

Temperature-dependent blockade of nucleocytoplasmic transport of newly synthesized RNA in neurons

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This study evaluates the temperature sensitivity of transport of recently synthesized RNA from the nucleus to the cytoplasm (nucleocytoplasmic transport) in CNS neurons. Rat hippocampal slices were incubated with [³H]uridine for 1 h to label recently synthesized RNA. Slices were then fixed immediately or maintained at 27°C or 37°C for chase intervals of 3, 4.5, and 6 h to allow for nucleocytoplasmic transport of recently synthesized RNA. The time-dependent translocation of recently synthesized RNA was evaluated autoradiographically. At the end of the 1 h pulse at either 27°C or 37°C, the label was localized exclusively over nuclei. In slices maintained at 37°C, labeling expanded to cover the cell body and proximal dendrites. However, in slices that were labeled and maintained at room temperature, labeling remained confined to the nucleus. In slices that were pulse-labeled at room temperature, and then transferred to 37°C medium, cytoplasmic labeling increased as a function of time. Nucleocytoplasmic transport of RNA in cultured rat hippocampal neurons showed a comparable temperature sensitivity. The inhibition of nucleocytoplasmic transport of RNA at room temperature provides an opportunity to evaluate neuronal function when no new RNA molecules can reach the cytoplasm.

INTRODUCTION

Brain slices have become a popular preparation for study of a wide variety of processes because they are easily manipulated and can be maintained for many hours *in vitro*. Brain slices are especially convenient for electrophysiological studies of synaptic transmission and modification as well as metabolic studies of protein synthesis, ischemia, and synaptic pharmacology^{5,14}. In many instances it is advantageous to maintain brain slices (particularly submerged slices) at reduced temperatures (24–32°C), since it is difficult to adequately oxygenate them at physiological temperatures⁹. Submersion is also important for some metabolic studies because the exchange of materials between the slice and the medium is uneven on the opposite sides of a slice maintained at an air/water interface. In the course of our studies of intracellular transport of recently synthesized RNA using brain slices, we unexpectedly found that the translocation of recently synthesized RNA from the nucleus to the cytoplasm (nucleocytoplasmic transport) was completely blocked when slices were maintained at room temperature. These results suggest a novel approach to prevent the expression of newly transcribed RNA in the cytoplasm of neurons. Our results also indicate that previous studies using slices maintained at room temperature were carried out under

conditions in which newly synthesized RNA would not be expressed.

MATERIALS AND METHODS

Slice preparation

Hippocampal slices were prepared from 19–21 day old Sprague-Dawley rats as described previously¹⁸. Animals were anesthetized with Halothane and decapitated. Slices 400 µm thick were cut with a tissue chopper and transferred to oxygenated artificial cerebral spinal fluid (CSF) composed of 2 mM KCl, 130 mM NaCl, 16.1 mM NaHCO₃, 3 mM KH₂PO₄, 1.3 mM MgSO₄, 1.8 mM CaCl₂, and 10 mM glucose, and aerated by bubbling with 95% O₂/5% CO₂. Slices were allowed to equilibrate for 1.5 h prior to labeling at either room temperature (approximately 27°C) or 37°C, corresponding to the temperature at which the slices were to be labeled.

Pulse labeling of recently synthesized RNA

Fifteen to 20 slices were transferred into 4 ml of aerated artificial CSF containing 1.2 mCi 5,6-[³H]uridine (New England Nuclear) and maintained for 1 h at either room temperature or 37°C. Slices were then washed 3 to 4 times with aerated artificial CSF containing 10⁻⁴ M unlabeled uridine at the proper temperature and then maintained for up to 6 h in CSF at 37°C or room temperature. Three to 4 slices were removed immediately following the end of the pulse or after 3, 4.5, and 6 h and fixed in 10% phosphate-buffered formalin.

To evaluate whether the inhibition of nucleocytoplasmic transport at room temperature was reversible, slices were equilibrated and labeled at room temperature, rinsed 3 to 4 times with media warmed to 37°C, and then maintained at 37°C for 45 min, 2 h, or 3 h, prior to fixation.

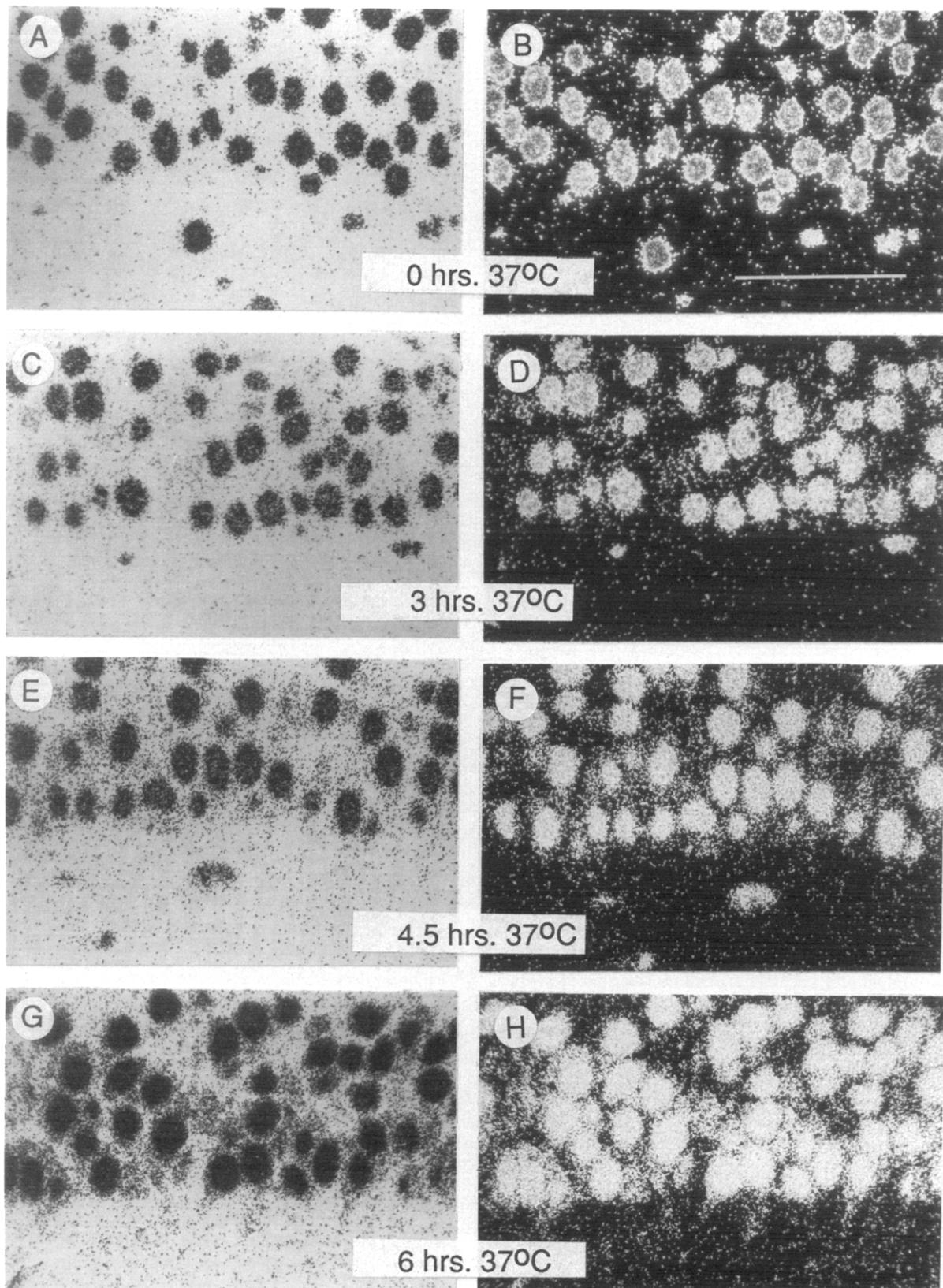


Fig. 1. Time course of nucleocytoplasmic transport of newly synthesized RNA in hippocampal slices maintained at 37°C. Left and right hand panels are bright- and dark-field photomicrographs of the same fields in CA1. A and B illustrate the pattern of labeling in a slice fixed after the end of the pulse labeling period. C and D illustrate the pattern of labeling in a slice fixed 3 h after the end of the labeling period. E and F illustrate the pattern of labeling in slices fixed 4.5 h after the end of labeling, and G and H represent slices fixed 6 h after the end of labeling. Bar = 100 μ m.

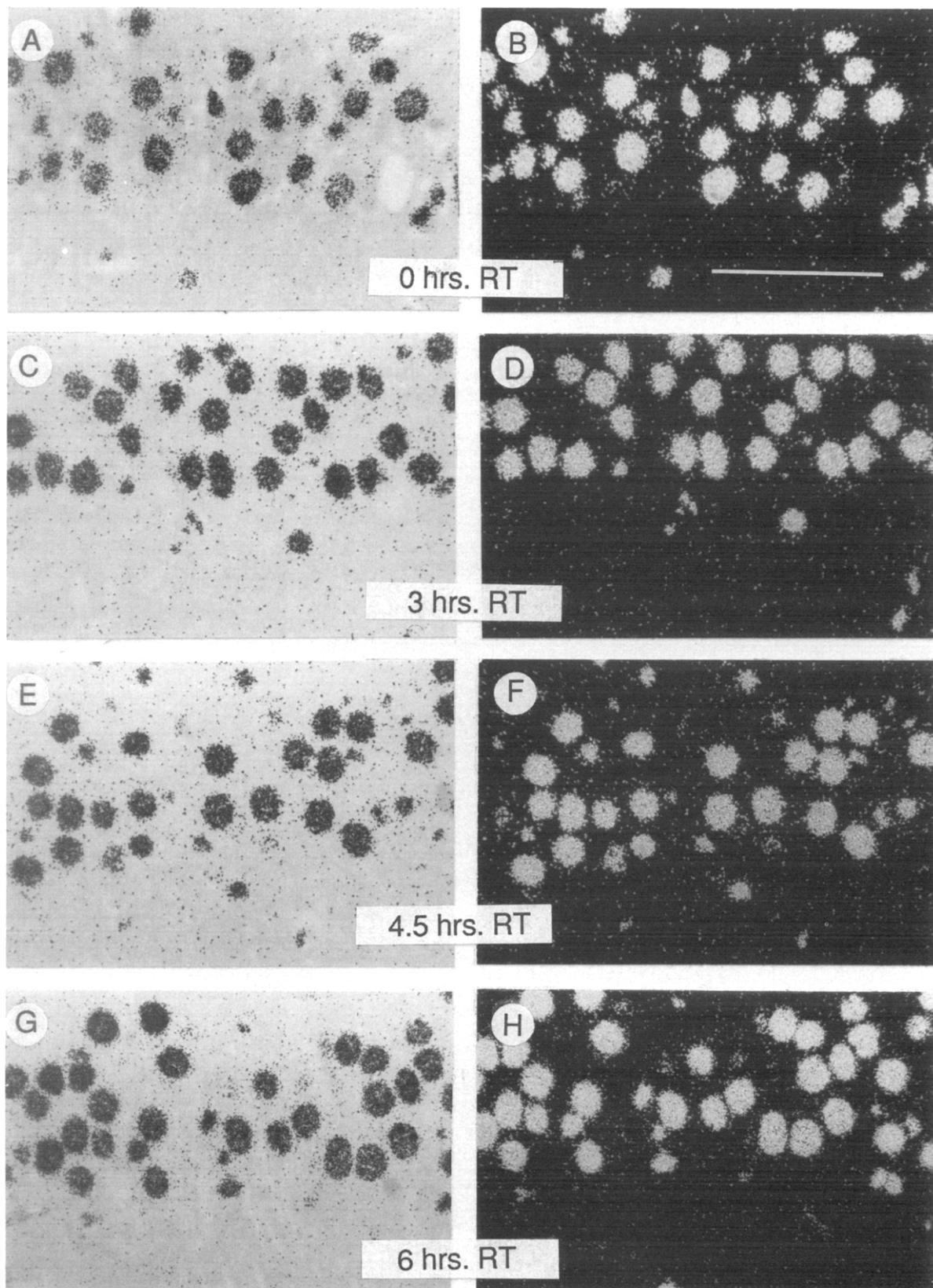


Fig. 2. Time course of nucleocytoplasmic transport of newly synthesized RNA in hippocampal slices maintained at room temperature. Left and right hand panels are bright- and dark-field photomicrographs of the same regions in CA1. A and B illustrate the pattern of labeling in a slice fixed immediately after the end of the pulse labeling period. C and D illustrate the pattern of labeling in a slice fixed 3 h after the end of labeling. E and F illustrate the pattern of labeling in a slice fixed 4.5 h after the end of labeling, and G and H illustrate labeling in slices fixed 6 h after the end of labeling. Bar = 100 μ m.

Labeling of cells in culture

Cultures of hippocampal neurons were prepared as described previously^{1,6}. Briefly, hippocampi from E18–E19 rat fetuses were collected and incubated at 37°C with 0.25% trypsin for 15 min. Neurons were dispersed by trituration and plated onto polylysine-coated glass coverslips at a density of 50,000 cells per 60 mm dish. After neurons had attached to the coverslip, they were transferred to dishes containing monolayer cultures of astroglia, and maintained in serum-free medium. Cultures were treated with cytosine arabinoside (5×10^{-6} M) on day 3 or day 4 to reduce glial proliferation. Experiments were performed on cells that had been maintained in culture for at least 15 days. All cultures were labeled with 40 μ Ci/ml of [³H]uridine for 45 min at 37°C. Following the labeling period, cells were washed 3 to 4 times with sterile saline

solution which was replaced with media containing 10⁻⁴M unlabeled uridine. Cells were then maintained either at 37°C or at room temperature for 3 or 6 h prior to fixation.

To evaluate the reversibility of the low temperature blockade of nucleocytoplasmic transport, cultures were labeled at 37°C, transferred to room temperature for 3 h, and then returned to 37°C for an additional 3 h prior to fixation.

Autoradiography

Fixed slices were osmicated and embedded in Poly/Bed 812 embedding media (Polysciences, Inc.). The slices were sectioned at 2 μ m using a glass knife and mounted on glass slides. Slides were dipped in Kodak NTB2 emulsion diluted 1:1 with distilled water. Cells labeled in culture were fixed in 4% paraformaldehyde, 4%

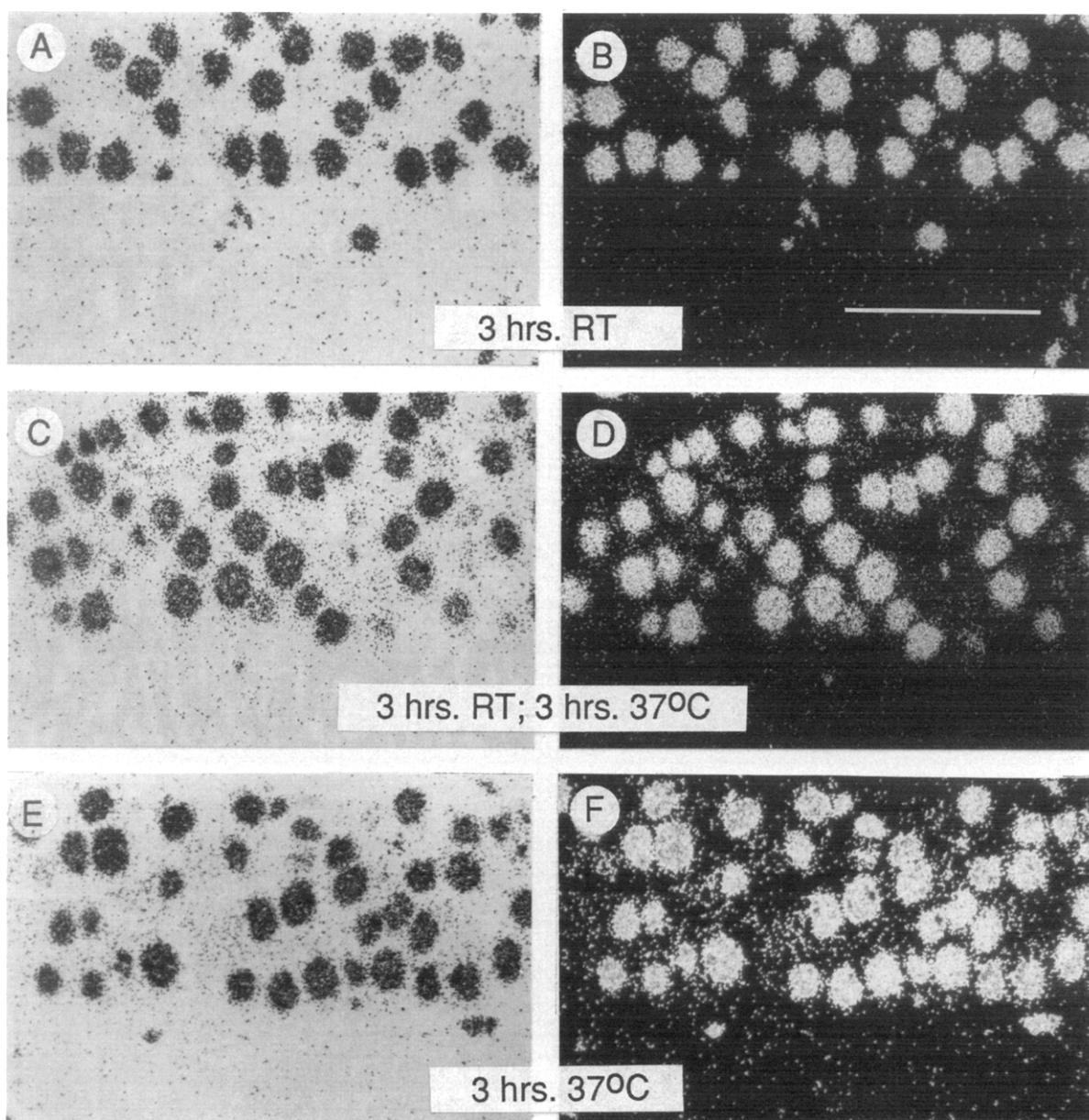


Fig. 3. Reversibility of nucleocytoplasmic transport. Left and right hand panels are bright- and dark-field photomicrographs taken from CA1. A and B illustrate the pattern of labeling in a slice that was pulse labeled, then incubated in unlabeled media for 3 h at room temperature. C and D illustrate the pattern of labeling in a slice that had been labeled and maintained for 3 h at room temperature, but then maintained at 37°C for an additional 3 h to reinstate nucleocytoplasmic labeling. E and F illustrate the pattern of labeling in a slice that was labeled and maintained for 3 h at 37°C. Bar = 100 μ m.

sucrose in phosphate-buffered saline, dehydrated, and air dried. Coverslips were mounted cell side up on microscope slides and dipped in undiluted emulsion. Autoradiographs were exposed for 10 or 30 days and developed at 15°C in Kodak D19.

RESULTS

Time course of nucleocytoplasmic transport at 37°C

Fig. 1 illustrates the distribution of labeling over neurons and glia in the CA1 region from slices fixed at the end of the labeling period or after 3, 4.5, and 6 h. Fol-

lowing the end of the pulse (0 h), silver grains were localized over neuronal and glial nuclei (Fig. 1A,B). Slices labeled and maintained at 37°C showed a gradual translocation of labeling from the nucleus to the cell body and into proximal dendrites. Cytoplasmic labeling was evident by 3 h after the pulse, and increased thereafter (Fig. 1C,D). By 4.5 h, the density of silver grains in the cytoplasm had increased dramatically (Fig. 1E,F). After 6 h, the cytoplasm was heavily labeled (Fig. 1G,H) and some labeled proximal dendrites could be discerned. It was not possible to trace individual dendrites from an

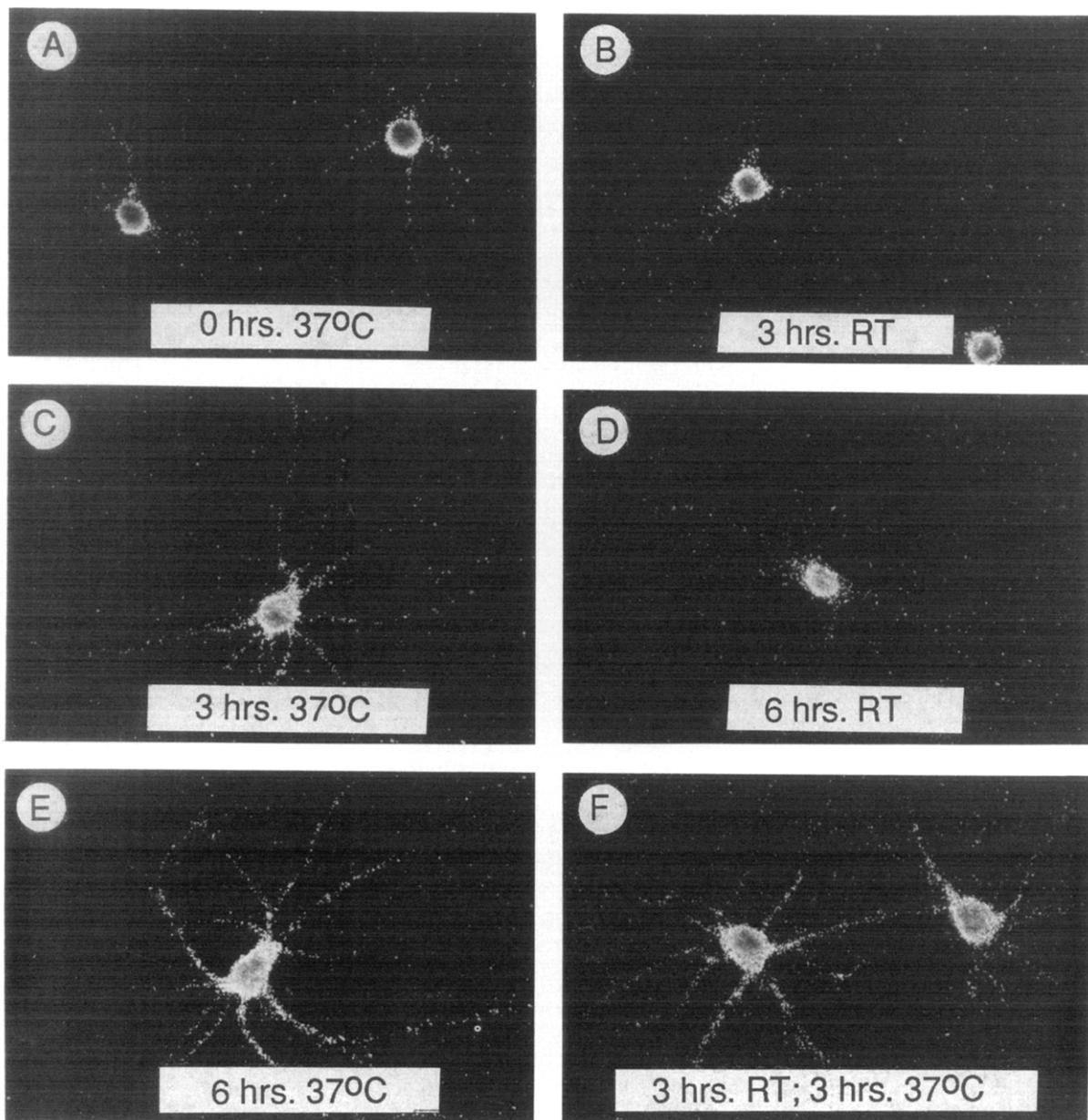


Fig. 4. Nucleocytoplasmic transport of newly synthesized RNA in cultured neurons. A, C, and E are dark-field photomicrographs of cultured neurons that were pulse-labeled and fixed or maintained at 37°C for 3, or 6 h. B and D are neurons that were labeled at 37°C and then maintained at room temperature for 3 or 6 h after the end of the pulse. F depicts neurons that were labeled at 37°C, but maintained at room temperature for 3 h, and then returned to 37°C for an additional 3 h to reinstate transport. Total magnification approximately 325 \times .

identified neuron into the more distal neuropil regions because the dendrites do not remain in the plane of the section. Glial cells present in these neuropil regions confound interpretation of labeling that is not directly visible over identifiable dendrites.

Nucleocytoplasmic transport is inhibited at room temperature

Slices that were labeled and maintained at room temperature (27°C) did not show any evidence of translocation of recently synthesized RNA from the nucleus to the cytoplasm (Fig. 2). At all time points examined, labeled RNA remained localized over the nucleus (see Fig. 2A–H). The amount of labeling after the 1 h pulse-labeling period appeared to be less than that produced during the same period at 37°C, suggesting that RNA synthesis was also reduced at room temperature. However, we made no attempt to quantify this difference. The level of labeling was clearly sufficient to detect nucleocytoplasmic translocation if any had occurred.

Reversibility of inhibition

To evaluate whether the low-temperature blockade of nucleocytoplasmic transport was reversible, slices were incubated at room temperature for the equilibration period and the 1 h labeling period, and were then transferred into 37°C chase medium for 45 min, 2 h, or 3 h. In slices that were fixed 45 min following the transfer to 37°C medium, there was no apparent cytoplasmic labeling. In slices that were fixed 2 h following the transfer there was some evidence of label just outside the nucleus (not shown). By 3 h, there was obvious translocation of the label into the cytoplasm (Fig. 3C,D). However, the extent of cytoplasmic labeling was not as great as in the slices labeled and maintained at 37°C for the entire time period.

Neurons in culture also display temperature dependent blockade of nucleocytoplasmic transport

As reported previously^{3,4}, when neurons in culture were pulse labeled with [³H]uridine, and then transferred to 37°C medium without labeled precursor, there was a time dependent translocation of recently synthesized RNA from the nucleus to cytoplasm and then into dendrites (see Fig. 4A,C,E). At the end of the 45 min pulse, label was localized almost exclusively over nuclei. Three h after the pulse, the cell bodies and proximal dendrites were labeled, and the extent of labeling increased further by 6 h. In contrast, autoradiographs of cultures maintained at room temperature for 0, 3, or 6 h following the pulse were indistinguishable. Label was localized almost exclusively over the nucleus, with a small percentage of the label appearing within the cell body.

Neurons that were pulse labeled at 37°C, transferred to room temperature media for 3 h, and then returned to 37°C for 3 more hours, exhibited labeling of the cell bodies and dendrites that was intermediate between that of the cultures maintained for 3 h and 6 h at 37°C for the entire chase period. These results indicate that blockade of nucleocytoplasmic transport is reversible.

DISCUSSION

The present results show that nucleocytoplasmic transport of newly synthesized RNA in neurons is temperature dependent. Specifically, the transport of recently synthesized RNA is reversibly inhibited at room temperature. In retrospect, these results are not surprising given results from studies in other cell types²². Autoradiographic studies have shown that embryonic chick retinal fibroblasts maintained in culture also do not transport newly synthesized RNA from the nucleus at 27°C⁸. In addition these studies revealed that synthesis of both protein and RNA at 27°C was reduced to 25–30% of that at 37°C^{7,8}. Thus, reduced temperature blocks nucleocytoplasmic transport of RNA, while other cellular processes are less affected. Other studies by this group have demonstrated altered processing of the 45S rRNA precursor at 27°C in HeLa cells, such that the 32S RNA intermediate accumulates, and virtually no 28S RNA could be found in the cytoplasm¹⁶. Related studies have shown that nucleocytoplasmic transport of RNA is inhibited at reduced temperature in HeLa cells, as well as in ovary cells of *Musca*² and in *Tetrahymena pyriformis*²⁰.

It is not clear whether low temperature blocks the nucleocytoplasmic transport of all types of RNA to the same extent. [³H]Uridine label is incorporated into all newly synthesized RNA and presumably the change in the distribution of label reflects the movement of all forms of RNA. However, when [³H]uridine is injected intracranially, approximately 80% of the counts are present in poly(A)⁻ fractions and represent rRNA while the remaining 20% of the counts represent poly(A)⁺ RNA (mRNA) (R. Kleiman, G. Banker and O. Steward, unpublished). The low level of cytoplasmic labeling observed in our experiments thus suggests that the transport of both rRNA and mRNA was blocked. Studies of retinal fibroblasts support this conclusion because when these cells are incubated with radiolabeled uridine at 27°C, neither newly synthesized rRNA nor poly(A)⁺ RNA appear in the cytoplasm⁸.

It is known that temperature can reversibly affect the physical properties of the nuclear membrane. Freeze fracture studies that examined the effect of temperature on nuclear and plasma membranes of lymphoid cells have shown that below 22°C nuclear membranes are

smooth and free of intramembranous particles on both inner and outer membranes. Returning the cells to physiological temperatures reversed the effect to produce normal particle distribution²¹. The authors did not observe this kind of reversible thermotropic phase separation in the plasma membrane. They also noted that while temperature has a dramatic effect on the distribution of intramembranous particles, there appeared to be no effect on the distribution of nuclear-pore complexes²¹. It may be significant that these kinds of dramatic physical changes parallel such substantial functional changes in transport through the nuclear membrane following decreases in temperature. Our studies add no new insights into the mechanism of this temperature dependent inhibition.

Maintaining tissue at room temperature rather than 37°C can slow a variety of metabolic functions, including protein and RNA synthesis, oxygen consumption and energy metabolism. Reduced temperature will selectively block vesicle traffic from the endoplasmic reticulum to the Golgi apparatus^{12,17}. Temperature can also alter the electrical properties of neurons and change their synaptic responses^{13,18,19}. Axonal transport of protein is also slowed at temperatures below 38°C, and can even be

stopped completely below 11°C in vitro¹¹. However, the blockade of nucleocytoplasmic transport seems to be quite profound at temperatures at which other cellular processes are maintained, albeit at reduced levels.

The present results suggest that temperature can selectively affect an important process in gene expression. This phenomenon may offer a novel approach to evaluate the functional significance of new gene expression in neurons as an alternative to using drugs to inhibit new RNA or protein synthesis. For example it has been shown that maintenance of long-term potentiation (LTP) is disrupted by protein synthesis inhibitors^{10,15}, suggesting that some protein must be synthesized to maintain the long term effects of LTP. By evaluating LTP in slices maintained at room temperature, one could determine whether new gene expression was required to maintain LTP, or whether the proteins responsible for maintenance of LTP can be synthesized using mRNA already present in the cytoplasm prior to induction of LTP.

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