

INDUCTION OF DNA SYNTHESIS IN CULTURED NEURONS BY ULTRAVIOLET LIGHT OR METHYL METHANE SULFONATE

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INTRODUCTION

Cellular DNA can suffer single-stranded chemical damage as a consequence of exposure to high-energy radiation or certain chemicals. Bacteria can repair this damage by excising a segment of the damaged strand and synthesizing a new segment complementary to the undamaged strand (14). Mammalian cells can also be induced to synthesize small amounts of DNA, when they would not ordinarily be doing so, by exposing them to ultraviolet light (12), X rays (10), or certain alkylating agents (5, 13). This "unscheduled DNA synthesis" has been demonstrated to be nonsemi-conservative repair synthesis in a number of cases (2, 10-13).

The cell types in which repair synthesis was originally studied were either actively proliferating (2, 5, 10-14) or capable of further proliferation (3). Recently, it was reported that unscheduled synthesis can also be induced in differentiated muscle nuclei, although the reported results were interpreted to indicate that a reduction in repair capability accompanies differentiation (4, 15). We report here that vertebrate sensory neurons in culture, though they never divide, can be induced to synthesize DNA by exposure to ultraviolet light (UV) or the alkylating agent methyl methane sulfonate (MMS).

MATERIALS AND METHODS

The experiments were conducted with cultures of dissociated neurons from pooled brachial, thoracic, and lumbosacral dorsal root ganglia of 8- to 10-day old chick embryos. Neurons were dissociated by trypsin digestion and mechanical agitation, separated from nonneuronal cells, and cultured on collagen-coated cover slips by methods which have been described in detail elsewhere (9). The culture medium employed was Leibovitz's L-15 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum, 0.06% glucose, 1 unit/ml nerve growth factor (Burroughs Wellcome and Co., Tuckahoe, N.Y.), 100 units/ml penicillin G, and 50 μ g/ml streptomycin sulfate.

Neurons in such cultures extend long, highly arborized, electrically excitable processes, recover from chromatolysis to assume a mature cytological appearance within about 2 wk, and remain healthy by morphological and electrical criteria for more than a month (9). In the routine preparative procedure, neurons are almost entirely freed of fibroblasts and glia; however, for the experiments reported here, incompletely purified cultures were chosen so that DNA synthesis in fibroblasts and neurons could be compared. Occasional glial cells were also present in the selected cultures but were ignored in these studies. The cultures, when used, contained approximately equal numbers of neurons and fibroblasts.

DNA synthesis was assayed by radioautography of culture cover slips which had been incubated in medium containing thymidine- 3 H (TdR- 3 H) as described below. After incubation, the cover slips were washed several times in isotonic buffer (Puck's saline G), fixed for 5 min in absolute methanol, washed successively in the buffer and water, dried, and dipped into Kodak NTB-2 liquid emulsion diluted 1:1 with water. Radioautographs were developed and examined after 15-25 days' exposure. Cells were considered to be labeled when the density of silver grains over them was at least twice the background level. Identification of the labeled material as DNA was based on the following observations: (a) The label was localized almost entirely over cell nuclei. Diffuse cytoplasmic labeling was seen only after cultures had been incubated in TdR- 3 H for 24 hr, 8 times the incubation period used in the experiments (see below). (b) Label remained confined to the nuclei when cells were incubated in cold medium for 2 days after their exposure to TdR- 3 H. In contrast, cytoplasmic labeling was evident in neurons fixed only 2-4 hr after an exposure to uridine- 3 H. (c) No grains appeared if the fixed cultures were incubated with DNase (500 μ g DNase I/ml for 20 min at 36°C) before radioautography. (d) Incubation of the fixed cultures with RNase (400 μ g beef pancreas RNase/ml for 20 min at 36°C) had no detectable effect on the distribution or quantity of silver grains. The same RNase treatment removed the label from cells which had been incubated for 2-3 hr with uridine- 3 H.

The source for UV irradiation was a germicidal

lamp (General Electric G15T8) emitting primarily at 2537 Å and set 55 cm above the cultures so as to deliver a nominal dose of 10 erg/mm² per sec. Irradiation was carried out at room temperature with the exposed cultures and controls immersed in a small amount of a saline buffer (Puck's G) having about the same absorption as water in the UV range of interest. Irradiated cultures and controls were then incubated for 3 hr at 36°C in culture medium containing TdR-³H (New England Nuclear Corp., Boston, Mass., 17.8 Ci/mole) at a concentration of 10 μCi/ml.

Treatment with MMS was carried out by incubating cultures for 45 min at 36°C in culture medium to which the drug (100–200 μg/ml) and TdR-³H (10 μCi/ml) had been added. The treated cultures were then washed in Puck's saline G and transferred to fresh medium containing TdR-³H (10 μCi/ml), but no MMS, for a further 2.5 hr of incubation before fixing and radioautography.

RESULTS AND DISCUSSION

In control cultures incubated for 3 hr in the TdR-³H medium, but not exposed to UV or MMS, about 10% of the fibroblasts had nuclei so

densely labeled that individual grains could not be distinguished; these cells were assumed to be the ones which were replicating DNA during the incubation with TdR-³H. 85% of the fibroblasts in these cultures were completely unlabeled, and the remaining 5% had intermediate levels of nuclear labeling. Neurons were never densely labeled by TdR-³H under any of the conditions examined, and more than 98% of them were completely unlabeled in the control cultures. This confirms a previous report that neurons do not normally incorporate TdR-³H in culture (16).

When cultures were exposed to UV before incubation with TdR-³H, almost no densely labeled (replicating) fibroblasts were seen. Instead, with increasing dosage of UV, an increasing percentage of fibroblast nuclei were lightly labeled (Fig. 1 *a*), and the neurons responded similarly (Figs. 1 *b-d*). Absolute dose-response relationships varied from experiment to experiment; the averaged results of several typical experiments are shown in Fig. 2.

The doses of UV employed in these studies were clearly detrimental to the neurons. For example

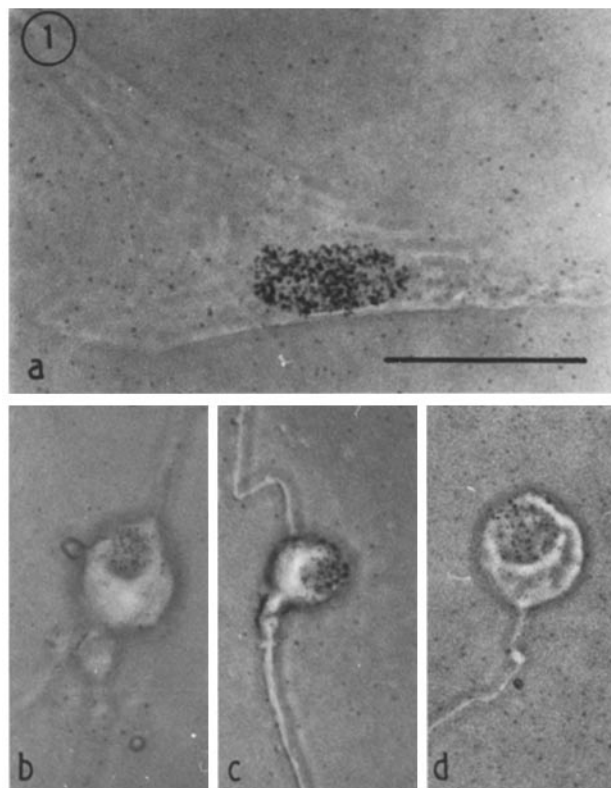


FIGURE 1 Representative cells after 100 sec of UV irradiation, incubation with TdR-³H, and radioautography. *a*, fibroblast; *b-d*, neurons. Magnification is the same for all photographs; scale in *a* is 50 μ. × ~ 520.

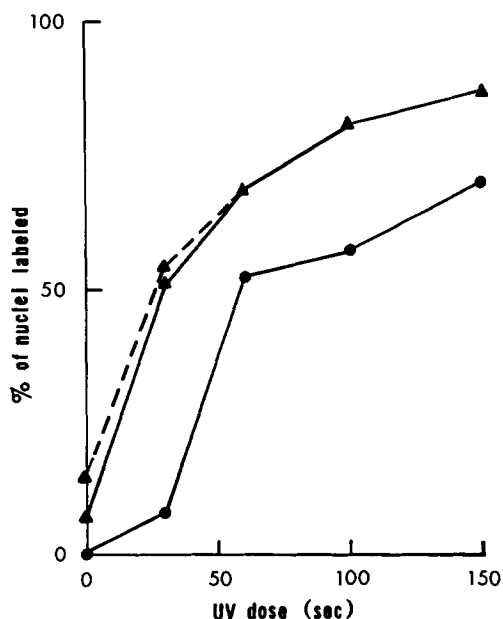


FIGURE 2 Per cent of nuclei labeled as a function of UV dose. —●—, neurons; ---▲---, fibroblasts; —▲—, fibroblasts, excluding those with heavily labeled nuclei (see text). Each point represents scoring of 50–200 cells.

in cultures irradiated for 60 sec, 40% of the neurons were lost during the following 2 days, while fewer than 5% were lost from control cultures over the same period. It was also noted that exposure to UV markedly inhibited the incorporation of uridine-³H by both neurons and fibroblasts. The following experiment was therefore performed to investigate the possibility that detectable DNA synthesis occurred only in those neurons that were lethally irradiated. Two sets of cultures were irradiated for 60 sec and incubated with TdR-³H for 3 hr. One set was then washed and fixed immediately; the other was washed and then placed in growth medium, without added thymidine, for an additional 2 days before fixing. After radioautography, 80% of the neurons fixed soon after irradiation were labeled, compared with 85% of the neurons that survived for 2 days. Since the percentage of labeled cells did not fall during the 2 days after irradiation, it may be concluded that unscheduled DNA synthesis was not restricted to an acutely moribund fraction of the irradiated population.

Histological studies indicate that in chick dorsal root ganglia (brachial), neuroblast division begins to decline on the seventh embryonic day and that mitotic activity ceases by day 9 (6). This suggests that the neurons employed in the above experi-

ments, having been explanted from 8-day embryos and cultured for 5–15 days in vitro before irradiation, were well beyond the proliferative stage. Further, there was no morphological or radioautographic evidence of replication in any of the cultured neurons. Nonetheless, it is possible that induced DNA synthesis was observed only because the neurons had not matured sufficiently by 8 days in vivo and did not continue normal maturation in vitro. We, therefore, performed an irradiation experiment with neurons obtained from 10-day embryos. When tested, at 4 days in vitro, UV induction of TdR-³H incorporation was as great in these neurons (70% of nuclei labeled after 120 sec of irradiation) as in neurons from 8-day embryos. Neurons explanted from embryos much older than 10 days did not survive well with the preparative and culture procedures employed and therefore could not be tested.

DNA repair synthesis induced by alkylating agents has been described in bacteria (7) and mammalian cells (5, 11, 13). The UV- and chemically-activated repair systems have been shown to be genetically identical in bacteria (1). Similarly, we found that unscheduled DNA synthesis could be induced in the neurons by MMS, a monofunctional agent which methylates DNA (8), as well as by UV irradiation. The absolute dose-response relationship varied considerably between experiments with MMS, but 10–30% of the neurons showed nuclear labeling by TdR-³H after incubation with 100 μ g MMS/ml and 20–60% were labeled after incubation with 200 μ g MMS/ml. In the same cultures, ~20% of the fibroblasts were labeled after exposure to 100 μ g MMS/ml and up to 90% were labeled after incubation with 200 μ g MMS/ml. Cells used in the MMS experiments were obtained from 8-day embryos and were cultured 4–6 days in vitro before being tested.

To extend these findings to another species, we have performed preliminary experiments with cultures of dorsal root ganglion neurons explanted from mouse embryos (~2 wk *in utero*). When challenged with UV irradiation (at 3 days in vitro), the mouse neurons displayed synthetic capability comparable to that of chick neurons.

After all treatments, the radioautographic grain density was higher over the nuclei of fibroblasts than it was over neuronal nuclei (the examples in Fig. 1 are representative). There are several plausible explanations for this difference: (a) UV and MMS may be less effective in reaching or damaging the DNA of neurons than they are the

DNA of fibroblasts. (b) With a given amount of damage, neurons may incorporate less TdR-³H than fibroblasts because of differences in synthetic capability or precursor pool size. (c) Radioautographic efficiency may be lower for the spheroidal neurons than for the virtually flat fibroblasts. The third alternative seems likely to account for at least part of the observed difference, but present information does not allow any of the possibilities to be excluded. In any case, it is clear from the results that vertebrate sensory neurons in culture, although they never replicate, are capable of unscheduled DNA synthesis. Whether other neurons possess this capability and whether it is maintained indefinitely by neurons in vivo remain open questions.

SUMMARY

Dorsal root ganglion neurons explanted from chick or mouse embryos do not replicate in culture but can be induced to synthesize DNA by exposure to ultraviolet light. The radiation dose need not be acutely lethal to induce radioautographically detectable amounts of synthesis. DNA synthesis has also been observed in chick embryo neurons treated with methyl methane sulfonate.

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REFERENCES

1. BOYCE, R. P., and P. HOWARD-FLANDERS. 1964. *Z. Vererbungsl.* **95**:345.
2. CLEAVER, J. E. 1969. *Radiat. Res.* **37**:334.
3. EVANS, R. G., and A. NORMAN. 1968. *Nature (London)*. **217**:455.
4. HAHN, G. M., D. KIND, and S. J. YANG. 1971. *Nature (New Biology) (London)*. **230**:242.
5. HAHN, G. M., S. J. YANG, and V. PARKER. 1968. *Nature (London)*. **220**:1142.
6. HAMBURGER, V., and R. LEVI-MONTALCINI. 1949. *J. Exp. Zool.* **111**:457.
7. HANAWALT, P. C., and R. H. HAYNES. 1965. *Biochem. Biophys. Res. Commun.* **19**:462.
8. LAWLEY, P. D., and P. BROOKES. 1968. *Biochem. J.* **109**:433.
9. OKUN, L. M. 1972. *J. Neurobiol.* In press.
10. PAINTER, R. B., and J. E. CLEAVER. 1967. *Nature (London)*. **216**:369.
11. PAINTER, R. B., and J. E. CLEAVER. 1969. *Radiat. Res.* **37**:451.
12. RASMUSSEN, R. E., and R. B. PAINTER. 1966. *J. Cell Biol.* **29**:11.
13. ROBERTS, J. J., A. R. CRATHORN, and T. P. BRENT. 1968. *Nature (London)*. **218**:970.
14. SETLOW, R. B. 1968. *Progr. Nucl. Acid. Res. Mol. Biol.* **8**:257.
15. STOCKDALE, F. E. 1971. *Science (Washington)*. **171**:1145.
16. UTAKOJI, T., and T. C. HSU. 1965. *J. Exp. Zool.* **158**:181.