Noninvasively Induced Degeneration of Neocortical Pyramidal Neurons

in Vivo: Selective Targeting by Laser Activation of Retrogradely
Transported Photolytic Chromophore

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Interactions among neuronal subpopulations determine brain development and function. The present study illustrates the ability to noninvasively and selectively lesion targeted subpopulations of neurons in a highly specific, temporally defined, and geographically localized manner. This method provides a fundamental advance toward rigorous investigation of the importance of identified neuronal subtypes. Projecting pyramidal neurons of rats and mice were targeted by retrograde transport of latex nanospheres from contralateral motor cortex containing a nontoxic chromophore (chlorin e₆) that produces cytotoxic singlet oxygen during photoactivation by deeply penetrating 670-nm light. Geographically defined regions of cortex were exposed to laser illumination at 670 nm to activate singlet oxygen production by the intracellular chromophore, and animals were sacrificed from 4 h to 7 days following laser exposure. Brains were processed to display degenerating neurons using sensitive silver-staining procedures. Selective damage occurred to pyramidal neurons with known callosal projections, solely within layers II/III and V of the illuminated region. Such subpopulation specificity provides models for neural transplantation and analysis of anatomically distributed neuronal networks. © 1993 Academic Press, Inc.

INTRODUCTION

One of the major goals of modern neuroscience is the understanding of the role of specific populations of neurons, from a molecular to a behavioral level, in relation to the overall organization and function of the brain. The ability to selectively lesion defined subpopulations of neurons in vivo in a highly specific, temporally defined, and geographically localized fashion would provide a powerful tool for studying the roles of cell-cell interactions during brain development, provide more precise animal models of central nervous system dysgenesis and degenerative diseases involving selective neuronal loss, and provide model "host" systems for studies of neural transplantation following cell-specific damage within the mammalian central nervous system. For instance, within the field of neural transplantation, many studies demonstrate partially successful integration of several different regions of brain and spinal cord into areas of host structural damage, with partial restoration of function (4, 17, 19, 21). There are many unanswered questions with these models of transplantation in terms of the possible modes of recovery; lesions to antagonistic systems in the host due to the grafting procedure itself, neurohumoral or neurotransmitter release without specific synaptic interaction, passive or active delivery of trophic factors, specific synaptic integration with the host brain, and action as a "pump" across the blood-brain barrier have all been suggested as possible mechanisms (3, 6, 16, 18).

We have recently reported the ability to noninvasively and selectively remove, in vivo, cells which were previously prelabeled in vitro and then grafted into rodent cortex (8). These in vivo studies took advantage of the fact that laser light at near infrared wavelengths penetrates effectively several millimeters through mammalian soft tissue, delivering up to megawatts of energy without nonspecific absorption or damage (1, 20). Most pertinent for our studies, recent studies have shown that near infrared light penetrates through human scalp and skull several centimeters into brain in vivo (11).

The in vivo experiments reported here extend prior studies in vitro using deeply penetrating long-wavelength light energy to effect specific neuronal injury to neurons targeted with cytolytic chromophores which are activated at these wavelengths (7, 9). Such studies are a step toward rigorous tests of the functional role, integration, and performance of neurons transplanted into damaged host brain. We have now refined this uniquely powerful noninvasive approach to produce selective cellular lesions in vivo to predetermined neuro-
nal subpopulations with a specificity which is unattainable by any existent methodology.

MATERIALS AND METHODS

One hundred nineteen adult rodents served as subjects for these studies. Seventy adult male C57B/6J mice and 8 Sprague-Dawley rats served as experimental subjects and under deep ketamine-based anesthesia received unilateral stereotactic injections, within middle and deep layers of motor cortex, of latex nanospheres carrying high concentrations of the singlet oxygen-producing chromophore chlorin e₆ and rhodamine. Homologous contralateral regions of motor cortex were later exposed to noninvasive laser illumination as described below. An additional 35 mice and 6 rats served as control animals which received either nanosphere injections but no laser exposure (N = 8 mice, 2 rats), laser exposure alone (N = 23 mice, 4 rats), or sham injections and sham laser exposure (N = 4 mice).

The nanospheres were fabricated as previously described (10), or the chromophore was attached to the nanospheres.

FIG. 1. Schematic of the experimental design utilizing the latex nanosphere delivery system (see Ref. 10). Nanospheres were injected unilaterally into motor cortex, and 4–12 weeks later contralateral homologous cortex within the retrogradely labeled hemisphere was exposed to laser illumination through intact skull or dura. Control tissue within each animal was located both adjacent to and within the area of laser exposure. Open triangles with dots represent damaged neurons in the lesioned region. Neurons which do not project contralaterally provide controls for laser exposure alone (without nanosphere labeling) because they will not retrogradely transport the nanospheres via callosal connections. The inset at the upper right shows the gross morphology of a mouse brain following laser exposure. The injection site of the nanospheres (arrow) appears red in this figure due to the rhodamine contained within the nanospheres. The homologous area of the contralateral cortex (black circle) was exposed to approximately 100 Joules/cm² transcranially using the tunable dye laser. Note that the cortical surface is grossly intact and uninjured. The pattern and size of two single laser pulses from the short-pulse tunable dye laser is indicated on black photographic paper where the black surface has been etched due to laser exposure. The bars of the ruler represent 1 mm.

FIG. 2. Silver-stained appearance of degenerating neurons in mouse brain 3 days after transcranial laser exposure. (A) This low-power view shows several beaded pyramidal neurons (arrow) in layer II/III of mouse cortex processed for silver staining 3 days following exposure to 100 Joules/cm² of pulsed-laser illumination. The overall integrity of the tissue has not been disrupted, and interspersed neurons, which did not contain retrogradely transported nanospheres, do not stain with silver. The dotted line indicates the cortical surface. (B) This higher power view of the same area shown above illustrates the beaded appearance of the degenerating pyramidal neurons, contrasted with the entirely unaffected interspersed neurons. The arrow points to the same neuron as that indicated in (A). (C) Computer reconstruction from the case above showing the location of silver-stained neurons. Note that only a few stained neurons are visible in any one section. The continuous upper lines represent the cortical surface and the lower lines represent the ventral most aspect of layer VI. A cluster of many degenerating layer II/III pyramidal neurons is indicated by the large arrowhead, with degeneration of fewer layer V pyramidal neurons indicated by the small arrow. The size bars in A, B, and C indicate 40 μm, 8 μm, and 1 mm, respectively.
FIG. 3. Degenerating neurons in rat brain 1 day after laser exposure. (A) Low-power view of cortex from a control rat following 100 Joules/cm² of transcranial continuous wave (CW) laser illumination, but no nanosphere injection. Note that there is no damage due to the laser illumination alone, as contrasted with laser illumination onto cortical neurons which contain retrogradely transported nanospheres containing the photosensitizer e₄ (see below). (B) Low-power view, same magnification as in (A), of cortex from an experimental animal which received the same amount of transcranial CW laser illumination as the control brain in (A). This animal received a contralateral injection of nanospheres 2 months previously to the laser exposure and was sacrificed 24 h after laser exposure. Note the many degenerating pyramidal neurons in layers V and VI and occasionally in layer II/III at this 24-h time point. Also note the lack of any injury to neurons within layers I or IV which also received laser exposure but were not labeled with the nanospheres. (C) Higher power view of layers IV–VI from the same animal pictured in (B). Note the many degenerating neurons and proximal dendrites in layer V, with overlying layer IV entirely unaffected. Size bars indicate 0.15 mm in A and B and 10 μm in C.
surface of rhodamine microspheres by a modification of previously published protocols (12) as follows. A fresh 1 mM solution of chlorin e₈-A (chlorine e₈ monoethylendiamine disodium, Porphyrin Products, Inc., Logan, UT) was made up with 3 ml of 0.01 M phosphate buffer pH 7.4, and activated with 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 30 min at 4°C. Then 50 µl of rhodamine latex microspheres (LumaFluo, Inc., New York City, NY) were diluted with 300 µl of 0.01 M phosphate buffer, pH 7.4, added to the activated chlorin e₈-A, and mixed at room temperature for 1 h. The reaction was stopped with 335 µl of 0.1 M glycine buffer at pH 8.0. This mixture was pelleted by serial high-speed centrifugation (60–90 min at 100,000g), washed, and resuspended until the supernatant was fully clear. The pellet was resuspended in 50 µl of phosphate buffer and the resulting chlorin e₈-A microspheres were injected typically within 14 days of the conjugation, although no effective shelf life has been determined.

Animals were exposed to near-infrared (670-nm wavelength) illumination 4 to 12 weeks later, by which time previous studies have shown there is dense retrograde labeling by the nanospheres in contralateral homologous areas of neocortex, particularly in lamina II/III, V, and VI (10). A tunable dye laser (Candela, MPDL-500) delivered 300 ns pulses of 670-nm light at a repetition rate of 0.1 Hz over exposure areas of 3 mm in diameter, or a continuous wave laser (Aurora Model 150-600, argon-pumped dye laser) delivered 670-nm light over an exposure area of 1 cm in diameter, with a power density of approximately 200 mW/cm².

The laser exposure was delivered via a flexible quartz optical fiber either directly onto the exposed dural surface through a small craniotomy or transcranially following a simple skin incision to expose the skull overlying the targeted region of neocortex (Fig. 1). Animals received laser exposures in three ranges, 12–50 Joules/cm² (N = 30 mice, 4 rats), 100–150 Joules/cm² (N = 62 mice, 4 rats), and 250–300 Joules/cm² (N = 5 mice). The remaining 14 animals served as controls without laser exposure and included nanosphere injections alone (N = 8 mice, 2 rats) or sham injections and sham laser exposure (N = 4 mice). Animals were sacrificed from 4 h to 7 days following laser exposure, with most animals at 24 h (N = 56) or 72 h (N = 17). All brains were processed to display degenerating neurons using sensitive silver stains (13, 14). We reconstructed three-dimensional images of two typical experimental brains processed for silver staining solely to aid in visual analysis. No quantitative analysis was performed from these reconstructions, and histologic assessments were made from direct microscopic observation. Serial 42-µm sections were used to enter digitized section outlines and positive staining cells into a VAX-based DEC graphics display system (Image Graphics Laboratory, The Children's Hospital). Reconstructions in three dimensions were computed, rotated for analysis, and used to capture partial reconstruction images showing relevant visual details. Section thickness was used to scale the reconstructions appropriately.

RESULTS

Both lasers and rodent species gave comparable results and in the experimental animals which received both nanosphere injections and laser exposure to homologous contralateral cortex, large degenerating pyramidal neurons were evident in layers V and VI, and smaller degenerating pyramidal neurons within layer II/III, only within the defined region of laser exposure. The degree and time course of cellular injury was dependent on laser dose and survival time after laser exposure. Experimental animals sacrificed at the longer survival periods (e.g., 72 h or more) displayed beaded staining of the soma and proximal dendrites of many layer II–III pyramidal neurons and an occasional layer V pyramidal neuron (Fig. 2). Animals sacrificed within 4–48 h displayed smooth “Golgi-like” staining of proximal dendrites of many layer V–VI pyramidal neurons in addition to the layer II–III neurons (Fig. 3). Experimental animals which received the lowest laser dose (25 Joules/cm²) directly onto the exposed dural surface displayed selective neuronal degeneration in layer II/III, V, and VI pyramidal neurons within the retrogradely targeted zone. At the higher laser doses, selective pyramidal neuron injury was evident following transcranial laser exposure (Figs. 2 and 3).

Control animals which received either a wide range of laser exposure alone (up to twice the typical dose for experimental animals), or nanosphere injection alone, did not display cellular injury (Fig. 3A). Additional control tissue is located within each experimental brain due to the fact that not all neurons within the area exposed to the laser will have retrogradely transported the nanospheres. This is the case for neurons in layers I and IV, and also those neurons in layers II/III, V, and VI, which lack callosal projections. Such neurons are not damaged by laser exposure alone. Similarly, neurons which are labeled with nanospheres but are outside the area of laser exposure provide controls for nanosphere labeling alone; they also are not damaged.

Previous studies from our laboratories using standard histological techniques have also shown no injury to unlabeled rodent cortex when exposed to similar doses of laser illumination and examined from 2 to 14 days later (8). At significantly suprathreshold laser doses (250–300 Joules/cm²) some nonspecific damage was evident to dura and superficial cortical layers, primarily in layer I.

DISCUSSION

In our earlier experiments we used the short-pulse tunable dye laser and mainly mice as experimental sub-
jects (7–10). We extended these experiments into the larger rodent (rat) to demonstrate the general applicability of the approach. We used the continuous wave laser to provide equivalent power densities over the larger anatomic regions in rat motor cortex. Additional technical considerations with regard to the choice of laser involve the desired effect(s) upon the target chromophore (thermal or photochemical or both) and duration of surgery and anesthesia. Recently, continuous wave sources at 670 nm have become more available and affordable compared to short-pulse lasers. In general, increased surface interactions, tissue scatter, and refraction at the high light energy fluxes present with pulsed lasers diminish the penetration of pulsed light energy at 670 nm through fresh, unfixed rodent brain compared with penetration by continuous wave energy, as is also seen with laser light interaction with skin (1, 5, 20, 21). Thus, for most experiments using photosensitizers such as chlorin e<sub>6</sub>, it would seem advisable to use a continuous wave laser.

We have previously shown that the photosensitizer chlorin e<sub>6</sub> contained within or on such nanospheres does not diffuse from the polymer base and offers superb photostability with less than 20% bleaching after exposure to 50 Joules/cm<sup>2</sup> in the 670 nm range; it continues to produce singlet oxygen (¹O₂) even after continual illumination over long periods of time (10). Prior to illumination at this specific wavelength, no ¹O₂ is produced and no cellular injury results; damage following ¹O₂ production is thought to be secondary to membrane disruption at the cell, organelle, or lysosomal level (15).

Due to the wide range and flexibility of laser and chromophore dose–response curves for effects irreversible cellular damage, fairly large differences in the intensity of neuronal labeling do not pose a practical problem. As long as a threshold level of labeling is present, sufficient damage results to kill the targeted neurons. One of the well-known characteristics of the silver stains used in the present studies to detect degenerating neurons is that only those cells at a specific stage in the process of degeneration will be visualized. Thus, these stains offer a “snapshot in time” of the total “integrated” number of eventually degenerating neurons. The fact that degenerating neurons were seen over a wide range of survival times demonstrates that there is a time course of cell death taking place from quite rapid (a few hours) to more delayed times (one to several days). Neurons which incorporate silver and are visualized at any one time after laser treatment are only a small portion of the total targeted population of neurons which have already degenerated or which will ultimately be affected. Variability in the rate and extent of population injury reflects variable or incomplete retrograde transport of the nanospheres containing photolytic chromophore, and variable geographic concor-

dance of the retrogradely targeted neurons with the region exposed to laser energy.

The results of these experiments show that it is possible to produce noninvasive lesions of such specificity that only a predetermined subpopulation of projection neurons is affected. The specificity of neuronal elimination provided by targeting via distant projections and geographic control of the laser illumination region allows control regions to be contained within immediately adjoining tissue within the same animal. This approach is applicable to models of neuronal degeneration, and toward selective “dissection” of neuronal networks in vivo, and will also allow the development of more precise animal models of human disease states which involve selective neuronal injury, death, or degeneration. The use of such selectively neuron-deficient degeneration models as hosts for neuronal transplantation will allow significant control over both host and graft cellular and structural conditions. Other exciting applications exist in biological and clinical settings in which the selective removal or reduction of a specific cellular population would be desirable, without injury to surrounding normal tissue (e.g., 2).

ACKNOWLEDGMENTS

We thank Bruce Bonsack, Tina Wilusz, and Steven Meadows for expert technical assistance. This work was supported in part by grants from the Whitaker Foundation (R.D.M. and J.D.M.), the Alzheimer’s Association (J.D.M.), the William Randolf Hearst Fund (J.D.M.), NIH Grants HD06859, HD28478 (J.D.M.), NS22404-07 (R.D.M.), and a Mental Retardation Center Grant HD18655 (Children’s Hospital). J.D.M. is a Rita Allen Foundation Scholar and R.D.M. is partially supported by the Veterans Affairs Merit Review program.

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