Focal Delivery of Neurotrophins into the Central Nervous System Using Fluorescent Latex Microspheres

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ABSTRACT

A new technique for in vivo and in vitro delivery of neurotrophins is described. The method is based upon the ability of certain fluorescent latex microspheres to adsorb large quantities of neurotrophin and then release the factor for at least three to four days. Injection of such neurotrophin-coated microspheres in vivo provides focal delivery of neurotrophins to distinct populations of neurons. The fluorescence of the microspheres accurately identifies the region of neurotrophin treatment in vivo; two independent methods indicate that microsphere-born proteins do not diffuse significantly beyond the site of injection. In addition to providing focal delivery and labeling of the site of application, labeled microspheres are retrogradely transported by neurons; thus, neurons whose distant terminals are exposed to neurotrophin can be identified and analyzed. This approach provides a powerful method for the introduction of neuroactive proteins into defined and localized regions of the central nervous system.

INTRODUCTION

Traditionally, the biological activity of proteins and other macromolecules has been investigated by testing the effects of exogenous application of the molecules in vivo or in defined in vitro systems. Among the challenges of such experiments, particularly in vivo, are controlling the region of application and determining which cells are exposed to the factor. This issue is of particular concern in the nervous system, where neighboring populations of cells often have very different biochemical and functional properties. With most current methods for in vivo delivery of biologically active agents in the nervous system—which rely upon diffusion of an agent from the cerebral ventricle, from a slow-release polymer or from a cannula connected to an osmotic mini-pump (for example, see References 2, 3 and 19)—it is difficult to limit application to restricted populations of neurons or their terminals and to reliably identify neurons that have been exposed to the agent. In a few studies, these difficulties have been largely overcome by immobilizing the agent of interest on or within latex microspheres. Microspheres in the range of 50–200 nm in diameter are large enough that they diffuse only short distances within tissues, yet they are small enough that, given appropriate surface chemistry, they can be taken up by neuronal processes by endocytosis and sometimes retrogradely transported (9,10). Incorporation of fluorescent dyes within such microspheres facilitates recognition of the site of applica-

tion and identification of the exposed cells. Latex microspheres have been used previously to deliver carbachol to discrete regions of the pontine brain stem in studies of sleep regulation (20) and to deliver light-activated molecules to selected populations of cells to permit their destruction by photothermolysis (12,14). We describe here a similar delivery system for the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). Microspheres coated with neurotrophins are stable and release active factor for at least 3–4 days. In vivo, the fluorescence of the microspheres provides an immediate and reliable marker of the spatial extent of neurotrophin application. Labeled microspheres remain competent to undergo endocytosis and retrograde axonal transport by neurons; thus, it is possible to apply neurotrophins to the terminal arbors of a population of neurons and examine the effects of neurotrophin treatment on their cell bodies at a distant site in the brain. This method should have utility in studying the effects of a variety of proteins in both in vitro and in vivo systems.

MATERIALS AND METHODS

Preparation of Neurotrophin-Coated Microspheres

Microspheres were coated with neurotrophin by passive adsorption based on the interaction of the positively charged proteins with negatively

charged groups of the surface of the microspheres. To remove residual surfactant and maximize the negatively charged surface available for protein binding, fluorescent latex microspheres (Lumafluor, Naples, FL, USA) were ion-exchanged for 6 h at room temperature with 10% (wt/vol) AG[®] 501-X8 Mixed Bed Resin (Bio-Rad, Hercules, CA, USA). The microspheres were then incubated overnight at 4°C with NGF (Collaborative Biomedical Products, Bedford, MA, USA), BDNF, NT-3 or NT-4/5 (human recombinant BDNF, NT-3 and NT-4/5 were the gift of Regeneron Pharmaceuticals, Tarrytown, NY). For each preparation of microspheres, 20 µL of microspheres (10% solids) were added to 100 µL of neurotrophin solution (100 ng/µL). The labeled microspheres were isolated by ultracentrifugation (195 000× *g* at 4°C for 60 min). The supernatant (containing any unbound neurotrophin) was then removed, and the microspheres were resuspended in 20 µL of sterile water. The supernatant and resuspended microspheres were stored at 4°C until assayed in vitro or used for in vivo experiments.

Analysis of Neurotrophin-Coated Microspheres In Vitro

We used several in vitro bioassays to characterize the properties and stability of neurotrophin-coated microspheres. We initially examined the neurotrophic activity of labeled microspheres using PC12 cells stably transfected with the *trkB* gene (11). PC12*trkB* cells (gift of Regeneron Pharmaceuticals) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 6% horse serum, 6% fetal calf serum, 1% penicillin/streptomycin and 1% G418 (Mediatech, Herndon, VA, USA). All cell culture reagents were obtained from Life Technologies (Gaithersburg, MD, USA) except as noted. Under these culture conditions, NGF or NT-4/5 elicited the extension of neuritic processes characteristic of neurotrophin-treated PC12 cells. Neurite extension provides a visual display of the neurotrophic activity of coated microspheres but is not easily quantified. For detailed characterization of the activity of neurotrophin-coated

microspheres, we tested their ability to promote the survival of PC12*trkB* cells in serum-free media (7,11). Cells were plated at high density (approximately 50% confluence) in 96-well plates in serum-free media. Control microspheres, neurotrophin-coated microspheres or free neurotrophin were added and the number of surviving cells was determined after 72 h using the CellTiter 96[™] AQ_{ueous} Non-Radioactive Cell-Proliferation Assay (Promega, Madison, WI, USA). We used this assay to test for direct effects of microspheres on cell survival, to estimate the concentration of neurotrophin that was carried by the microspheres and to determine the time period that the microsphere-bound neurotrophin remained active. To estimate the time period that labeled microspheres could deliver active neurotrophin at physiological temperatures, aliquots of labeled microspheres were incubated in media at 37°C for 1–4 days. The microspheres were removed by ultracentrifugation and resuspended; both the microspheres and the media in which they had been incubated were then tested using the survival assay.

PC12*trkB* cells express only TrkA and TrkB receptors and could not be used to test NT-3-coated microspheres. To verify the activity of NT-3-coated microspheres, we tested their ability to induce phosphorylation of the immediate early gene cyclic AMP response element binding protein (CREB) in a human neuroblastoma cell line. SK-N-SH cells, which express TrkB and TrkC after treatment with retinoic acid (RA; D. Lo, unpublished observations), were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. The cells were plated at high density in 96-well plates and treated with 10 µM RA for four days before being used for the assay. RA-treated cells were then exposed to free NT-3 or NT-3-coated microspheres for 7–60 min. As a positive control, cells were stimulated for 30–60 min with 10 µM forskolin.

Phosphorylation of CREB was assayed by immunocytochemistry using a polyclonal antibody specific for the phosphorylated form of CREB (anti-

pCREB, Reference 6) obtained from Upstate Biochemical (Lake Placid, NY, USA). After stimulation as described above, the cells were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Nonspecific binding was blocked with 5% bovine serum albumin with 0.4% Triton[®] X-100 (BSA/TX); the cells were then incubated in the anti-pCREB antibody (diluted 1:1370 in BSA/TX) for 1 h at room temperature. After rinsing, the cells were incubated for 1 h with an alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (1:500 in BSA/TX; Boehringer Mannheim, Indianapolis, IN, USA) and rinsed. The cells were then incubated with an alkaline phosphatase-labeled rabbit antibody against alkaline phosphatase (alkaline phosphatase anti-alkaline phosphatase [APAAP], 1:200; Boehringer Mannheim) for 1 h, washed in Tris-buffered saline and finally incubated in the dark with *p*-nitrophenylphosphate (pNPP, 1 mg/mL in Tris buffer; Pierce Chemical, Rockford, IL, USA) for 1–4 h. Absorbance was read at 410 nm using a Model MR5000 Plate Reader (Dynatech, Chantilly, VA, USA).

Neurotrophins were bound to the microspheres by passive adsorption (not by covalent linking); thus, we expected they would be gradually released. We used a coculture system and the survival assay to determine whether the activity of the coated microspheres was due to the release of neurotrophin into the media or direct activation of Trk receptors on the cell membrane by microsphere-bound neurotrophin. PC12*trkB* cells were plated in serum-free media in 24-well plates. Polycarbonate 0.2-µm pore size filter inserts (Falcon[®]; Becton Dickinson Labware, Bedford, MA, USA) were placed in the wells, and additional media containing either free NT-4/5 (100 ng/mL) or an equivalent concentration of NT-4/5 on microspheres were added to the inserts. After three days, the filter inserts were removed, and cell survival was determined as described above. The microspheres could theoretically pass more or less freely through the filters (given their average diameter of 50 nm compared to the pore size of 200 nm), but examination (by fluorescence microscopy) of the media above and beneath

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the filters three days after addition of microspheres revealed the vast majority of the microspheres remained above the filters, with no possibility of direct contact with the cells.

Injection and Efficacy of Neurotrophin-Coated Microspheres In Vivo

To investigate the diffusion of neurotrophins following in vivo injection of coated microspheres, small (approximately 200 nL) injections of NGF-coated microspheres were placed in the cortex of young ferrets (P40). Three to four days later, the animals were anesthetized and perfused with 4% paraformaldehyde. Frozen tangential sections of the cortex (50 μ m) were cut on a sliding microtome and collected in phosphate buffer. Free-floating sections were reacted to demonstrate NGF-like immunoreactivity using a rabbit-anti-NGF antibody (Collaborative Biomedical Products) visualized using the avidin-biotin-peroxidase method (Vector Laboratories, Burlingame, CA, USA). Sections were mounted onto gelatin-subbed slides, air-dried, cleared briefly in xylene and cover slips were applied.

For an independent measure of the diffusion of proteins in vivo, microspheres were coated with horseradish peroxidase (HRP; Sigma Chemical, St. Louis, MO, USA) using the same method (passive adsorption) and concentration of protein (500 ng per μ L microspheres) used to prepare neurotrophin-coated microspheres. HRP-coated microspheres were injected into the visual cortex of two young adult rats (5 weeks of age). The rats were allowed to survive for one or three days, after which they were deeply anesthetized and perfused with fixative (1% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffer). Frozen sections (50- μ m thick) of the visual cortex were cut on a sliding microtome and collected in buffer. Alternate sections were reacted histochemically to demonstrate peroxidase activity using diaminobenzidine (DAB; 20-min incubation in 0.05% DAB followed by 20-min additional incubation after addition of 0.01% H_2O_2). The remaining sections were reacted using tetramethylbenzidine (TMB) according to the

method of Mesulam (16) (20-min preincubation in 0.05% TMB in acetate buffer with 0.1% sodium nitroferrocyanide, 20-min incubation after addition of 0.01% H_2O_2). Following the re-

action, the sections were washed in several changes of buffer, mounted onto gelatin-subbed slides, air-dried, cleared briefly in xylene and cover slips were applied.

Figure 1. Efficacy of neurotrophin-coated microspheres in vitro. The plots in Panels A–F demonstrate the relative efficacy (measured as the ability to promote survival of PC12TrkB cells) of free neurotrophins, neurotrophin-coated and control microspheres, and the supernatant remaining after the preparation of coated microspheres. For free neurotrophin, data points indicate survival (relative to parallel cultures containing 100 ng/mL NGF) after addition of neurotrophin at the indicated concentration. For coated microspheres, data points indicate survival after addition of the quantity of microspheres that would have delivered the indicated concentration of neurotrophin if all of the neurotrophin in the initial preparation was bound to the microspheres. The close approximation of the dose-response curve for coated microspheres indicates virtually all of the neurotrophin was, in fact, adsorbed. The curve for control microspheres was generated by substituting equal quantities of uncoated microspheres for the neurotrophin-coated microspheres. For the supernatant, data points indicate survival after addition of the amount of supernatant that would have delivered the indicated concentration of neurotrophin if all of the neurotrophin in the initial preparation remained in the supernatant and none was adsorbed. The substantial right-shift of the growth curve (at least two log units) indicates most neurotrophin was bound to the microspheres and, in fact, only 1% or less remained in the supernatant. Two independent experiments are shown for each neurotrophin; for BDNF and NGF experiments in which the neurotrophin was carried by red (left) and green (right) microspheres are shown. Values are mean $\pm$ standard deviation for triplicate wells.

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Microspheres were used as an *in vivo* delivery system to examine the effects of neurotrophins on neurons that were deprived of activity in the developing visual system. These experiments have been reported in detail elsewhere (18) and are summarized here only briefly. To determine whether neurotrophin-coated microspheres modulate the effects of monocular deprivation (MD) in the developing visual system, we closed one eye of 21 ferrets (postnatal day 38–42) and, at the same time, made highly localized injections of microspheres coated with NGF, NT-4/5, NT-3 or BDNF into the primary visual cortex (V1) of the contralateral hemisphere. The microspheres were taken up by nerve terminals and retrogradely transported; thus, after histological preparation, we could identify and measure projection neurons in the lateral geniculate nucleus (LGN) that were exposed to, bound and internalized neurotrophin. Within individual animals, adjacent injections of control microspheres permitted comparison of these neurons with others that projected to V1 but were not exposed to neurotrophin.

RESULTS

Efficacy of Neurotrophin-Coated Microspheres *In Vitro*

Microspheres coated with NGF, NT-4/5 or BDNF promoted the survival of PC12 $trkB$ cells in a dose-dependent manner. There was no evidence that adsorbing neurotrophins onto the surface of the microspheres diminished their activity. Neurotrophin-coated microspheres were prepared such that the maximum concentration of factor on the microspheres (given the volumes of factor and microspheres used) was 500 ng of neurotrophin per μ L of microspheres. The actual concentration of each neurotrophin carried by the microspheres appeared to be close to that maximum, that is, almost all of the neurotrophin bound to the microspheres. Coated microspheres had activity approximately equal to, and sometimes exceeding, that of free neurotrophin at the concentration used to label the microspheres, while the supernatant that remained after the microspheres were coated and removed by

centrifugation retained little (if any) measurable neurotrophic activity (Figure 1). In those instances in which the supernatant retained neurotrophic activity, it constituted only about 1% of the initial activity (Figure 1, A and F). Both red (rhodamine-containing) and green (fluorescein-containing) microspheres were coated with neurotrophin and tested in the survival assay. Each type of microsphere delivered neurotrophin with similar efficacy (Figure 1).

At the highest concentrations of neurotrophin delivered on microspheres, a decrease in cell survival *in vitro* was apparent that was not seen with equivalent concentrations of free neurotrophin. Since the microspheres were always prepared with the same concentration of neurotrophin, higher concentrations of factor were provided to the cultures by adding more microspheres. The microspheres themselves, at high concentrations, appeared to be toxic to the PC12 $trkB$ cells. Control (uncoated) microspheres at low con-

centrations had no effect upon the survival of PC12 $trkB$ cells, but when the volume of microsphere solution was 0.1% or more of the total volume of media, a decrease in cell survival was observed (Figure 1E). Therefore, cell survival at the highest concentration of neurotrophin delivered on microspheres reflects a balance between the survival-promoting effects of the factor and a detrimental effect of high concentrations of microspheres in the cultures. No evidence of toxic effects of *in vivo* injections of the microspheres were apparent in this study or in previous experiments that utilized the microspheres as retrograde tracers (for example, see References 9 and 10).

NT-3, either in solution or carried by microspheres, induced phosphorylation of CREB in a time-dependent manner (Figure 2). The optimal period for stimulation was approximately 15 min with free NT-3, but CREB phosphorylation continued to increase for 1 h in response to NT-3-coated microspheres.

Figure 2. CREB phosphorylation in response to NT-3 and NT-3-coated microspheres. The level of phosphorylated CREB (mean $\pm$ standard deviation relative to unstimulated parallel cultures) is shown for RA-treated SK-N-SH cells stimulated with forskolin (10 μ M), NT-3 (100 ng/mL) or NT-3-coated microspheres (nominal equivalent concentration 100 ng/mL). The dashed line indicates the level of CREB phosphorylation in the absence of stimulation.

The response to NT-3-coated microspheres appeared to exceed that to free NT-3 at an equivalent concentration (Figure 2).

NT-4/5- and NGF-coated microspheres were stored at 4°C for at least four weeks without measurable loss of activity (Figure 3, A and B). Experiments to test the release from, and stability of, neurotrophin-coated microspheres at physiological temperatures indicated NT-4/5- and NGF-coated microspheres retained measurable neurotrophic activity after at least four days at 37°C (Figure 3, C and D; BDNF and NT-3 were not tested). After four days, activity appeared to be reduced approximately two orders of magnitude.

When neurotrophin-coated microspheres and PC12 $TrkB$ cells were placed on opposite sides of polycarbonate filters (cocultures), there was no significant loss of the ability of the coated microspheres to promote cell survival, despite the fact that under these conditions all but a small percentage of the

microspheres remained above the filter and out of contact with the cells. A slight (10%) decrease in survival (relative to parallel cultures without filter inserts) was evident under these culture conditions, but the same decrease (11%) was seen in cocultures in which free NT-4 rather than NT-4/5-coated microspheres were added. This slight loss of efficacy was probably due to binding of NT-4/5 to the filters. Taken together, these observations indicate it was not necessary for cultured cells to have direct access to labeled microspheres for the neurotrophin initially bound to the microspheres to exert its effect.

Efficacy of Neurotrophin-Coated Microspheres In Vivo

The in vitro studies confirmed the fluorescent microspheres can deliver neurotrophin that remains biologically active for at least several days at physiological temperatures. The observation that released neurotrophin could ac-

count for the activity in vitro led us to investigate the degree to which proteins diffuse from the microsphere injection site in vivo. NGF-like immunoreactivity (NGF-IR) was evident at injection sites containing NGF-coated microspheres but not at injections of control microspheres (Figure 4). NGF-IR was limited to the injection site; there was no evidence of significant NGF diffusion within the limits of detection. Similarly, after injection of HRP-coated microspheres, HRP activity was always coincident with the microsphere injection sites (Figure 5). There was no evidence that HRP diffused beyond the injection site, regardless of whether peroxidase activity was visualized using DAB or the more sensitive TMB method. Taken together, these experiments indicate there is little diffusion of protein away from the microsphere injection site. Thus, it appears that the fluorescence of the microspheres is a reliable marker of the region of neurotrophin application in vivo.

Coating the microspheres with

Figure 3. Stability of neurotrophin-coated microspheres. (A and B) NT-4- and NGF-coated microspheres maintained full efficacy in the survival assay after at least one month at 4°C. (C and D) Each type of coated microsphere continued to deliver active neurotrophin after at least four days at 37°C. Decay in efficacy of neurotrophin-coated microspheres at physiological temperatures reflected both release of factor into the supernatant and some loss of activity, since activity in the supernatant was always less than the activity lost from the coated microspheres. Values are mean $\pm$ standard deviation for triplicate wells.

Figure 4. Diffusion of NGF from microspheres in vivo. (A) A cross-section through the ferret visual cortex is shown with adjacent injections of NGF-coated (left) and control microspheres (right). (B) Immunocytochemical labeling with an anti-NGF antibody reveals NGF-like immunoreactivity associated with NGF-coated microspheres but not control microspheres. There is no indication of significant diffusion of NGF from the injection site. Scale bar = 100 μ m.

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protein did not interfere with the ability of neurons to take up the microspheres at their terminals and transport them retrogradely to the cell body (Figure 6). Thus, it was possible to identify neurons with terminals that were exposed to neurotrophin after *in vivo* injection of coated microspheres. Experiments combining microsphere injection with monocular deprivation demonstrated that neurotrophin-coated microspheres were active *in vivo* (18). After four days of monocular deprivation, the cell bodies of neurons in the deprived layer of the LGN labeled with control microspheres were 20%–30% smaller than control neurons in the adjacent region of the non-deprived layer. Cells in the deprived layer containing NT-4-coated microspheres, however, did not show such shrinkage. NT-4-coated microspheres prevented the shift in cell somal size distribution towards smaller somata that was caused by MD. The other neurotrophin-coated microspheres (NGF, BDNF or NT-3) did not block the atrophy that followed MD. Since *in vitro* assays demonstrated the microspheres carried these factors as effectively as NT-4, the results *in vivo* suggest biological specificity.

DISCUSSION

These experiments demonstrate fluorescent latex microspheres can deliver biologically active neurotrophin, mark the region to which factor was applied and label neurons whose distant terminals were exposed to neurotrophin. Not only is neurotrophic activity maintained, it appears neurotrophins delivered on microspheres may have somewhat greater efficacy than the same concentration of neurotrophin delivered free in solution, since, in some cases, coated microspheres produced a greater response than free factor at an equivalent concentration (e.g., Figure 1C) or the sum of the survival-promoting activity of coated microspheres and the supernatant remaining from their preparation exceeded the total activity expected from the neurotrophin initially used to coat the microspheres (e.g., Figure 1F). In addition to such evidence of increased efficacy in the survival assays, the level of phosphorylation of CREB obtained in response to NT-3-coated microspheres was clearly greater than that seen in response to free NT-3. Increased efficacy could arise from a stabilizing effect such that

immobilization of the neurotrophin on the microspheres makes it less subject to degradation. Alternatively, the effect of a given amount of neurotrophin may be greater when the factor is supplied more gradually. The results of NT-3 stimulation of SK-N-SH cells suggests neurotrophins delivered on the microspheres are made available with a slower time course (Figure 3).

The delivery system described here provides at least two significant advantages over other methods for *in vivo* delivery of neurotrophins and other agents. First, although the concentration of neurotrophin delivered to neurons at any given distance from the *in vivo* injection site remains to be determined, it appears the microsphere-based delivery system provides dramatically more focal application of neurotrophin than previously used methods. In other studies, immunocyto-

Figure 5. Diffusion of HRP from microspheres *in vivo*. (A and B) Cross-sections through two rat visual cortices are shown containing injections of HRP-coated microspheres. (C and D) HRP activity, demonstrated here using DAB, was limited to the injection site; there was no evidence that HRP diffused significantly from the microspheres. Scale bar = 100 μ m.

Figure 6. *In vivo* delivery of neurotrophin-coated microspheres. (A) A tangential section through Area 17 of ferret cortex is shown containing one injection of red, NT-4-coated microspheres (which appear yellow in this double exposure) and one of green, control microspheres. (B) Groups of retrogradely labeled neurons extend across the LGN after such injections in V1 (the border between the A and A1 laminae of the LGN is indicated by the dotted line). The adjacent regions of red- and green-labeled cells projected to adjacent injection sites in the visual cortex and generally contained similar numbers of retrogradely labeled neurons. Red-labeled cells appear to predominate in this particular horizontal section, since it passes through the center of one group of cells and the margin of the other. Scale bar = 250 μ m (A); 150 μ m (B).

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chemical and functional assays indicated neurotrophins diffuse throughout the hemisphere after intraventricular injection (8,17). Immunocytochemical assays following infusion of NT-4 or NT-3 by osmotic mini-pump revealed detectable neurotrophin over an area several millimeters in diameter (2). In contrast, similar immunocytochemical analysis in the present study indicated the spread of NGF was restricted to the site of microsphere injection. More importantly, we have seen effects of NT-4 treatment on one population of geniculate neurons in the absence of effects upon neighboring populations that project to adjacent sites in V1, sites separated by only a few hundred micrometers. Such limited diffusion of neurotrophins from labeled microspheres, despite the fact that neurotrophins were bound by passive adsorption (by ionic interactions) rather than by covalent coupling, is consistent with

earlier reports that the conjugation of carbachol to latex microspheres significantly reduces its diffusion in vivo (13).

In addition to providing for focal delivery, the microsphere-based delivery system may decrease the amount of factor that must be delivered to produce effects in vivo. Notably, the effects we have described in vivo were produced by significantly less neurotrophic factor than has been delivered in earlier in vivo studies. Given the maximum concentration of factor carried by the microspheres and the size of the injections (approximately 200 nL), a single injection of neurotrophin-coated microspheres delivered approximately 100 ng of factor, which was sufficient to block the effects of MD. Previous studies have involved the delivery of tens of micrograms of neurotrophin, usually over days or weeks (for example, see References 1–4, 8 and 15). Due perhaps to differences in the amount of NGF

used or in the method of delivery, the failure of NGF to affect geniculate neurons when delivered to their terminals in V1 in ferrets stands in contrast to previous reports that NGF injected in the ventricles blocks the effects of MD in rats and cats (1,3,4,15). Since NGF infused into the ventricles influences NGF-responsive neurons throughout the brain (8), it may alter the effects of MD after such administration by acting outside of the LGN and V1, perhaps affecting modulatory circuits arising from the basal forebrain (5). The more focal delivery of neurotrophin using the present method provides greater confidence in determining the site of activity.

We have used the microspheres experimentally only for the delivery of neurotrophins, but the method should be useful for examining the effects of a variety of neuroactive factors in the intact nervous system. This study demonstrates that, at least for some proteins,

simple adsorption onto the surface of the microspheres is sufficient to provide focal delivery of the protein and thus simplify interpretation of experimental results. While the method cannot provide the long-term, stable delivery of reagents obtained with slow-release polymers or osmotic methods, the potentially great reduction in the amount of factor required to elicit biological effects can provide significant conservation of rare or expensive reagents. In addition, the availability of both red and green microspheres, coupled with the capacity for focal application, facilitates comparison within individual animals of treated and control neurons or of populations of neurons treated with different agents. In general, the ability to restrict the application of experimental factors to small regions of the brain and to specific populations of neurons to differentially expose specific domains of neurons (axons or dendrites, for example) and to identify treated neurons, should benefit studies in many areas of neuroscience.

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