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Neurotrophin-3 signals redistribute RNA in neurons

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ABSTRACT **The translocation of specific mRNAs to dendrites and their potential for locally regulated translation are likely to serve as an effector in neuronal plasticity. Whether translation in dendrites is regulated by delivery of the RNA to sites of plasticity or a stationary pool of localized RNA undergoes enhanced translational efficiency is not clear. We show that RNA can translocate into dendrites in response to NT-3. RNA granules were visualized in cultured rat cortical neurons using the dye SYTO 14, which labels poly-ribosome complexes. Long before the morphological effects of NT-3 appeared, there was increased distal translocation of labeled complexes. This effect was blocked by K252a, a potent inhibitor of tyrosine kinase receptors. Therefore, neurons can utilize extracellular signals to alter the distribution of protein synthetic machinery via the active transport of RNA granules.**

Increasingly, a role for the neurotrophins in neuronal plasticity has become evident. Neurotrophins can serve as selective retrograde messengers that regulate activity-dependent, long-term functional changes, particularly in the visual cortex (reviewed in ref. 1). Long-term functional changes such as long-term potentiation are dependent on protein synthesis (2). Effecting these prolonged changes, therefore, requires alterations in RNA expression patterns. The presence of polyribosomes beneath postsynaptic sites in dendrites (3, 4) suggested an anatomical locus for the synthesis of proteins in conjunction with synaptic enhancement. A heterogeneous population of RNAs are selectively sorted to dendrites, many of which are involved in intracellular signaling (reviewed in ref. 5). For example, during the maintenance phase of long-term potentiation in the rat dentate gyrus of the hippocampus, specific isoforms of several serine/threonine kinases increase their expression in a characteristic spatial and temporal profile (6).

Several mechanisms may serve neurons to regulate the synthesis of proteins at specific sites. Neurotrophins can enhance spontaneous synaptic activity in hippocampal cultures (7). Increased synaptic activity can induce the translocation of RNAs to dendrites as shown for the immediate-early gene, Arc mRNA (8, 9); and in chicken embryo fibroblasts, extracellular signals can induce the translocation β-actin mRNA out to the lamella (10, 11). Alternatively, locally stationed mRNAs, which are delivered and turnover in a constitutive manner, may undergo modulation of their translational efficiency. The translocation of mRNAs appears to be associated with granules as observed in many different systems including cultured oligodendrocytes (12), fibroblasts (13), neurons (14), and Drosophila embryos (15). Applying the cell permeant nucleic acid dye, SYTO 14, to cultured cortical neurons, it is possible to observe motile granules that contain key components of translation and translocate along microtubules at rates consistent with fast transport (14). We report that RNA granules directly visualized in living neurons respond to NT-3 signaling by translocating to neurites well before the appearance of any morphological changes induced by the growth factor.

MATERIALS AND METHODS

Cell Culture. The method of cell culture has been described in detail by Banker (for review see ref. 16) and modified for use with cortical neurons in our laboratory (17). The cerebral cortex was dissected from embryonic day 18 rats and digested with 0.25% trypsin in Hanks' balanced salt solution. Tissue was washed twice in Hanks' balanced salt solution, placed in MEM with 10% fetal calf serum, and mechanically dissociated by pipetting. Neurons were plated at low density (1,000 cells per cm^2) on poly-L-lysine coated coverslips (1.0 mg/ml; overnight). After 2 h, when neurons had attached to the substrate, coverslips were inverted onto a monolayer of astrocytes. Astrocytes were prepared from postnatal day 1 rat cortex by culturing dissociated cortex in MEM with 10% fetal calf serum on untreated tissue culture plates. The coculture was maintained in glutamate-free MEM with N2 supplements, which include transferrin (100 μg/ml), insulin (5 μg/ml), progesterone (20 nM), putrescine (100 μM), and selenium dioxide (30 nM). In addition, extra glucose (600 mg/l) and sodium pyruvate (1 mM) were used.

Labeling of Cultures. For live cell analysis, cells were incubated with 50 nM SYTO 14 for 10 min in MEM with N2 supplements plus 10 mM Hepes at 37°C, then washed twice in media before evaluation. Under these conditions, neurons appeared healthy for the 2 h period of observation as determined by neuronal morphology, viability, and organelle movement. The spectral characteristics of this dye in the presence of RNA are an absorption maximum of 521 nm and a fluorescence emission maximum of 547 nm. To subtract out mitochondria that were labeled with SYTO 14, cells were colabeled with a mitochondrion selective dye, Mitotracker CMXROS (Molecular Probes) for 15 min at 37°C. This dye has an excitation and emission maximum of 579 nm and 599 nm at 50 nM.

Growth Factors and Inhibitors. Cells, labeled with dyes as described above, were starved for 45 min in MEM without N2 supplements at 37°C. NT-3 (Austral Biological, San Ramon, CA), diluted in PBS containing 100 μg/ml BSA, was added to media. Control cells had conditioned media with N2 supplements added back. For inhibition experiments, 30 nM K252a (Kamiya Biomedical, Thousand Oaks, CA) in dimethyl sulfoxide was added 1 h before labeling the cells. Control cells had the same concentration of dimethyl sulfoxide (1:10,000) added 1 h before labeling.

Immunocytochemistry. For immunocytochemistry, cells were incubated with 500 nM SYTO 14 and 200 nM Mitotracker for 15 min in MEM with N2 supplements at 37°C, then washed twice in media before fixation. Neurons were fixed using 4% paraformaldhyde (in PBS with 5 mM $MgCl_2$) for 15 min. After fixation, cells were permeabilized with 0.5% Triton-100 for 10

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min. Cells were incubated with a rabbit polyclonal antibody raised against trkC (antibody no. 798, Santa Cruz Biotechnology), which did not cross-react with trkA or trkB sequence, and detected with a secondary antibody conjugated to Cy3. Cells were also labeled with an antibody against a neuronal specific tubulin and detected with a secondary antibody conjugated to fluorescein.

Video Microscopy. An image taken with a Cy5 filter that detected Mitotracker label but not SYTO 14 label, was compared with one taken with a fluorescein filter that detected SYTO 14 label. Signal between the two images were compared using a line scan that generated signal intensity profiles for each image. Average pixel intensities of labeled structures were then compared between each image. Signal that colocalized in both the Cy5 and fluorescein images were considered mitochondria. Signal that only appeared in the fluorescein image was considered SYTO 14-labeled RNA granules. A series of images were taken every 1 min for 10 min and movement was visualized as an animated loop with time compression. The instantaneous rate of RNA granule translocation was determined by taking a series of images every 5 sec for 1 min and calculating $d(x)/d(t)$ as t approaches zero for the two largest $d(x)$ from each moving granule. Cells were viewed with a Zeiss Axiovert 135TV microscope and 63×/1.4 objective on a stage kept at 37°C by a Thermomix BU water bath (B. Braun, Melsungen, Germany). Fluorescent light was generated by a 100-watt mercury arc lamp and filtered using Chroma HiQ bandpass filters. The images were captured with a cooled CCD camera (Photometrics, Tucson, AZ) using a 35 mm shutter and driver (Uniblitz, Rochester, NY) for 10–50 msec exposure times through a 3% neutrodensity filter. Images were processed using METAMORPH 2.0 (Universal Imaging, Media, PA) running on an Intel 486DX2 processor and viewed on a Viewsonic 17 monitor. Images were recorded on a Panasonic Optical Disk drive.

Quantification of RNA Granules. By 4 d in culture, most cortical neurons have an identifiable axon as judged by its length, which significantly exceeds the other neurites, and its untapered appearance. In fixed cultures, the remaining nonaxonal neurites were scored for the number of SYTO 14-labeled RNA granules in a 40 μm stretch of process beyond the proximal shaft. For each experimental group, 10 random fields were chosen and all nonaxonal neurites in each field were scored. Each group represents the summation of three separate cell culture experiments.

RESULTS

Tyrosine Receptor Kinase in Cultured Cortical Neurons. To determine the number of neurons in the cortical cultures expected to respond to NT-3, its receptor, TrkC (18), was labeled immunocytochemically. In 74% of 4-day-old neurons, the neurites labeled in a punctate pattern and the somata stained more intensely. The remaining 26% of neurons had little or no TrkC antibody staining (Fig. 1).

Increase in RNA Granules in Neurites in Response to NT-3. NT-3 application clearly increased the number of RNA granules in those neurites that did not have an axon-like morphology (Fig. 2). The rapid increase in RNA granules following NT-3 administration could be due to their local assembly or their translocation from the cell body and proximal neurites out to the neurite shaft. To distinguish between these two mechanisms, changes in RNA granule distribution were evaluated by time lapse video microscopy. Neurons double labeled with SYTO 14 and Mitotracker were observed after stimulation with NT-3. The granules in neurites present before the addition of NT-3 (Fig. 2, arrowheads) were joined by additional granules 15 min after NT-3 treatment. New granules first appeared just beyond the proximal shaft at 9 min after addition of NT-3 (Fig. 2*c*, arrows). These new granules translocated

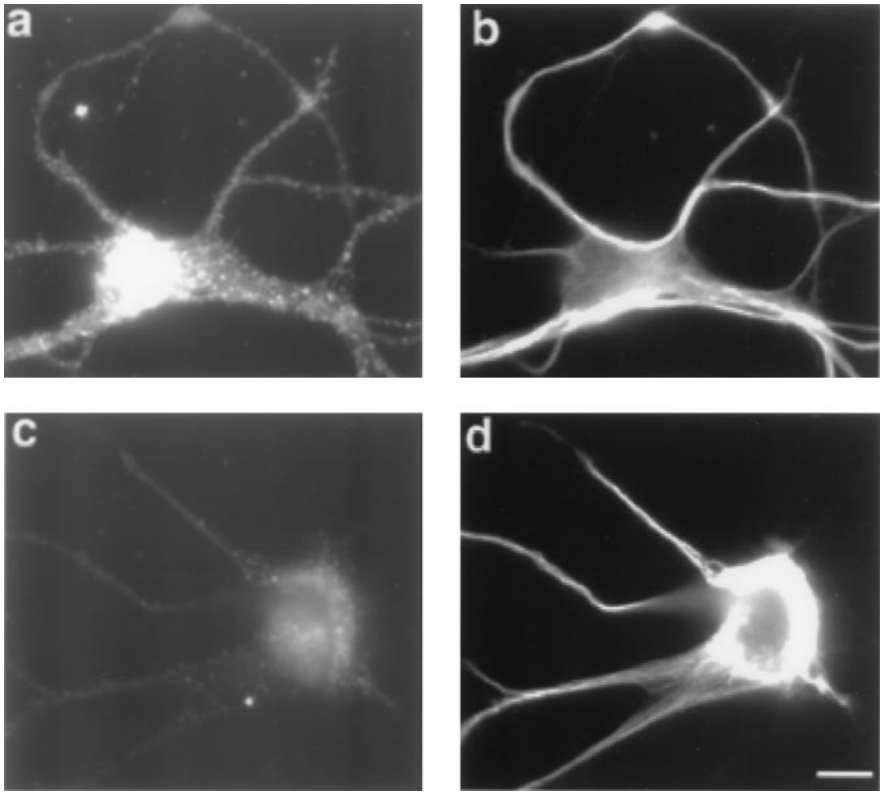

FIG. 1. Immunofluorescence micrographs of 4-day-old cultured cortical neurons double-labeled with trkC and a neuron specific tubulin monoclonal antibody. (*a*) Positive neuronal staining with trkC antibody and (*b*) corresponding staining with the tubulin antibody. (*c*) A negatively stained neuron with trkC antibody and (*d*) corresponding staining with neuronal specific tubulin antibody. (Bar = 10 μm.)

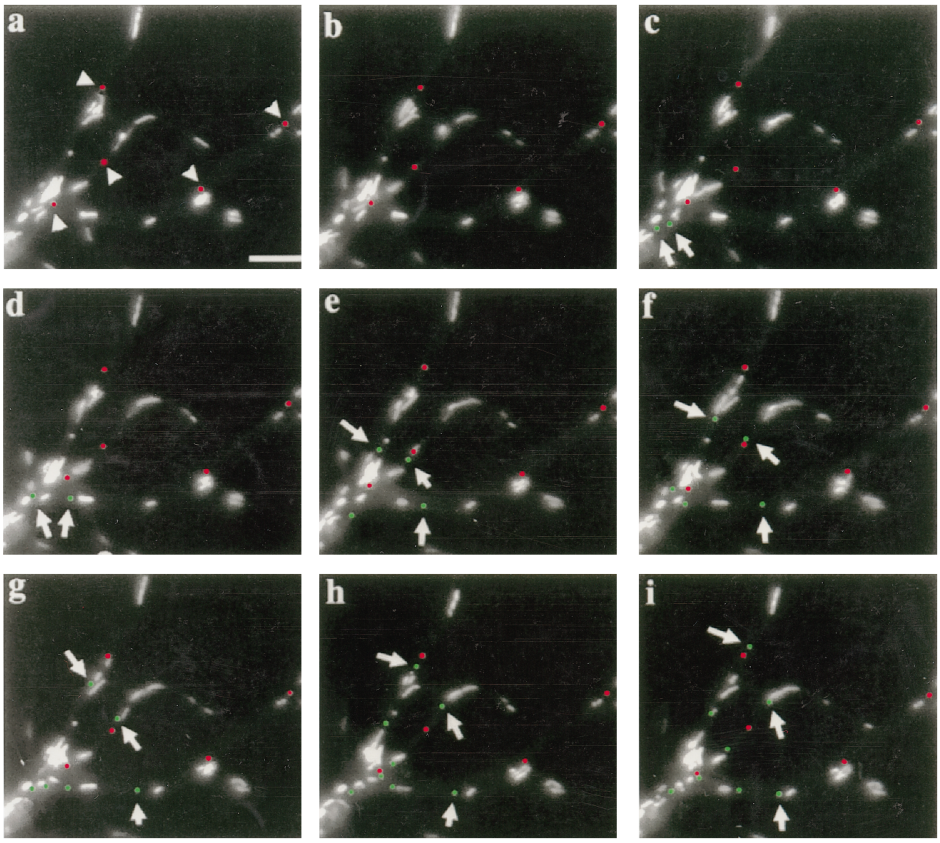

FIG. 2. Time lapse of RNA granule transport in neurites of cultured cortical neurons stimulated by NT-3 (*a*) SYTO 14-labeled neurites 5 min after NT-3 treatment in which arrowheads point to the baseline locations of those RNA granules present at the initiation of the experiment (colorized in red). The remaining labeled intracellular structures are mitochondria. The end of the proximal neuritic shaft is located at the bottom left hand corner. (*b–i*) Micrographs show neurites at 1 min intervals from 8 to 15 min after NT-3 treatment. Arrows point to some of those RNA granules that appeared during the interval (colorized in green). (*c*) New granules first appeared close to the proximal shaft at 9 min and (*d–i*) are observed to move distally into the neurites (arrows). (Bar = 10 μm.)

distally over the remaining observation period (Fig. 2 *d–i*, arrows) with an average velocity of 0.10 μm/sec. The estimated instantaneous rate 0.12 ± 0.01 μm/sec (n = 10) did not significantly differ from the average velocity. Additional granules appeared over the next few minutes and some were observed moving distally (Fig. 2 *e–i*, green granules). These results indicate that NT-3 can induce the translocation of RNA granules and thereby increase their numbers.

Those granules present in the neurites before the administration of NT-3 were not observed to move suggesting that most of the granules entering the neurite originated from a very proximal site either in the cell body or proximal shaft where granule density was very high and granules may be more motile. Rarely, granules appeared *de novo* in a neurite shaft (Fig. 2*e*, left arrow). These granules may have assembled locally in the neurite, a phenomenon that may be comparable to the aggregation of polyribosomes observed *in vitro* following stimulation of a synaptoneurosome preparation (19), or emerged from an obscuring mitochondrion. Overall, there was a 9-fold increase in the number of moving granules from 5 to 15 min after NT-3 treatment. This increase in moving granules declined after 15 min; however, 2 h after treatment there remained a small increase in the number of moving granules (Fig. 3*a*).

Rapid Increase in RNA Granule Density Because of NT-3 Treatment. After just 10 min in NT-3 (50 ng/ml), the number of RNA granules had increased significantly and by 1 h, when the effect plateaued, the accumulated density of granules in neurites from the entire culture had nearly doubled (Fig. 3*b*). Because not all cells responded to NT-3 (some because they lack the TrkC receptor) the increased density of granules in the responsive cells was even higher. When RNA granule density was measured on a per cell basis, about half of the neurons in culture responded to NT-3. This degree of responsiveness is similar to the number of neurons in which NT-3 induced c-fos immunoreactivity and in which morphological changes occurred (20–22). After 1 h in NT-3, the 4-day-old cortical cultures were fixed and the number of RNA granules were counted in a 40 μm segment in each nonaxonal neurite. Fifty-six percent of neurons examined had neurites in which the number of granules did not significantly differ from the control group. The remaining 44% of neurons examined had at least one neurite with a nearly 3-fold increase in RNA granule density ($P < 0.001$, n = 50). In neurons that had at least one neurite with an increased density of granules, 88% had at least one other neurite, and 66% had more than two neurites with an increased density of RNA granules. Therefore, although all the neurites of a single cell did not show a uniform response to NT-3, an increased density of granules in one neurite strongly predicted an increase in the other neurites. Although the number of motile events declined rapidly by 1 h, the increased numbers of granules remained stable at 2 h after the addition of NT-3.

RNA Granule Response to NT-3 Is Dose-Dependent and Blocked by Trk Inhibitor. This increase in the number of RNA granules present in neurites due to NT-3 treatment was dose dependent with an IC_{50} of 2 ng/ml of NT-3 (Fig. 4) and the dose range was similar to that used for eliciting other functional responses to NT-3 (20–22). K252a, a general inhibitor of protein kinases including tyrosine kinases, blocked the NT-3-induced reorganization of the RNA granules (Fig. 4) when used in nanomolar doses. K252a alone had no effect on RNA

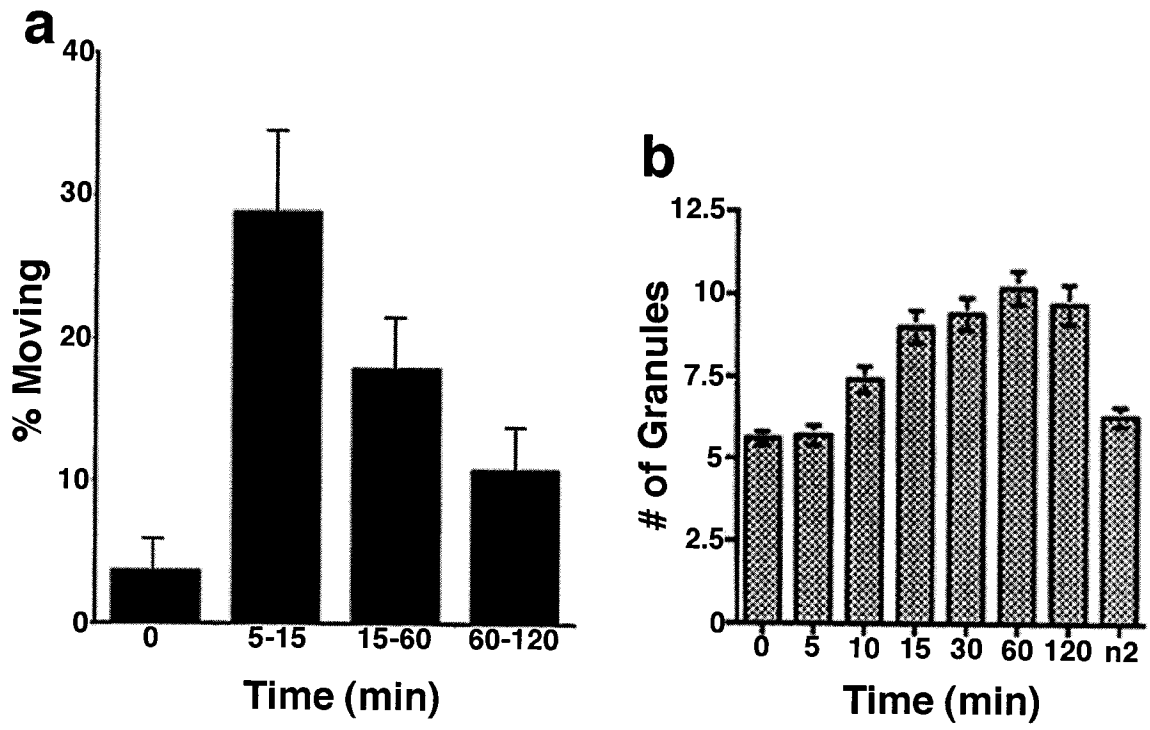

FIG. 3. Rapid increase in RNA granule movement and density in neurites of cultured cortical neurons due to NT-3 treatment. (*a*) The percent of SYTO 14-labeled RNA granules that were observed moving over a 10 min period at varying times after NT-3 treatment (50 ng/ml) ($n = 25$). Between 5–15 min after adding NT-3 there was a 9-fold increase in the number of granules moving ($P < 0.001$). Between 15 and 60 min, the number of moving granules decreased, but was still 4-fold higher than baseline ($P < 0.01$). (*b*) Number of SYTO 14-labeled RNA granules per 40 μm of nonaxonal neurite of 4-day-old cortical cultures were measured at different time points after treatment with NT-3 (50 ng/ml). Time 10 min is significantly different from time 0 ($P < 0.01$); F test demonstrated inequality of variances for control vs. time points 15 through 120 min ($P < 0.05$) and Satterwaite approximation for samples of unequal variances demonstrated means significantly different for these time points ($P < 0.001$). Control lane N2 only received N2 supplements for 2 h. ($n = 50$ for all lanes).

granule density. As shown previously, without NT-3 only a small percentage of RNA granules moved within the observed time periods; thus blocking endogenous NT-3 would not be expected to have a detectable impact on the distribution of

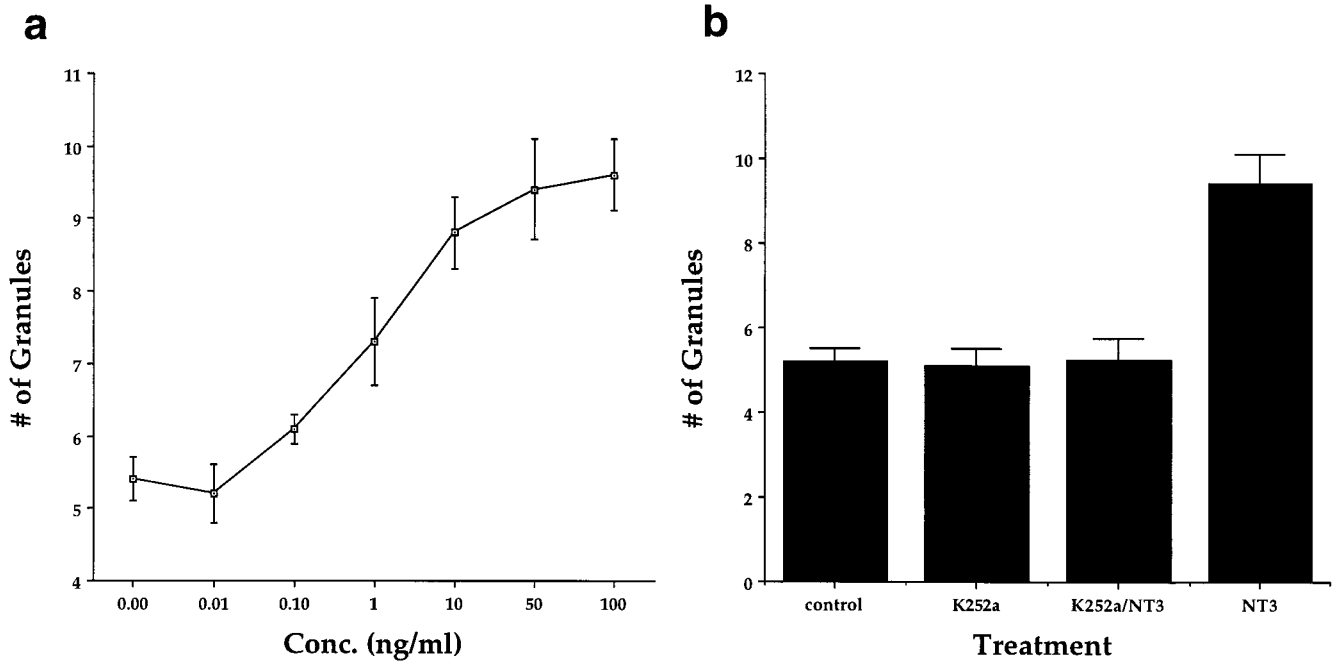

FIG. 4. Increase in RNA granule density due to NT-3 treatment is dose dependent and blocked by a trk inhibitor. (*a*) Number of SYTO 14-labeled RNA granules per 40 μm of nonaxonal neurite in 4-day-old cortical cultures after 1 h of treatment with varying concentrations of NT-3 ($n = 50$); (*b*) Similar measurements in cells treated with 0.01% dimethyl sulfoxide (control), 1 h of 30 nM K252a; 1 h of pretreatment with 30 nM K252a then 1 h of 30 nM K252a and 50 ng/ml NT-3; and 1 h of 50 ng/ml NT-3 ($n = 50$ all lanes). Treatment with K252a blocked the effect of NT-3 ($P < 0.001$).

RNA granules over the course of 1 h. Neither NT-3 (50 ng/ml) nor K252a (30 nM) caused any morphological changes in the cells over the course of the experiment.

DISCUSSION

The redistribution of the RNA granules to dendrites occurs very rapidly after NT-3. It is unlikely that the response depends upon transcription because the RNA granule density begins to increase within 15 min, but there is no induction of c-fos in the first 15 min after NT-3 administration (21). The redistribution of RNA granules occurred well before any detectable morphological changes occurred in the neurites. An increase in RNA granules within neurites may play a role in the implementation of the enhanced neurite outgrowth, increased neurite complexity, spreading of growth cones, and accelerated development of neuronal polarity induced by NT-3 (22). Candidate proteins that may be synthesized in newly transported RNA granules include microtubule-associated protein 2 (23–25), the α subunit of calcium/calmodulin-dependent protein kinase II (26), Arc (10), the glutamate receptor (27), and probably many others (27, 28). The number of neurons that responded to NT-3 by increasing the density of RNA granules was similar to the number of cells that became c-fos immunoreactive or developed morphological changes after NT-3 administration (20–22).

Based on several observations, the translational competence of RNA granules can be hypothesized. Components of protein synthesis are spatially organized together. At 4 days in culture, cortical neurons segregate RNA into nonaxonal neurites (29), suggesting that these cells have developed a mechanism regulating the transport of RNA to specific subcellular targets. In oligodendrocyte processes, granules containing myelin basic protein mRNA colocalize with ribosomal RNA, arginyl-tRNA, and elongation factor 1α (30). In neurons, RNA granules colocalize with polyA mRNA, elongation factor 1α, and the 60S ribosomal subunit (14). Local protein synthesis occurs in dendrites isolated from their cell bodies (31) and in growth cones (32). In fibroblasts, the delocalization of β-actin mRNA leads to the loss of β-actin protein from the leading edge (33). Finally, in CA3 and CA1 isolation experiments, neurotrophin-induced synaptic potentiation required protein synthesis at sites distinct from the pyramidal cell bodies (34). Estimating the CA1/CA3 dendritic fields to be $\approx$100–150 μm from the cell bodies, and given our measured rate of RNA granule translocation at 0.1 μm/sec (1) as well as a 10 min lag period before the onset of NT-3-induced RNA granule translocation, RNA granules would arrive at sites of synaptic activity after 30 min.

Mounting evidence, including the requirement for protein synthesis in neurotrophin-induced synaptic potentiation (37), suggests a link between localized RNAs and neuronal plasticity. Using *N*-methyl-D-aspartic acid receptor-dependent synaptic stimuli in the adult rat hippocampus, Arc (activity-regulated cytokeleton-associated protein) mRNA is strongly induced in granule cell neurons and the interruption of synaptic input to the primary visual cortex reduced Arc mRNA in the deafferented cortex (10). Transgenic mice in which the promoter of the α subunit of $Ca2^{++}$-calmodulin-dependent protein kinase drives the expression of a *lacZ* transcript fused to the 3′ untranslated region of CaMKIIα contain β-galactosidase reaction product localized to dendrites (35). Importantly, the levels of the β-galactosidase protein are variable within the denditic shaft suggesting differential localization of the mRNA within the shaft or local control over translational efficiency. By activating ras-mitogen-activating protein and phosphoinositide pathways (36), NT-3 may signal specific RNAs via RNA binding proteins that contain SH2 and SH3 domains (37) or by coupling to a phosphatidylinositol second messenger system (19).

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