POSTNATAL NEUROGENESIS IN THE CEREBELLUM OF THE CAT AND TRITIATED THYMIDINE AUTORADIOGRAPHY

GOPAL D. DAS AND JOSEPH ALTMAN

Laboratory of Developmental Neurobiology, Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907 (U.S.A.)

(Accepted January 19th, 1971)

INTRODUCTION

In autoradiographic studies on mammalian neurogenesis intraperitoneal injection of [^{3}H]thymidine is the commonly employed route of administration of the labeled precursor of DNA. Studies on the postnatal neurogenesis in mammals, like the mouse[13,14,16], rat[3,5,6] and guinea pig[7] bear testimony to the success and reliability of this technique. In our attempts to study postnatal neurogenesis in the cat it was found that, unlike in the mouse and rat, the standard dose of [^{3}H]thymidine (10 μCi/g body weight) when administered intraperitoneally failed to show any labeling of the precursors of neurons in the central nervous system, although histologically postnatal neurogenesis was evident in this species also. Since the cat is used very widely in neuroanatomical, neurophysiological and behavioral research, it was felt necessary to determine the factors that may be contributing to the apparent failure of the technique of tritiated thymidine autoradiography in this species.

MATERIAL AND METHODS

Six-day-old kittens were injected intraperitoneally or intracisternally with [^{3}H]-thymidine (spec. act. 6.7 Ci/mmole; 1 mCi dissolved in 1 ml of sterile saline solution). In 3 kittens the intraperitoneal route was used. The first of these received 2.0 mCi (dose: 10 μCi/g body weight), the second 3.8 mCi (dose: 20 μCi/g body weight), and the third 6.4 mCi (dose: 40 μCi/g body weight). These animals were allowed to survive for 6 h following the injections.

In four 6-day-old kittens the intracisternal route was employed for the administration of the radiochemical. These animals were anesthetized with Nembutal (30 mg/kg body weight) and the cisterna magna was exposed surgically. In each animal about 300 μl of cerebrospinal fluid was withdrawn using a Hamilton syringe prior to injection of the radiochemical. In 2 animals 100 μCi of [^{3}H]thymidine was injected in the cisterna magna and in 2 other animals 250 μCi. During the survival period (6 h) the animals were kept on a heating pad in prostrate position. The intent of

having 6 h as the survival period was to obtain maximum number of cells with maximum intensity of labeling on them.

All the animals were sacrificed by cardiac perfusion with 10% neutral formalin and their brains were removed. These brains, after further fixation for 2 weeks in 10% neutral formalin, were embedded in Paraplast and sectioned serially in the coronal plane at 6 μm. Following standard histological processing the sections were treated for autoradiography. The slides were coated with Kodak NTB-3 nuclear emulsion by the dipping technique. The autoradiograms were exposed at 5°C in light proof boxes containing Drierite as the desiccant for 13 weeks. At the end of the exposure period they were developed in the standard manner and stained with cresyl violet[2].

For comparative evaluation we used autoradiograms from the existing material in our laboratories of the brains of 6-day-old rats each of which had received 0.14 mCi of [^{3}H]thymidine intraperitoneally (dose: 10 μCi/g body weight; spec. act. 6.7 Ci/mmole, 1 mCi dissolved in 1 ml of sterile saline solution) and were killed 6 h later. The histological and autoradiographic preparation of this material was identical with that used for the kittens.

Evaluation of the autoradiograms

With intracisternal injection of [^{3}H]thymidine it was found that the posterior lobes of the cerebellum which were close to the surface showed a maximum number of cells with high intensity of labeling. Therefore in all the kittens the uvula of the cerebellar vermis was selected for quantitative evaluation. With the aid of an eye-piece grid the total number of labeled cells was counted in 25 unit areas (64 μm $\times$ 320 μm) in the proliferative zone of external granular layer and in the same number of larger unit areas (320 μm $\times$ 320 μm) in the internal granular layer. From these data the average number of labeled cells in unit areas in the proliferative zone of external granular layer and the internal granular layer was calculated. In the material from the rats the total number of labeled cells was counted in 25–30 unit areas (32 μm $\times$ 320 μm) in the proliferative zone of external granular layer and in the same number of unit areas (160 μm $\times$ 320 μm) in the internal granular layer. The total number of labeled cells was reduced to an average per unit area, and these values were doubled to permit a comparison with the data from the kittens.

RESULTS

Quantitative observations

Histologically two zones may be distinguished in the external granular layer, an outer zone which is typically composed of round proliferative cells, and an inner zone, composed of spindle-shaped migrating cells. The former is known as the proliferative zone and the latter as the migratory zone. Following injections, in all the cases if any labeled cells were found these were seen exclusively in the proliferative zone. In the kittens that received [^{3}H]thymidine intraperitoneally at the rate of 10 μCi/g body

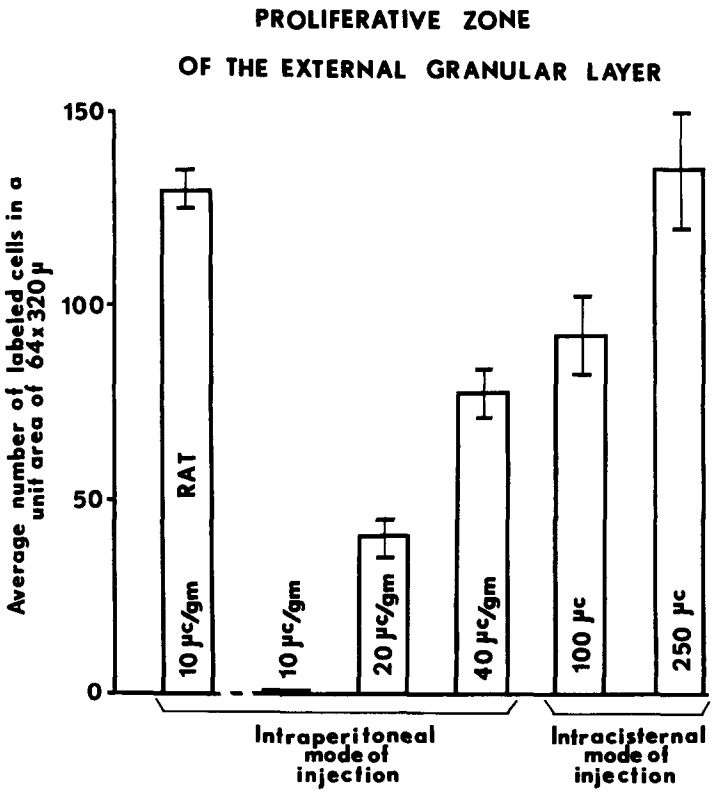


Fig. 1. Graph showing average number of labeled cells in a unit area of 64 μm $\times$ 320 μm in *the proliferative zone* of the external granular layer in 6-day-old kittens. On the extreme left data from 6-day-old rats are presented for comparison.

weight, no labeled cells were found. By increasing the dose to 20 μCi and then to 40 μCi/g body weight a corresponding increase in the number of labeled cells occurred. The average number of labeled cells with 40 μCi/g body weight was approximately twice that obtained with 20 μCi/g body weight. However, in the rat the standard dose of 10 μCi/g body weight yielded 75% more cells than in a kitten with a dose of 40 μCi/g body weight (Fig. 1).

With intracisternal administration of the radiochemical the average number of labeled cells was higher than that following intraperitoneal injection but is was restricted to the vicinity of the injection site. In this case we found that by increasing the amount of radiochemical by 150% (from 100 μCi to 250 μCi) a relatively small (45%) increase in the labeled cells was achieved (Fig. 1).

In the internal granular layer no labeled granular cells were seen when the radiochemical was administered at the rate of 10 μCi/g body weight intraperitoneally. Labeled granular cells were seen in the kittens that received [^{3}H]thymidine in the doses of 20 μCi/g and 40 μCi/g body weight. With a 100% increase in the dose from 20 μCi to 40 μCi/g body weight there was nearly 300% increase in the number of labeled granular cells. However, in rats the standard dose of 10 μCi/g body weight

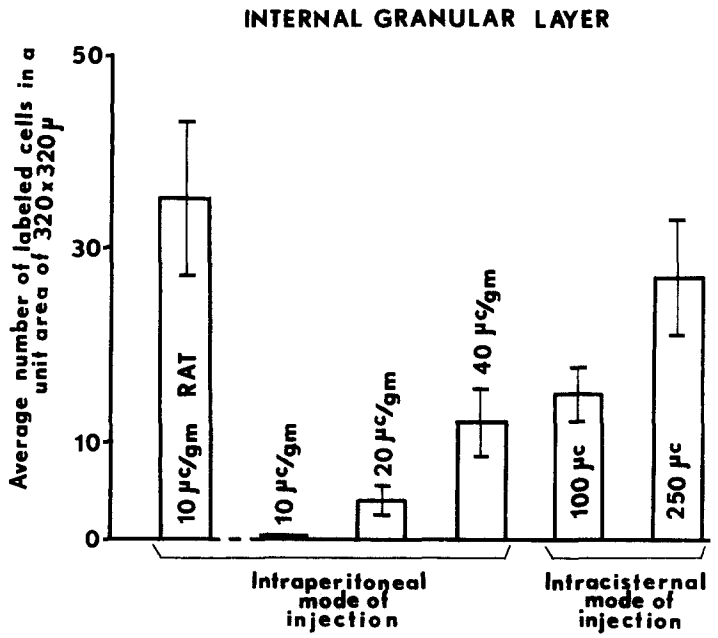


Fig. 2. Graph showing average number of labeled cells in a unit area of 320 μm $\times$ 320 μm in the internal granular layer in 6-day-old kittens. Data presented on the extreme left are from 6-day-old rats.

yielded a larger number of labeled granular cells, about 300% more than what was achieved in the kitten with the dose of 40 μCi/g body weight (Fig. 2).

Following the intracisternal route of administration of [^{3}H]thymidine the average number of labeled granular cells was higher than that obtained after intraperitoneal injection but again cell labeling was restricted to the vicinity of the injection site. However, with a 150% increase in the quantity of [^{3}H]thymidine (from 100 μCi to 250 μCi) there was 60% increase in the average number of labeled granular cells (Fig. 2).

Qualitative observations

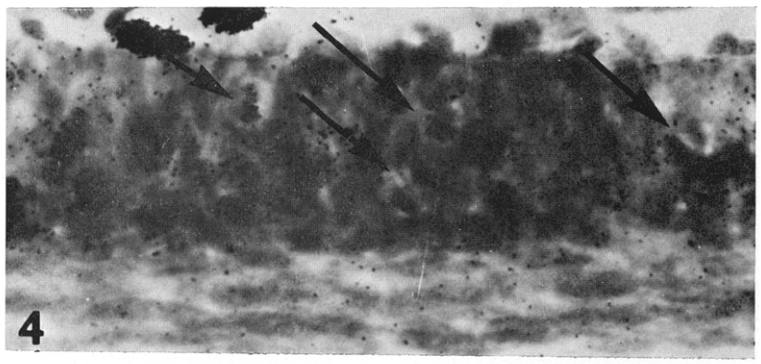
The dosage of [^{3}H]thymidine, when employing the intraperitoneal route, in addition to influencing the total number of labeled cells had an influence on the grain

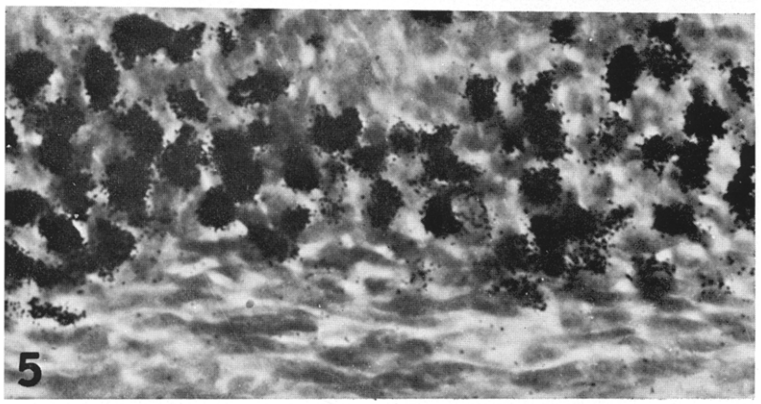
---

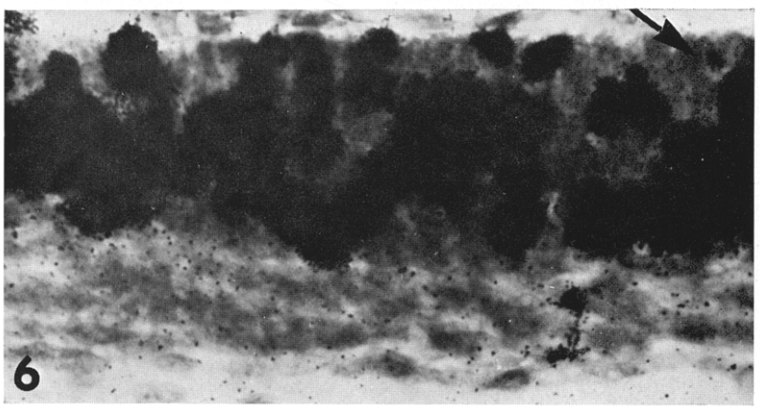
Figs. 3–6. High-power photomicrographs of autoradiograms of the *external granular layer* in the uvula of cerebellum in 6-day-old kittens. Note the labeled cells found in the proliferative zone only. Underneath proliferative zone is the zone of spindle-shaped migratory cells. *The photomicrographs are focused to show the reduced silver grains lying over the nuclei of cells and therefore the cells are out of focus.* Arrows indicate mitotic cells. Six μm thick sections, cresyl violet stain. $\times$ 1,250.

Fig. 3. Intraperitoneal injection, dose: 20 μCi/g body weight; total quantity injected: 3.8 mCi.
Fig. 4. Intraperitoneal injection, dose: 40 μCi/g body weight; total quantity injected: 6.4 mCi.
Fig. 5. Intracisternal injection, total quantity injected: 100 μCi/ (0.10 mCi).
Fig. 6. Intracisternal injection, total quantity injected: 250 μCi (0.25 mCi).


3


4


5


6

concentration over the labeled cells. In the proliferative zone of the external granular layer and in the internal granular layer the label concentration over the cells was barely a few grains above the background level (Fig. 3) when [^{3}H]thymidine was administered at the rate of 20 μCi/g body weight. When the dose was increased to 40 μCi/g body weight the cells in these very regions showed higher label concentration (Fig. 4). The intracisternal mode of injection of the radiochemical yielded not only a large number of labeled cells but also a very high label concentration. With increasing amount of the radiochemical administered the number of labeled cells as well as the intensity of labeling appeared to increase (Figs. 5 and 6).

DISCUSSION

The observations made in this study lead us to the following general conclusions. When [^{3}H]thymidine was injected intraperitoneally to the young kittens using a dose of 10 μCi/g body weight, which is the standard dose employed in work with rodents, the radiochemical did not seem to reach the brain. If this dose was doubled, that is 20 μCi/g body weight, some cells synthesizing DNA were found labeled. With further increase in dosage, that is 40 μCi/g body weight, the number of labeled cells as well as intensity of labeling on them were increased. Even with such a high dose of [^{3}H]-thymidine the number of labeled cells and the intensity of labeling on them appeared far less than what could be achieved in rodents with a standard dose of 10 μCi/g body weight. These facts suggest that the radiochemical when injected intraperitoneally in the kittens does indeed reach their brains, but there may be some special blood–brain characteristics in this species which seem to restrict the availability of [^{3}H]thymidine to the neural tissue. What these special characteristics in the kittens are, is unknown; but certainly, it may be concluded, they are not present in the young rodents. It may be possible to label many more mitotically active cells in the proliferative zone of the external granular layer and in the internal granular layer of cerebellum of kittens by injecting [^{3}H]thymidine intraperitoneally using very high doses, but this may be an undesirable procedure considering that a dose of 20 μCi/g body weight causes radiation damage to the cells of various tissues[10,11,15].

Furthermore, one could argue that failure to label as many mitotically active cells as would be expected from the histological evaluation of brain sections of the kittens, may be due to less extensive postnatal neurogenesis in the cerebellum of kittens. That this is not the case was established by achieving the labeling of a very large number of proliferative cells when [^{3}H]thymidine was administered intracisternally, and this confirmed our observations made on histological evaluation of the brain sections. However, use of the intracisternal mode of administering [^{3}H]thymidine is fraught with a number of limitations which make it impracticable, if not impossible, for normative as well as experimental studies.

When [^{3}H]thymidine is administered intraperitoneally it is available for about 30–60 min[8–10,17], providing pulse labeling whereas when it is injected intracisternally it may be available for longer duration since circulation of the cerebrospinal fluid is known to be sluggish, taking about 8 h for one circulatory cycle[12]. Furthermore, we

found that there was a sharp gradient in the number of labeled cells and in intensity of labeling in the proliferative zone of the external granular layer from superficial regions to the deep regions of different lobules of the cerebellum, presumably because of the sluggish and non-uniform circulation of the cerebrospinal fluid. Under these circumstances the radiochemical seemed to be available only in a few regions of the cerebellum for a long duration and not at all in other regions. These considerations limit the usefulness of [^{3}H]thymidine autoradiography for the quantitative as well as qualitative studies of cell proliferation in the brains of kittens. Similar conclusions were arrived at by Altman[1], and Altman and Chorover[4] in studies in which various radiochemicals were injected into the third ventricle of adult cats.

SUMMARY

In the cerebellum of 6-day-old kittens evidence for extensive postnatal neurogenesis was established by administering [^{3}H]thymidine intracisternally. When the radiochemical was administered intraperitoneally, it did not appear to reach the central nervous system sufficiently. For both the routes of administration of the radiochemical, namely intracisternal and intraperitoneal, limitations on the use of tritiated thymidine autoradiography for the study of neurogenesis in the cat were brought out.

ACKNOWLEDGEMENT

This research is supported by the U.S. Atomic Energy Commission and the National Institute of Mental Health.

REFERENCES

1 ALTMAN, J., Autoradiographic investigation of cell proliferation in the brains of rats and cats, *Anat. Rec.*, 145 (1963) 573–592.
2 ALTMAN, J., The use of fine-resolution autoradiography in neurological and psychobiological research, In T. J. HALEY AND R. S. SNIDER (Eds.), *Response of the Nervous System to Ionizing Radiation*, Little, Brown and Co., Boston, Mass., 1964, pp. 336–359.
3 ALTMAN, J., Autoradiographic and histological studies of postnatal neurogenesis. II. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in infant rats, with special reference to postnatal neurogenesis in some brain regions, *J. comp. Neurol.*, 128 (1966) 431–474.
4 ALTMAN, J., AND CHOROVER, S. L., Autoradiographic investigation of the distribution and utilization of intraventricularly injected adenine-^{3}H, uracil-^{3}H and thymidine-^{3}H in the brains of cats, *J. Physiol. (Lond.)*, 169 (1963) 770–779.
5 ALTMAN, J., AND DAS, G. D., Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats, *J. comp. Neurol.*, 124 (1965) 319–336.
6 ALTMAN, J., AND DAS, G. D., Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions, *J. comp. Neurol.*, 126 (1966) 337–390.
7 ALTMAN, J., AND DAS, G. D., Postnatal neurogenesis in the guinea pig, *Nature (Lond.)*, 214 (1967) 1098–1101.
8 BLENKINSOPP, W. K., Duration of availability of tritiated thymidine following intraperitoneal injection, *J. Cell Sci.*, 3 (1968) 89–95.

9 BRESCIANI, F., Effect of ovarian hormones on duration of DNA synthesis in cells of the C3H mouse mammary gland, *Exp. Cell Res.*, 38 (1965) 13–32.
10 CRONKITE, E. P., BOND, V. P., FLIEDNER, T. M., AND RUBINI, J. R., The use of tritiated thymidine in the study of DNA synthesis and cell turnover in hemopoietic tissues, *Lab. Invest.*, 8 (1959) 263–277.
11 CRONKITE, E. P., FLIEDNER, T. M., KILLMANN, S. A., AND RUBINI, J. R., Tritium-labelled thymidine (H^3-TdR): Its somatic toxicity and use in the study of growth rates and potentials in normal and malignant tissue of man and animals. In *Tritium in the Physical and Biological Sciences, Vol. 2*, International Atomic Energy Agency, Vienna, 1962, pp. 189–206.
12 CROSBY, E. C., HUMPHREY, T., AND LAUER, E. W., *Correlative Anatomy of the Nervous System*, MacMillan, New York, 1962, p. 576.
13 FUJITA, S., Quantitative analysis of cell proliferation and differentiation in the cortex of the postnatal mouse cerebellum, *J. Cell Biol.*, 32 (1967) 277–287.
14 FUJITA, S., SHIMADA, M., AND NAKAMURA, T., H^3-Thymidine autoradiography studies on the cell proliferation and differentiation in the external and internal granular layers of the mouse cerebellum, *J. comp. Neurol.*, 128 (1966) 191–208.
15 JOHNSON, H. A., AND CRONKITE, E. P., The effect of tritiated thymidine on mouse spermatogonia, *Radiat. Res.*, 11 (1959) 825–831.
16 MIALE, I. L., AND SIDMAN, R. L., An autoradiographic analysis of histogenesis in the mouse cerebellum, *Exp. Neurol.*, 4 (1961) 277–296.
17 RUBINI, J. R., CRONKITE, E. P., BOND, V. P., AND FLIEDNER, T. M., The metabolism and fate of tritiated thymidine in man, *J. clin. Invest.*, 39 (1960) 909–918.