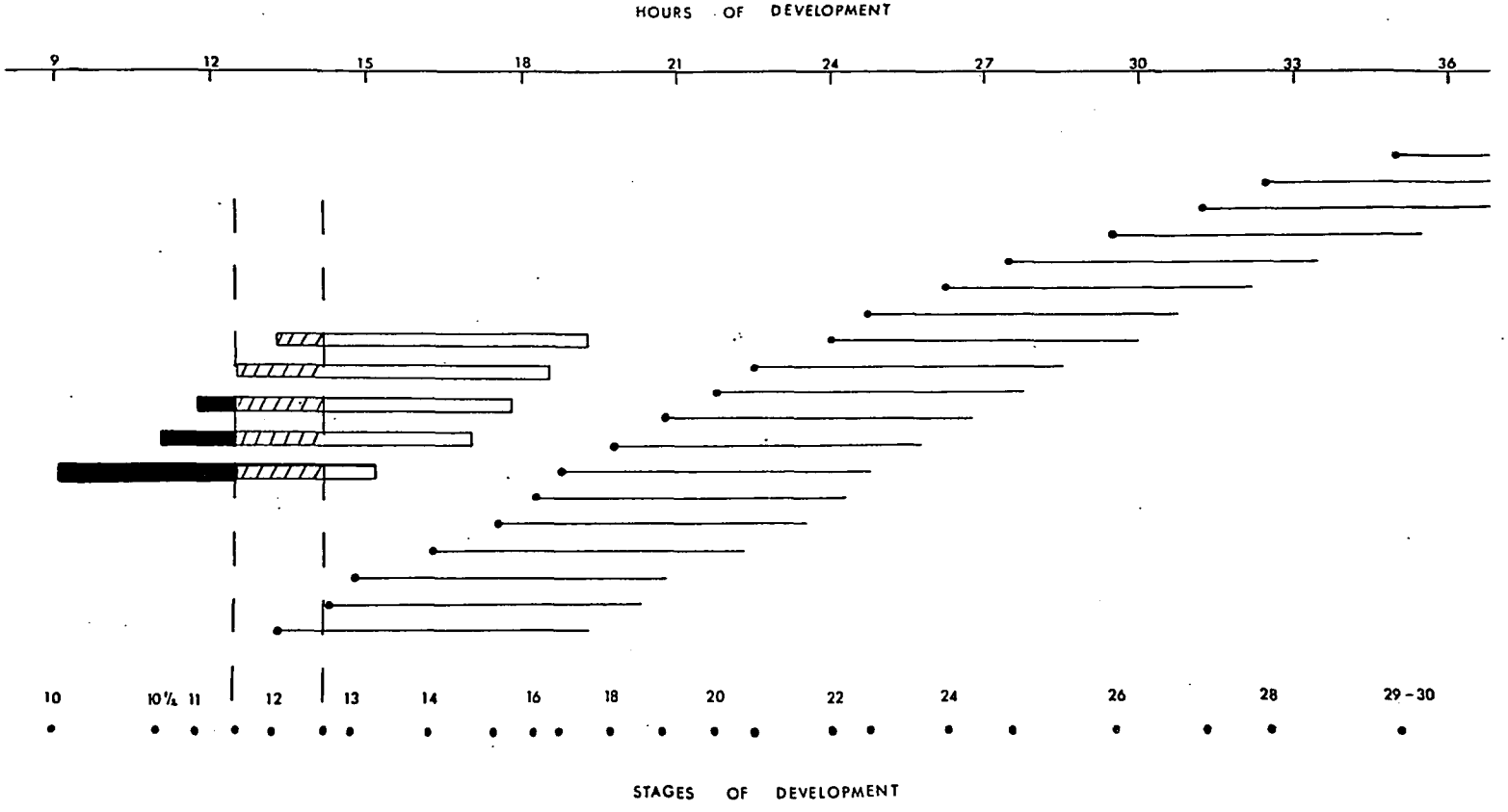

25a
b
c
d

Fig. 26. Diagram of experimental design and results. The lines represent the periods of labeling duration for groups of embryos injected at specific stages. Each line represents at least five embryos, all of which had unlabeled Mauthner cells. The bars represent periods of labeling of groups of four embryos which proved to have labeled Mauthner cells. Clear portion: no incorporation of label into DNA. Hatched portion: probable period of incorporation of the label. Dark portion: period probably preceding the last S phase.


HOURS OF DEVELOPMENT
9
12
15
18
21
24
27
30
33
36
10
10¼
11
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16
18
20
22
24
26
28
29 - 30
STAGES OF DEVELOPMENT

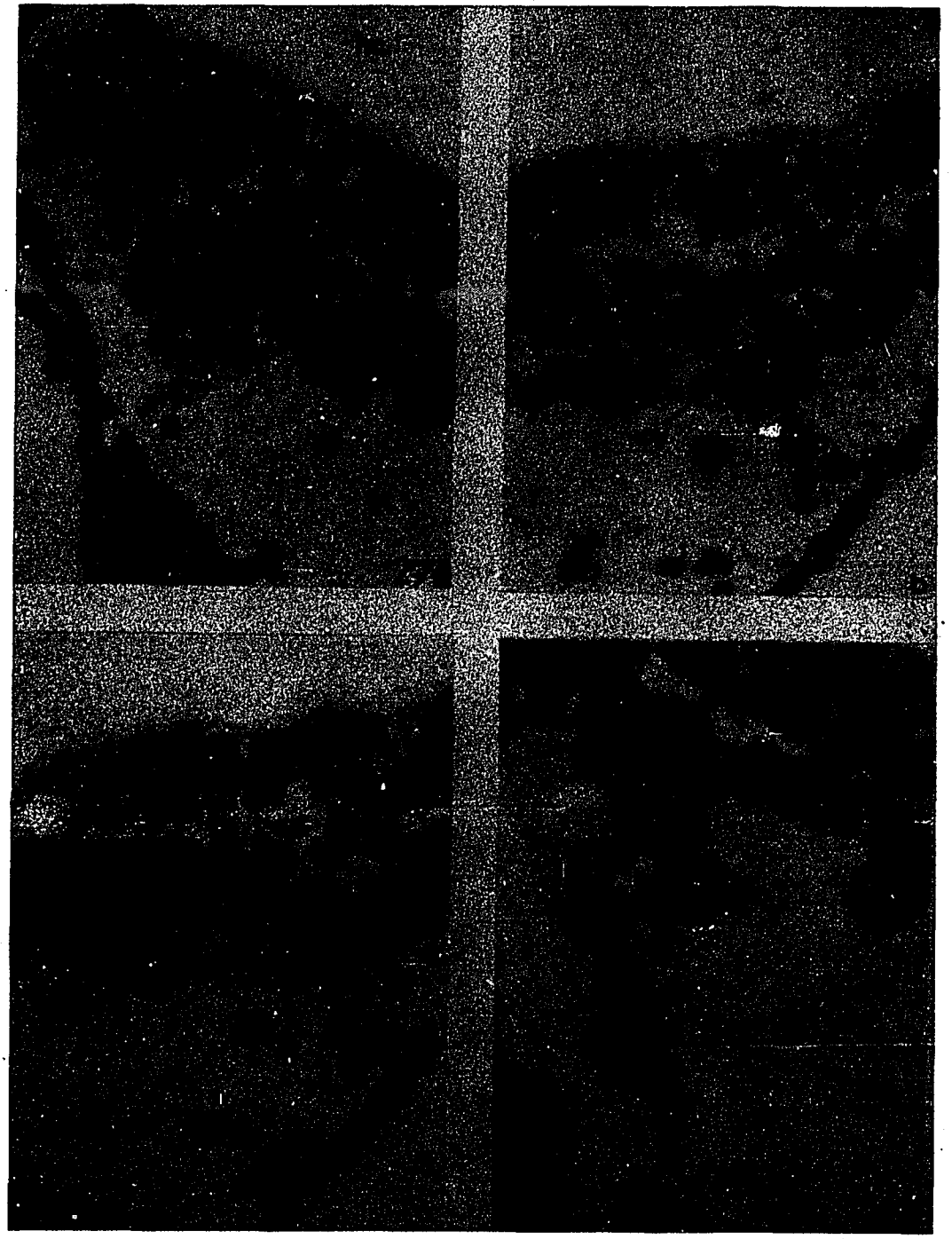
Fig. 27. Radioautographs of an embryo injected with tritiated 5-Bromodeoxyuridine (BrdU) at stage 10-1/2.

Fig. 27a. Mauthner's cell nucleus (N) is labeled. Magnification x 690

Fig. 27b. Contralateral Mauthner's cell. The cell is more clearly seen in this section. The nucleus (N) and nucleolus are heavily labeled. Magnification x 690

Fig. 27c. Following serial section again showing labeled nucleus (N) of Mauthner's cell (M). Magnification x 710

Fig. 28. Radioautograph of Mauthner's cell (M) from an embryo injected at stage 13-1/2 with tritiated BrdU. The cell's nucleus is unlabeled. Following serial sections failed to show any label in the cell. Magnification x 980



Figs. 29-33. Silver stained sections from embryos injected with BrdU. Embryos were fixed in Lawdosky's solution and stained in Bodian's protargol reduced with hydroquinone.

Fig. 29a. Mauthner's cell (M) in an embryo injected at stage 10-1/4. The nucleolus seems to be "vacuolated" (n). Following sections showed the rest of the nucleolus. The cytoplasm is heavily impregnated with the silver stain. The BrdU concentration in this embryo was 67 µg/ml. Magnification x 1500

Fig. 29b. Contralateral Mauthner's cell from the same embryo. This nucleolus was not "vacuolated." Note disarray of cells of the neural tube. The marginal layer is scant. Magnification x 1540

Fig. 30a. Mauthner's cell in an embryo injected at a stage before stage 10. BrdU concentration inside of embryo 33 µg/ml. The Mauthner cell's nucleus (N) seems of normal size as well as its nucleolus. Magnification x 1350

Fig. 30b. The axon's (Ax) course through the marginal layer towards the midline of the neural tube can be followed. Following sections showed the axon's decussation at the midline. The marginal layer is scant. A more than normal number of nuclei are seen as compared to neural tubes from control embryos fixed at this stage (stage 37-38). Magnification x 1350


n
29a


n
b


N
30a


n
Ax
b

Fig. 30c. Section through the telencephalon of same embryo as a and b. The distorted neural tube "seems" to be enveloping the somites (S) and the notochord (Not). Magnification x 310

Fig. 30d. Section through medulla at the level of the Mauthner cell's location. The cell and its axon can be seen at the right side of the neural tube. Note the cellularity of the medulla. Magnification x 310

Fig. 31. Section through the structure that developed in place of an eye in embryo shown in Fig. 29. Only pigment (P) from pigmented cells of the retina distinguish these cells from cells of other structures in the embryo. G = gut. Magnification x 530

Fig. 32 Section through the somite of an embryo injected at stage 10-1/4. This embryo had an internal concentration of BrdU of 28 μg/ml. The somites seemed to be in disarray when compared with control embryos. Magnification x 530

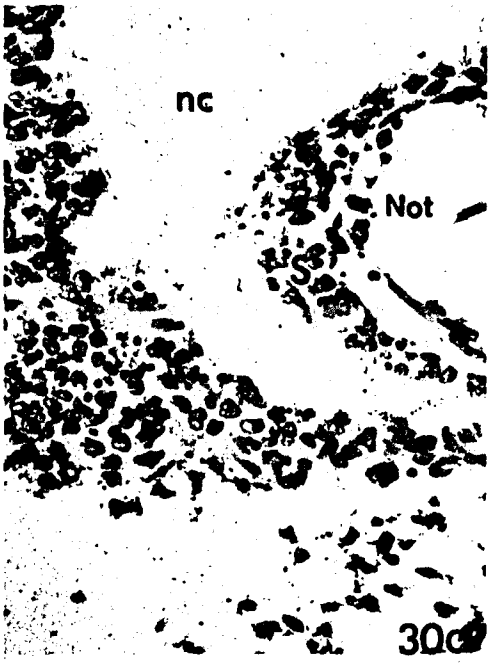
e = ear

S = somites

nc = neurocoel

Not = notochord

P = pigment



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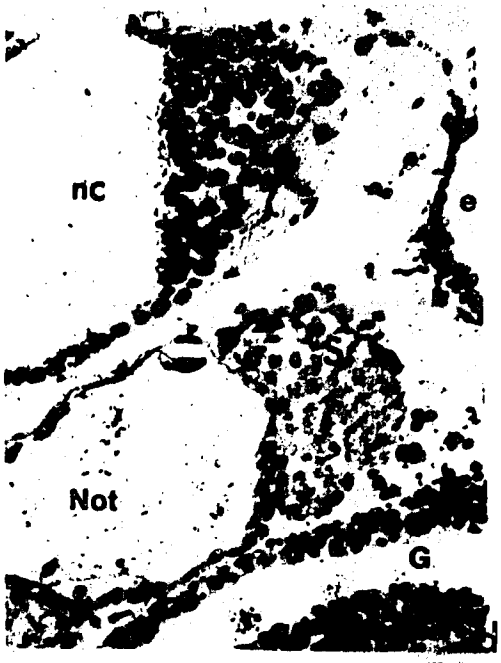

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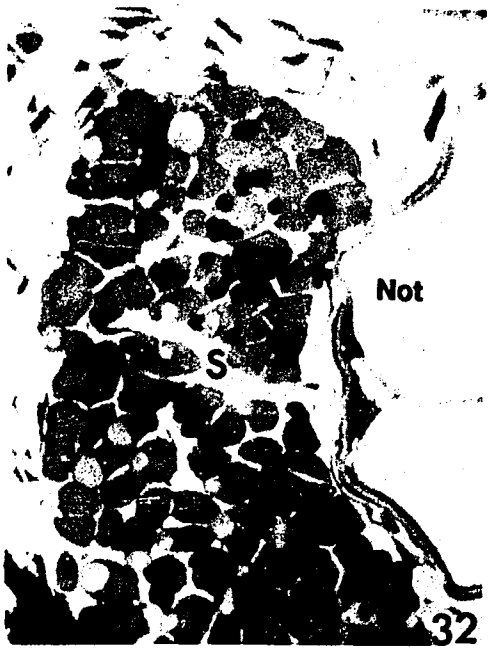


32

Fig. 33. Radioautographs of an embryo injected at stage 15. This embryo had an internal concentration of 50 μm/ml.

Fig. 33a. The Mauthner cell is shown with its large nucleus (N) and nucleolus (n). Its processes are well stained with silver. The neural tube is in total disarray. Magnification x 1130

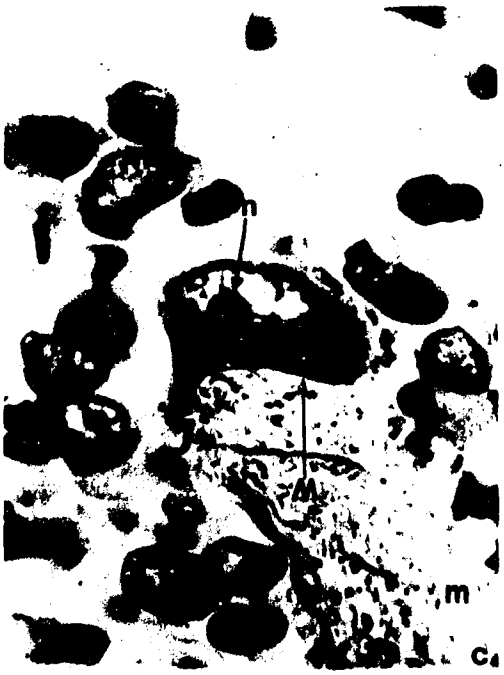
Fig. 33b. Mauthner's cell (M) showing its relationship to other cells in the area. Part of the axon (Ax) is also seen. Magnification x 850

Fig. 33c. Contralateral Mauthner's cell (M). Magnification x 1310

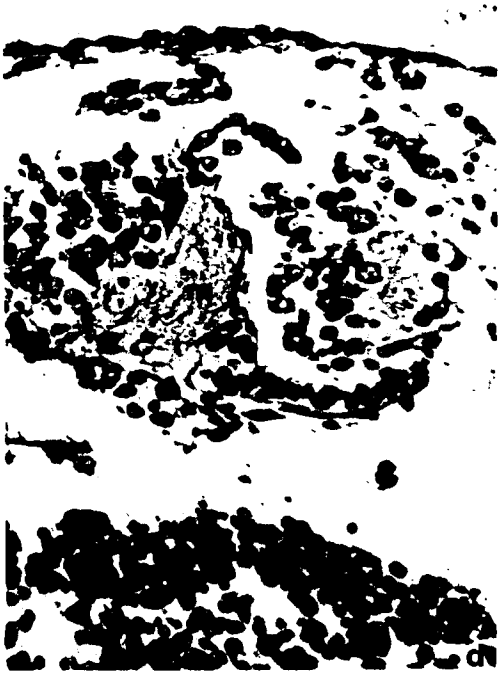
Fig. 33d. Section through neural tube in the location of the medulla. The normal shape of the neural tube cannot be found. Some marginal layer (m) can be appreciated. Magnification x 590

33a

b



c



d

Fig. 34. Overview of a transverse section at the level of the medulla. A Mauthner's cell (arrow) can be seen at the left margin of the neural tube. Note its size in relation to the rest of the structures in the neural tube and other organs. Magnification x 140

Fig. 35a. Section through a control embryo fixed for electron microscopy. This is a 1 micron section stained with Toluidine blue. Both Mauthner cells (M) can be seen in the same section. Magnification x 340

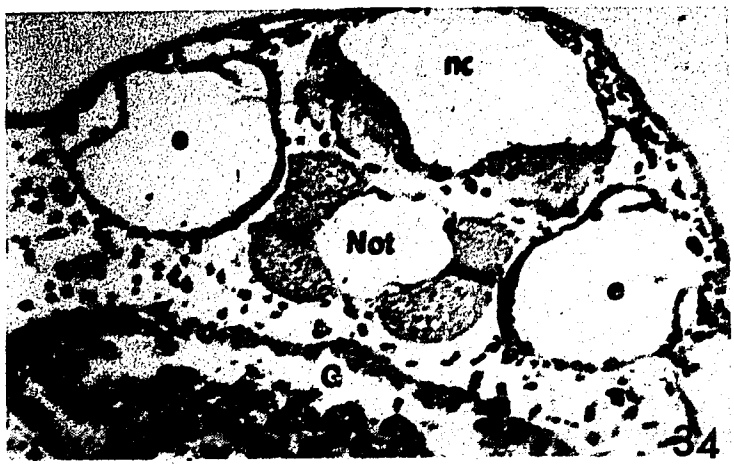
Fig. 35b. High magnification of section 35a, showing the Mauthner cell with its axon (ax) and ventro-medial dendrite (vd). Magnification x 1350

N = nucleus

m = marginal layer

Cy = cytoplasm

nc = neurocoel



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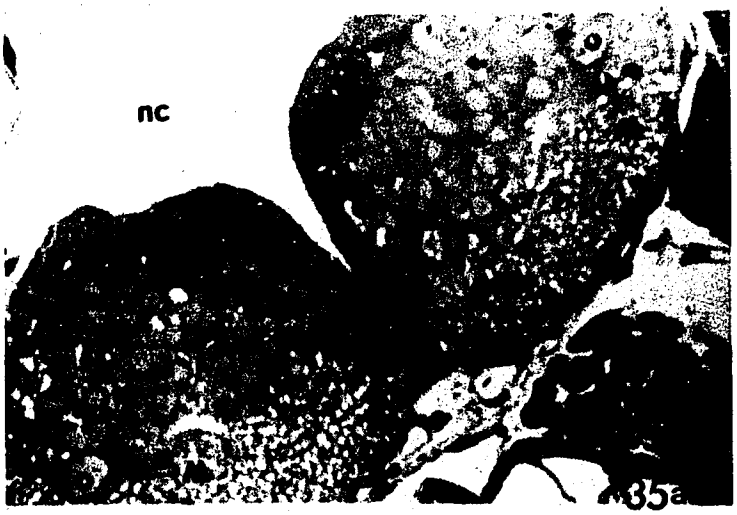
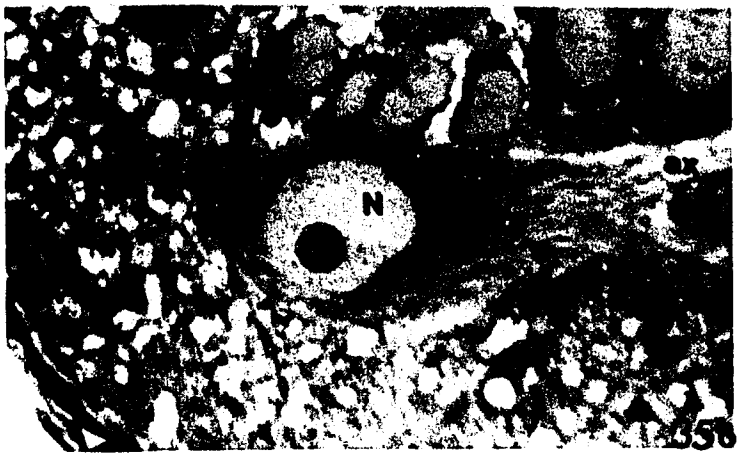


35a



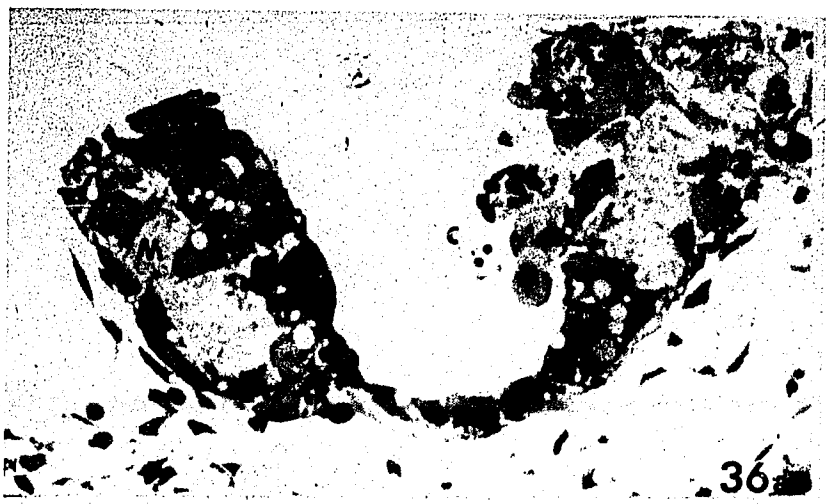
35b

Fig. 36. Light microscope section (1 micron thick) stained with Toluidine blue. This embryo was injected at stage 10 and had an internal concentration of BrdU of 28 µg/ml.

Fig. 36a. The Mauthner cell (M) and its processes are seen on the left of the neural tube. Some cells (c) attached to the neural tube can be seen protruding into the neurocoel. Magnification x 540

Fig. 36b. High magnification of section 36a. Magnification x 1320

Fig. 36c. Contralateral Mauthner cell (M) of same embryo. Yolk granules (y) near the nucleus can be seen. Magnification x 1500


M
C
36


M
M

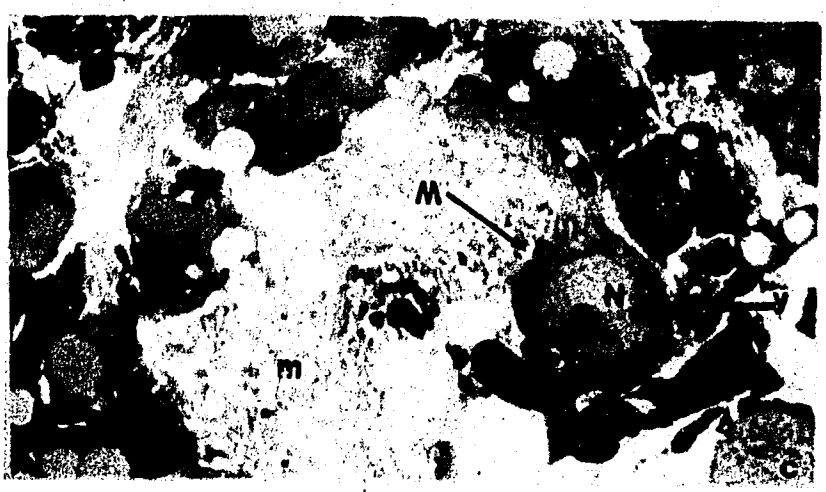

M
N
m

Fig. 37. Light micrographs of an embryo prepared for electron microscopy. Embryo was injected at stage 24 and had an internal concentration of 105 µg/ml.

Fig. 37a. The Mauthner cell is shown (arrowhead) in a well-developed neural tube. There is some disruption of the cellular organization in the neural tube. Aggregates of cells (c) are seen protuding into the neurocoel. This phenomenon has never been observed in any other experimental or control animal used in this study. Magnification x 600

Fig. 37b. Higher magnification of section a. Magnification x 900

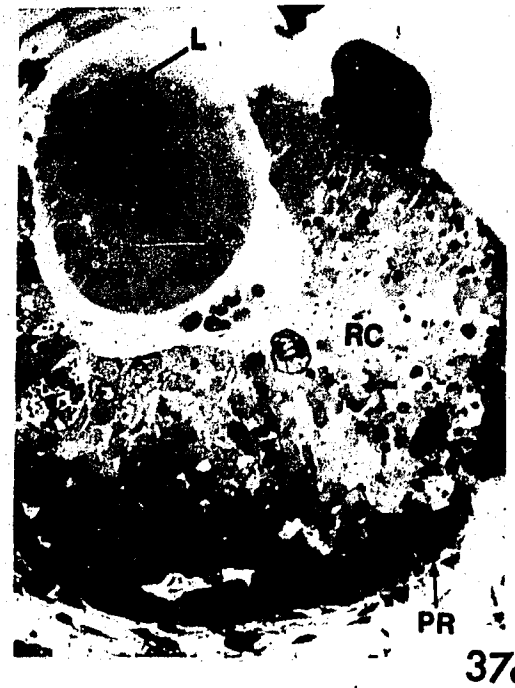
Fig. 37c. Contralateral Mauthner's cell. Only part of the nucleus is seen in this section but its cytoplasm (cy) is very abundant. A big lipid granule is seen in the dorsal portion of the cell. Magnification x 1310

a
b
c
d

Fig. 37d. Light micrograph (1 micron thick) of retina from same embryo as 37a, b, c. The lens has developed and is highly cellular; there is no indication of fiber lens formation. The different neural retina cells are pleomorphic and show no specific pattern of layers. Magnification x 330

Fig. 37e. Another section through the neural retina. The pigmented retinal cells (PR) have formed a layer and pigment can be seen. No plexiform layers are seen. Magnification x 510

Fig. 38. This is a section through a normally differentiated eye from an embryo which was chosen for radioautography. The rods and cones (rc) are clearly seen as well as the pigmented cells of the pigmented retina (PR) and the ganglion cells (rg). The plexiform layers are seen (PL). There is fiber formation as well as cells in the lens (L). Magnification x 340



37d

e

38

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