ter-saturated ethyl acetate; a 500-μl portion was then mixed with 75 μl of 1*M* K_2CO_3. The organic phase was discarded and 50 μl of K_2CO_3 was used for assay. The VMA was converted to vanillin by adding 15 μl of 2 percent $NaIO_4$ and incubating at 50°C for 30 minutes. After cooling on ice, the reaction was stopped with 15 μl of 10 percent $Na_2S_2O_5$ and the sample was neutralized with 30 μl of 5*N* acetic acid followed by 60 μl of 3*N* $K_2HPO_4 \cdot 3H_2O$ (*p*H 7.5). Vanillin was extracted with 800 μl of toluene (scintillation grade) and 600 μl was mixed with 150 μl of 1*M* K_2CO_3. A 100-μl portion of this phase was analyzed with a Gilford spectrophotometer at 360 nm. Urinary VMA was determined on 1 ml of urine. Internal and external standards were routinely used, and recovery was between 80 and 90 percent; the results are corrected for percentage recovery. The adaptations of both methods demonstrated linear relationships between the amount of tissue or urine assayed and the concentration of VMA and HVA detected. Specificity was checked against a mixture containing norepinephrine, epinephrine, dopamine, tyrosine, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and l-(3,4-dihydroxyphenyl)alanine. Possible interference by high amounts of lead and δ-aminolevulinic acid in urine from lead-exposed children and mice was also investigated and found not to occur.

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Induction of Mitosis in Mature Neurons in Central Nervous System by Sustained Depolarization

Abstract. *DNA synthesis and mitosis have been induced in vitro in fully differentiated neurons from the central nervous system by depolarization with a variety of agents that produce a sustained rise in the intracellular sodium ion concentration and a decrease in the potassium ion concentration. Depolarization was followed in less than 1 hour by an increase in RNA synthesis and in 3 hours by initiation of DNA synthesis. Apparently normal nuclear mitosis ensued, but cytokinesis was not completed in most cells; this resulted in the formation of binucleate neurons. The daughter nuclei each contained the same amount of DNA as the diploid preinduction parental neurons; this implies that true mitogenic replication was induced.*

The hypothesis has been advanced (*1*, *2*) that intracellular cation levels associated with generation of electrical transmembrane potentials in somatic cells may be functionally involved in control of mitogenesis and, hence, of cell division. Results from studies with a variety of cell systems have supported this premise (*3*, *4*). As a corollary of the hypothesis, it was proposed (*2*) that mitogenesis might be activated in highly polarized, nondividing cells such as neurons in the central nervous system (CNS) and muscle by treatments that would produce and maintain a substantial increase in the level of intracellular Na^+ and a decrease in K^+. This proposition was recently investigated for the case of mature neurons from chick spinal cord depolarized with ouabain (*5*); DNA synthesis was induced in a significant percentage of the neuron population by a range of ouabain concentrations. The question remained, however, as to whether the observed synthesis was truly mitogenic, rather than the result of an anomalous thymidine exchange or repair activity. We now report the induction of full DNA synthesis and subsequent mitosis in much larger percentages of chick spinal cord neurons by use of more favorable ouabain treatments, and an equally effective induction of mitotic activity in such neurons by other agents that depolarize by quite different mechanisms. The daughter nuclei from the induced mitoses each contain an amount of DNA identical to that of the mitotically quiescent G_1 (or G_0) neurons; this implies that true mitogenic replication has been induced by the imposed ionic changes.

The neurons were obtained by trypsin dissociation of spinal cords from chick embryos 7 to 10 days old and cultured as described (*5*). Mature 16- to 20-day cover slip cultures of well dispersed, fully differentiated neurons were used in the experiments. The cultures contained numerous small neurons and similar-sized neuroglial cells in addition to the large, distinctive motoneurons, but primarily the latter were utilized in the present investigation to preclude any possible confusion with the glial cells; fibroblasts were also quite numerous. The motoneurons were readily identified by their large size, angular cytons with long axon and dendrites, extensively granular cytoplasm, distinctive vesicular nucleus with prominent nucleolus, and differential staining properties (*6*). The glial population in the cultures increased continuously by mitotic division up to 14 days after plating but declined thereafter, although many glial mitoses were still observable; the ratio of glia (including the numerous microglia) to neurons at the time of culture use in experiments was approximately 10 to 1. In general, the glial cells tended to aggregate in clumps while the motoneurons were well dispersed. The cultured neurons had resting potentials of −35 to −60 mv, and were entirely devoid of mitotic activity after plating.

The ability of ouabain, veratridine, and the ionophore gramicidin to induce neuronal DNA synthesis and mitosis was determined over a range of concentrations for each agent. For DNA synthesis determinations, cultures were incubated for 6 hours with medium containing the test agent and [^{3}H]thymidine (0.1 μc/ml). The cover slips were rinsed, fixed, coated with Kodak NTB-3 nuclear track emulsion, and exposed for 14 days as described (*5*). The time of onset of DNA synthesis following exposure to ouabain was determined by pulse labeling for 1 hour prior to serial fixations at 1 to 6 hours. Similar pulse labeling with [^{3}H]uridine (2.5 μc/ml) was used to study changes in RNA synthesis rate following ouabain treatment; the same autoradiographic procedures were used as with DNA, but with a 7-day exposure period. For mitotic activity determinations, cultures were incubated for 24 hours with medium containing the test agent, then fixed, stained, and examined for binucleate neurons (*7*). Control cultures were assayed as were the test cultures, but were incubated in normal culture medium without addition of the depolarizing or other agents. Several supplementary time-lapse film studies were made of treated neurons to elucidate features of the mitotic process and the behavior of daughter cells. Relative amounts of DNA in the nuclei of control phase (G_1) neurons and in the daughter nuclei resulting from induced mitoses were determined by Feulgen microspectrophotometry of individual nuclei (*8*). The nuclear DNA content of G_1 fibroblasts in the cultures was similarly determined by using an excess-thymidine blocking procedure for accumulating G_1 cells, to establish the basic diploid nuclear DNA level. The neuron cultures were Feulgen-stained by the procedure of DeCosse and Aiello (*9*), and the relative absorbency of each nucleus was determined at 550-nm wavelength by using a Zeiss fluores-

Table 1. Percentages of CNS neurons induced to initiate DNA synthesis and nuclear division by various depolarizing agents. From 300 to 700 neurons were assayed at each test condition; four to six separate cultures were examined per condition. Grain density of labeled control cells (DNA synthesis) was one to two grains per nucleus, only slightly above background; that of labeled induced cells was severalfold higher (five to six grains per nucleus). The binucleate cells found in control cultures (mitotic activity) apparently resulted from neuroblasts in mitosis at the time of cord removal; the binucleate level in control cultures remained constant or decreased after 2 days in vitro. Data presented are for near-optimum concentrations of activating agent. A few cultures treated with $10^{-6}M$ ouabain plus NaCl had labeling frequencies as high as 58 percent.

Agent	Agent (M)	DNA synthesis (% of cells labeled)		Mitotic activity (% of binucleate cells)	
		Test	Control	Test	Control
Ouabain	10^{-5}	34	4	20	3
Ouabain	10^{-4}	34	2		
Ouabain + NaCl	10^{-6}	31	3	23	3
Veratridine	5×10^{-5}	63	3	30	8
Gramicidin	10^{-8}	32	6	29	6

cence microscope integrated with a model 240 Gilford monochrometer and digital photometer.

The percentages of neurons induced by the various agents to initiate DNA synthesis and to complete nuclear division are given in Table 1. All agents tested were effective in inducing both DNA synthesis and nuclear division. Since significant electroosmotic swelling (*1*) was found to accompany neuron depolarization in our initial studies with ouabain (*5*), an attempt was made to reduce the swelling (and to increase the Na^+ influx) by increasing the total medium osmolarity 5 percent by addition of NaCl. This addition not only reduced the swelling but also lowered the ouabain concentration required for mitogenesis activation. Depolarization by the alkaloid veratridine produced less rapid swelling and detachment of neurons, and yielded the highest percentage of activated cells (*10*). Like ouabain, the ionophore gramicidin produced substantial swelling and detachment upon depolarization, and was as effective as ouabain in its activation of neurons. Since both glial cells and fibroblasts showed spontaneous mitotic activity in control and test cultures, no quantitative assay was made of their mitotic activity in treated cultures. However, in some gramicidin-treated cultures a pronounced increase in the number of fibroblasts labeled with [^{3}H]thymidine and in the grain density of such labeling occurred in portions of tightly confluent fibroblast monolayer areas in the neuron cultures. Numerous mitotic figures of fibroblasts and glia were seen in both control and test cultures, but very few binucleate cells of either type were found, which indicates completion of full cytokinesis in these cells. Phase-contrast photomicrographs of two binucleate neurons resulting from ouabain treatment are shown in Fig. 1.

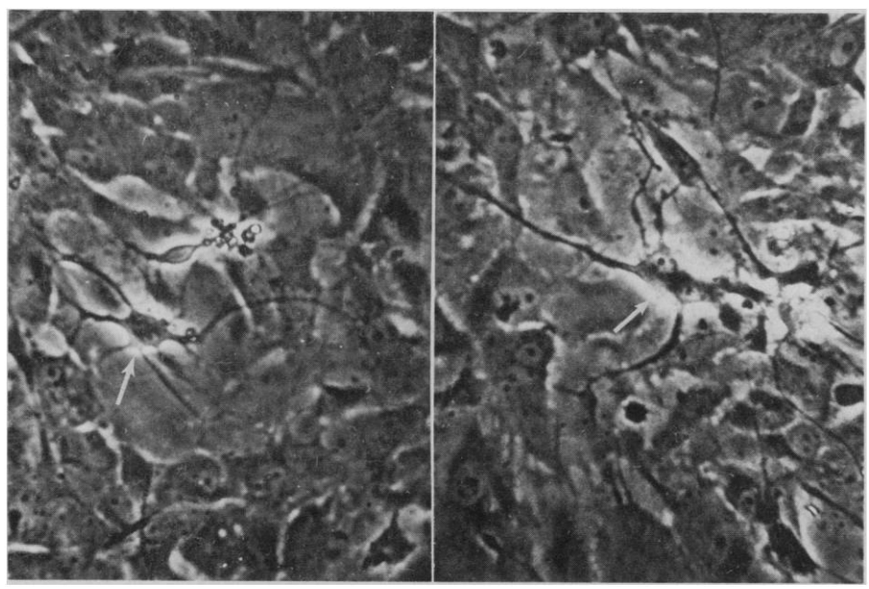

Fig. 1. Phase-contrast photomicrographs of two live binucleate neurons resulting from the ouabain treatment of 15-day test cultures. Ouabain was removed from the cultures after a 24-hour treatment. Several other binucleate neurons appear in these photographs, but are not so clearly discerned as those indicated by the arrows.

Although the present experiments were concerned with assay of binucleate induction and the treatment time used was intended to permit full completion of nuclear division, a number of clearly discernible neuronal mitotic figures was found in the fixed test cultures. Metaphase, anaphase, and even prophase figures were clearly distinguished in neurons whose processes had not detached during treatment and which retained characteristic morphology and cytoplasmic staining properties. Also, a few neuronal mitotic figures on stained slides were independently identified by the periodic coordinate location procedure (*7*).

As shown in Fig. 2, DNA synthesis began in an appreciable fraction of the ouabain-treated cells within 3 hours. DNA synthesis was preceded by a significant rise in the RNA synthesis rate, which essentially reached its maximum level within 1 hour; this indicates that mitogenesis activation was induced quite rapidly with the ouabain concentrations used. A frequency distribution of the [^{3}H]uridine grain count per nucleus for control neurons and those treated with ouabain plus NaCl indicated that 78 percent of the treated cells had rates of RNA synthesis significantly greater than the mean level for the control neurons. In the time-lapse films of binucleate neuron formation, clear observation of individual chromosomal dispersion and movements was generally precluded because of the pronounced refractiveness of the rounded cyton, but the time of chromosome separation and formation of two nuclear masses could be discerned in several films. The elapsed time from beginning of treatment through completion of cytokinesis in one film of a gramicidin-treated neuron undergoing full cytokinesis was 12 hours. Although maximum swelling and "rounding up" (*1*) of the cell occurred within 2 to 3 hours after gramicidin addition, the actual onset of mitotic prophase did not begin until approximately 10 hours, and mitosis was completed by 12 hours. In cases where full rounding of the neuron took place with absorption of its process-

es, complete cytokinesis usually occurred; the resulting daughter cells were quite transient in shape and initially not morphologically identifiable as neurons.

Measurements of the DNA content of each of the daughter nuclei in 62 binucleate neurons resulting from ouabain-induced divisions revealed that each of the 124 daughters contained identical amounts of DNA. The DNA content of each daughter nucleus from the induced divisions was found to be identical also to that of the mononucleate neurons in control cultures. Since each of the 101 control neurons assayed contained the same amount of nuclear DNA as the G_1 (diploid) fibroblasts in the cultures, the control and test neurons were diploid cells prior to treatment.

The premise is generally accepted that mitotic activity ceases in fully differentiated neurons of the CNS and dorsal root ganglia, both in vivo and in vitro (*11*), although observations of divisions in such neurons in vitro have been reported (*12*). In the present experiments, neurons that were apparently fully differentiated (*13*) were obtained by using cords in which neuronal mitotic activity had essentially ceased (*5*) and by maintaining the cultures for several days after attainment of full morphological and electrophysiological development before use in experiments. Neuronal mitotic activity was completely absent in preexperimental cultures, as demonstrated by (i) constancy of the number of binucleate neurons after culture establishment (that is, beyond 2 days in culture; see Table 1 legend), and (ii) direct observations of some 200 individual neurons on a daily basis during 3 weeks of culture. Consequently, the mitoses reported herein were induced in neurons which had achieved the state of complete mitotic quiescence characteristic of the fully differentiated cells. An obvious and important extension of the present experiments, however, is determination of the mitotic response of neurons from adult animals and humans of various ages to these treatments.

The observation of neuronal mitotic figures in test cultures, along with the finding that daughter nuclei of binucleate neurons induced from diploid mononuclear parental neurons also contain the exact diploid level of DNA, strongly implies that the induction treatment activated true mitogenic replication of the neuronal nucleus. It is highly unlikely that anomalous nuclear fragmentation would result consistently in the production of daughter nuclei having equal amounts of DNA, corresponding exactly to the normal diploid level. Likewise, it is unlikely that the daughter nuclei resulted from nuclear amitosis, in view of the presence of neuronal mitotic figures in the test cultures and the dearth in observations of true amitosis in higher somatic cells. It is clear that the daughter nuclei did not result from the mitosis or fission of tetraploid parental nuclei already existing in the preexperimental cultures, since both the daughter and parental cell groups possessed the diploid level of DNA. Rather, the results indicate that the treatments induced an exact doubling of parental DNA followed by an exact halving to produce identical daughter nuclei, as in normal mitosis. The fact that most of the treated neurons retained some degree of process attachment and did not complete cytokinesis, while those few which fully rounded up underwent complete division (*7*), suggests the possible involvement of the neuronal microtubule system in preventing completion of cytokinesis, thus fostering binucleate rather than individual daughter neuron formation.

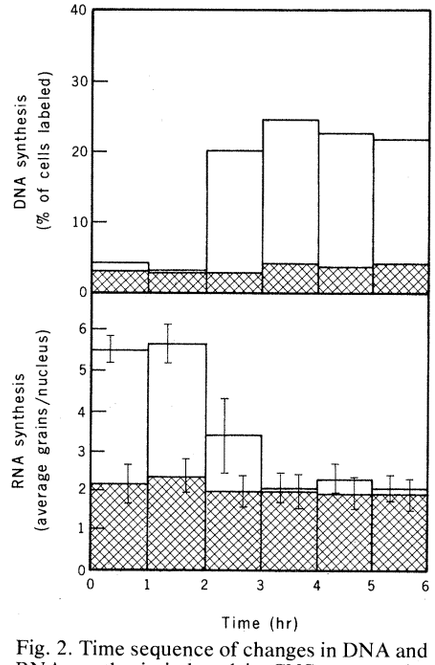

Fig. 2. Time sequence of changes in DNA and RNA synthesis induced in CNS neurons by treatment with $5 \times 10^{-5} M$ ouabain plus NaCl, added to the culture medium at zero hour. Shaded bars refer to control cultures, and open bars to treated cultures. The labeling pulse was given 1 hour before culture fixation; approximately 350 neurons were assayed per time interval. Cells were considered to be synthesizing DNA only if their nuclear grain count was three times background (two to three grains per nucleus). Autoradiographic grain counts (RNA synthesis) are the mean per nucleus, averaged over 350 randomly chosen neurons in each time interval. The range of the mean at the 95 percent confidence level is indicated for each interval (the range for test neurons is indicated at left of interval bar, that for controls at right).

In regard to the validity of the basic hypothesis on ionic modulation of mitogenesis activity which the present experiments were primarily designed to test, it appears particularly significant that apparently normal nuclear mitosis has been induced in such highly differentiated and mitotically refractory cells as CNS neurons by three distinct agents whose only basic commonality of action is to effect an increase in cellular Na^+ and decrease in cellular K^+ (*5, 14*). The present results strongly imply that the induced mitoses follow the usual sequence of mitogenic events and reinforce the previously advanced view that activation of mitogenic RNA synthesis by significant shifts in the intracellular Na^+ level or Na^+ to K^+ ratio (or both) may be a key event in the initiation of mitogenesis (*1, 2, 4*). Time-lapse film and direct visual observations have demonstrated that viable daughter cells result from such induced division when cytokinesis is completed. Whether the daughter cells of such neuron divisions still possess full neuronal phenotypic properties and functionality remains to be determined, but this question obviously constitutes a subject of considerable basic, and ultimately perhaps clinical, importance. In this regard, elucidation of those conditions that favor completion of cytokinesis, rather than binucleate neuron formation, is essential.

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rounded up fully during treatment, with detachment and absorption of their processes; the small daughter cells were also lost during fixation. In the present study, only clearly identifiable binucleate neurons (remaining on the fixed culture slides) were included in the assay of mitotic activity; the percentages of mitotic neurons given herein (Table 1) are thus minimums. The true percentages are possibly double those indicated, as estimated from the periodic observations. A similar situation existed in the case of the DNA synthesis determinations; detachment and loss of activated cells also occurred in substantial numbers, but with somewhat less frequency than in the mitotic cells, due perhaps to the shorter treatment period. The possibility that the binucleate cells observed arose from induced or spontaneous fusion of adjacent neurons, rather than from nuclear division, was precluded by use of cultures in which the individual test neurons were well dispersed.

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Neural Transport of Tetanus Toxin

In figure 2 of their report, Price *et al.* (*1*) suggest that ^{125}I-labeled tetanus toxin is taken up at neuromuscular junctions and carried in motor axons by retrograde axonal transport to the central nervous system. With the nerves not crushed but intact one should then be able to demonstrate intraaxonal radioactivity at the level of the ventral roots. We succeeded in doing so (*2, 3*). Moreover, in intact (noncrushed) peripheral nerves we (*2, 3*), as well as Gardner and Fedinec (*4*), found a high amount of tetanus toxin in the epineurium. If the epineurium is opened, tetanus toxin leaks out (*5*). Transport of ^{125}I-labeled tetanus toxin also in sensory axons cannot be ruled out: after intramuscular injection of labeled toxin we (*3*) and Stöckel *et al.* (*6*) found radioactivity in cell bodies of pseudounipolar cells of the spinal ganglia. In the spinal cord, the perikarya of some motoneurons were heavily labeled (*2, 3, 6, 7*). Whether radioactivity found in the vicinity of the perikarya has been released from the motoneurons into the neuropil is still an open question.

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14 July 1975

Price *et al.* (*1*) show the presence of a ^{125}I-labeled product of tetanus toxin within the axoplasm of motor nerves supplying muscles into which the labeled material has been injected. The authors "think it likely that tetanospasmin can reach the CNS [central nervous system] by retrograde intraaxonal transport, but we cannot exclude some contribution by the circulatory system." Thus they offer evidence in support of the hypothesis first advanced by Marie and Morax (*2*) that tetanus toxin ascends from the wound site via the motor nerves to the CNS where it produces the signs of tetanus intoxication. This hypothesis was proposed to explain the clinical phenomenon of local tetanus. Briefly stated, this is the development of muscle stiffness localized solely to the site of a wound containing toxin. Because Marie and Morax did not consider that the toxin might also act on the striated muscle, it was necessary to postulate intraneural centripetal spread to the corresponding innervating neurons to explain the specificity and localization of the initial local tetanus. It was recognized that if the naturally acquired or experimental dose of toxin was large enough, generalized tetanus, involving all the striated musculature, would follow the local tetanus.

In their emphasis on demonstrating intraaxonal ascent of the toxin and thus finally confirming the Marie-Morax hypothesis, Price and associates ignore the evidence indicating that the centripetal theory does not account for the principal features of tetanus intoxication. First, while the authors do not compare the toxicity of their preparations before and after iodination by testing animals via the same route of injection, their data indicate that the iodinated product is less toxic than the original material. Despite this, they provide no evidence to show that the radioactive material observed within axons is labeled active tetanus toxin rather than largely nontoxic labeled protein following a pathway common to many proteins. Several investigators using various methods have shown that active (toxic) tetanus toxin has such a high affinity for neural membranes that it would be expected to be immobilized within the terminal axons (*3*). Also in direct contrast with the work of others is the absence of label in the perineural space (*4*). Second, the authors give no quantitative data on recovery of the ^{125}I-labeled toxin; that is, how much toxin was actually found within the nerve at the time of sampling and how much was bound elsewhere either nonspecifically (in such organs as spleen and kidney) or specifically within the CNS? Was any attempt made to localize the toxin in those animals that had been killed with intraperitoneal doses of radioactive toxin and how did this distribution compare with the distribution of the toxin in the animals in which the toxin was injected intramuscularly before nerve crush? It is also important to consider the time intervals following intramuscular injection of the toxin because if a dose sufficient to produce intoxication is absorbed via lymphatics and blood vessels before the "hours later" of table 1 of (*1*), it is irrelevant whether labeled active or inactive toxin can be demonstrated in the axoplasm after this time. The lack of control data bearing on these points leads us