Unfocused Laser Illumination Kills Dye-Targeted Mouse Neurons by Selective Photothermolysis

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Selective photothermolysis (SP) is a novel technique by which brief, unfocused laser pulses are selectively absorbed by, and cause selective thermal damage to, endogenously pigmented structures. The present experiments demonstrate the feasibility of using an exogenous non-fluorescent chromophore (procion blue) to effect cellular damage by SP. Dorsal root ganglia neurons in vitro were selectively labeled with procion blue and subsequently damaged by unfocused laser illumination. Progressive cellular damage was assessed by propidium iodide (PI), a fluorescent dye that leaks through damaged membranes and binds to nucleic acids. Graded scores of intracellular PI fluorescence demonstrated a highly significant difference in amount of damage between groups of experimental and control cells. Selective photothermolysis is discussed as an experimental tool for neurobiologists in particular and for general use within the biomedical field.

INTRODUCTION

Miller and Selverston13 were the first to describe the general technique of photodamaging cells via injections of fluorescent dyes and subsequent exposure to focused fluorescent light. There are limitations to lesioning techniques using fluorescent light. Fluorescent light by itself can cause damage to cells, and light at these wavelengths does not penetrate well through soft tissue. Therefore, only line of sight surface cells can be lesioned, and care must be taken to not expose surrounding cells to the fluorescent light to guard against non-specific damage. Although Miller and Selverston13 chose to study Lucifer Yellow, they predicted that many optically dense dyes might allow cellular destruction by irradiation.

Anderson and Parrish1-4 first described 'selective photothermolysis' for use within the field of dermatology. They studied the optical properties of human skin, soft tissue and microvasculature in great detail. Mathematical models of penetration, absorption and radiation transfer were developed3,16. Study of the absorption spectra of the major endogenous chromophores, melanin and hemoglobin, allowed selection of laser wavelengths that could penetrate optimally through mammalian tissue in order to cause specific, thermal damage to pigmented structures4. At long wavelengths in the range of 650–850 nm, an 'optical window' exists where penetration depths through soft tissues of many millimeters are possible15. Megawatts of laser energy at these wavelengths can penetrate several millimeters of unpigmented tissue without absorption or damage2. By varying wavelength and pulsewidth, short pulses of high energy laser irradiation have been shown to selectively damage microvessels, leaving nearest-neighbor cells intact. Shorter, higher energy pulses have been used to destroy melanosomes leaving the parent melanocytes intact3.

Selective photothermolysis is quite different from any existent method of effecting cellular damage using laser microbeams, fluorescent dye injections, or focused fluorescent beams7,10,11. Unfocused laser energy generates heat specifically confined to structures that have a greater optical absorption at some wavelength other than the surrounding tissue; thermal
damage results. The localization of damage can be as precise as with microbeam techniques, but thousands to millions of targets can be damaged simultaneously without precise aiming. It is unnecessary to focus on cells to be lesioned with this unique technique because optical, absorptive, and thermal properties of target cells and neighboring tissue provide selectivity of damage by selective photothermolysis.

During a period of laser illumination, absorption and de-excitation without radiation convert laser energy into heat within each target in the exposed field. Targets transfer heat to cooler, neighboring tissue mainly by thermal diffusion, a process that takes time and allows most of the generated heat to remain within targets for many milliseconds. Following a very brief pulse of laser energy (ns or µs), the temperature within targets may readily exceed that necessary for denaturation of protein, membrane disruption, or other thermal damage while surrounding tissue retains its relatively cool temperature.

Such a highly selective targeting and lesioning of cells would be especially useful within complex tissues such as the nervous system where single cells are the functional units. Lesioning of neuronal subgroups and axonal pathways would allow study of development and organization. Powerful, generalized experimental and therapeutic use of SP may be achieved, building on the existing theoretical and practical framework by: (1) choosing appropriate chromophores; (2) developing methods of selectively delivering chromophores to target cells; and (3) demonstrating that SP is practical with exogenous dye-targeting. We report here the first demonstration of SP using an exogenous chromophore.

We selectively delivered an exogenous chromophore (procion blue) to mouse dorsal root ganglia cells in vitro by conjugating the chromophore to wheat germ agglutinin. Following laser exposure we judged cellular damage due to selective photothermolysis by the incorporation of propidium iodide. Propidium iodide is a fluorescent dye that leaks through damaged membranes. We rated propidium iodide incorporation on a scale from 0 to +3. The results of our studies suggest that selective photothermolysis is feasible within the mammalian nervous system using methods to deliver exogenous dye specifically to neuronal subgroups.

MATERIALS AND METHODS

Conjugation of wheatgerm agglutinin (WGA) and procion blue (PB)

Wheatgerm agglutinin (WGA) and fluorescein-labeled WGA (WGA-FLC) were purchased from Vector Laboratories. Procion blue HB was purchased from Sigma Chemicals and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluene-sulfonate ('carbodiimide') was purchased from Aldrich Chemical Company. N-acetyl-D-glucosamine (NADG) was purchased from Pfaustiehl Chemical Company. Activated chromatography gel with NADG attached and protein determination reagents were purchased from Bio-Rad Laboratories. Procion blue (PB) was covalently linked to either wheatgerm agglutinin (WGA-PB) or fluorescein-conjugated wheatgerm agglutinin (WGA-FLC-PB) with modifications of techniques used commercially to derivatize active protein species with the carbodiimide class of reagents (Vector and Bio-Rad Laboratories, personal communication).

Procion blue was dissolved in 10 mM sodium acetate buffer at pH 5.0 to form a 10% solution of the dye by weight and filtered using sterile 0.22 µm filters. Carbodiimide was added to this solution at a concentration (20 mg/ml) that is expected to yield near-quantitative coupling to lectins. Carbodiimide was added to this solution at a concentration (20 mg/ml) that is expected to yield near-quantitative coupling to lectins. The reaction mixture was then placed on ice for 30 min.

Lyophilized WGA was dissolved in sterile 10 mM sodium acetate buffer at pH 5.0 to a concentration of 40 mg/ml. Fluorescein-conjugated WGA (WGA-FLC) was purchased as a solution of 8.3 mg/ml in buffer at pH 8.3 and was dialyzed for 48 h against sterile 10 mM sodium acetate buffer at pH 5.0. Either WGA or WGA-FLC was used in a single conjugation reaction. In order to saturate the active sugar-binding sites and to minimize the risk of steric alteration to these areas via derivatization, 200 mM NADG was added to the WGA or the WGA-FLC. This solution was allowed to stand at room temperature for 1 h. Equal volumes of the activated PB solution and the lectin solution were combined at room temperature and allowed to stand for 4 additional hours. The final solution contained 5% PB, 10 mg/ml carbodiimide, 20 mg/ml lectin, and 100 mM NADG in 10 mM sodium acetate buffer at pH 5.0. The solution was then...

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dialyzed under high vacuum against 100 mM sodium phosphate buffer at pH 7.4 for 3–4 days. The conjugate was continuously washed with phosphate buffer and the dialysate was changed daily.

**In vitro system**

Dorsal root ganglia were removed from C57BL/6J mice and cultured as monolayers using standard techniques. Some cultures were exposed to WGA-PB in the medium for 1 h, washed, and returned to the incubator for an additional 40 h. Control cultures were treated similarly except that they were not exposed to WGA-PB.

Experiments were performed at 37 °C in medium buffered for standard incubator conditions. Dishes were placed on an inverted microscope and fields with 5–20 neurons were located, their X–Y coordinates recorded, and their phase contrast appearances videotaped with a silicon-intensified camera. Fields of cells were centered within areas approximately 0.1 cm² and exposed to 1 μs pulses of laser energy at 600 nm from a tunable dye laser. These pulses delivered 5.0 J of energy to the fields (50 J/cm²). Multiple fields within a dish were successively treated in this way until all laser exposure was complete. Within each field the locations of presumptive neurons, as judged by phase contrast microscopy, were traced onto a clear acetate sheet attached to the video monitor. The neurons to be followed during a time lapse experiment were thus chosen at time zero.

Cellular injury was assessed by propidium iodide leakage through damaged membranes. Laser irradiation was performed with PI in the dish at a concentration of 0.05 mg/ml. Control experiments demonstrated no difference whether or not PI was in the dish at the time of laser exposure. In some control experiments fields of cells were exposed to the laser, and PI then added to the dish at varying times after exposure. In other control experiments PI was added to the dish, washed out prior to laser exposure, and then added again at varying times after laser exposure. These control experiments gave results exactly similar to experiments carried out with PI in the dish at the time of laser exposure. We chose to have PI in the dish at the time of laser exposure to check for immediate damage, and because it simplified the identification of cells that were not healthy prior to actual laser exposure. Each field of cells was relocated using the X–Y coordinates and its phase and fluorescence appearances were videotaped at succeeding times after lasing. During the time-lapse experiments we minimized the chance of non-specific fluorescence damage by exposing fields of interest to no more than 5 s at each observation time, for a total of 30 s or less during a 24–48 h experiment. Image acquisition at 30 frames per second allowed documentation of all focal planes within a matter of seconds. Between observations, cultures were returned to the incubator.

**Scoring system**

After all fields were observed, data analysis was performed in a double-blind fashion. The videotape was set to an unknown frame, the index number recorded, and the index counter set to zero. A second investigator started at that point. Using the clear acetate sheets with the outlines of traced neurons, each neuron was scored for propidium iodide fluorescence on a scale from 0 to 3. A score of 0 indicated an absence of fluorescence over the image of that particular cell, in all focal planes. A score of 1 was assigned for faint cytoplasmic fluorescence, 2 for distinct cytoplasmic fluorescence, and 3 for bright nuclear fluorescence (Figs. 2, 3). This scoring system was found in early trials to be highly reproducible by any single observer and between observers. We believe the scores indicate the relative level of cellular damage.

**RESULTS**

**Conjugation chemistry**

No changes in the absorption spectrum of PB were observed following our conjugation procedures. All showed a peak absorption between 605 and 610 nm, with a wide range of absorption both above and below this wavelength. Affinity chromatography and quantitative analysis demonstrated that the active carbohydrate-binding sites on the WGA molecules were not altered by the conjugation chemistry.

Because any cell containing PB would suffer damage from SP, it was critical to prove the selectivity of PB delivery. Control experiments were carried out with fluorescein labeled WGA-PB (WGA-FLC-PB). Bright intracellular staining was visible in a large fraction of DRG neurons in monolayer cultures 40 h after exposure to WGA-FLC-PB (Fig. 1). Background and non-specific fluorescence on the surfaces of Schwann cells and fibroblasts were almost non-existent.
**Statistical analysis**

Averages and standard error of the mean (S.E.M.) values were calculated for cells in each group at each time point. Because the scores for intracellular fluorescence are non-parametric data we performed both non-parametric and parametric statistical analysis. Non-parametric analysis yielded the same results as parametric analysis. For simplicity, only parametric statistics are reported. See figure legend for details (Fig. 4).

Experimental cells that were labeled by procion blue conjugated to wheat germ agglutinin reacted quite differently compared to the unlabeled control cells. The propidium iodide scores (representing leaky membranes) for these cells increased early and rapidly (see Fig. 4). As early as 9 h following laser exposure the experimental cell group had sustained significantly more damage than all of the control groups combined. Qualitatively, the phase contrast appearances of cells which were labeled and lased deteriorated at a faster rate than control fields; these appearances correlated with the observed increases in intracellular PI fluorescence.

Statistical analysis of control cells reveals no difference between scores for: (1) unlabeled cells in fields exposed to 50 J/cm\(^2\) of laser energy; (2) unlabeled and unlased cells; and (3) cells labeled with WGA-PB but not exposed to laser illumination (Fig. 4). Laser illumination with energy densities of 50 J/cm\(^2\) and with 1 μs durations were found to be almost entirely innocuous to healthy dorsal root ganglia cells in vitro. Unlabeled cells exposed to laser illumination (Fig. 4H) caused no damage above that of unlabeled and unlased control cells (Fig. 4C). These pulses delivered megawatts of power to the exposed areas over these short periods, but the unlabeled tissue did not absorb enough energy to cause damage by thermal or photochemical means. As judged by propidium iodide incorporation, neither surface cells nor underlying Schwann cells or fibroblasts were damaged. This demonstrates that energies of this magnitude are capable of penetrating at least two cell layers without significant absorption. This finding agrees

Fig. 1. Active transport of wheatgerm agglutinin-fluorescein-procion blue (WGA-FLC-PB). a: monolayer of dorsal root ganglia (DRG) neurons 40 h after 1 h exposure to WGA-FLC-PB. Note the complete absence of background staining and the bright intracellular staining of DRG neurons. Fluorescence micrograph, ×225. b: dorsal root ganglion explant culture treated the same as in a. Although only one plane of focus is shown, it is evident that some cells deep in the explant are labeled. It can also be seen that not all cells are labeled, which demonstrates the selective uptake of WGA-FLC-PB. Fluorescence micrograph, ×187. c: phase micrograph of DRG neurons in mono-
well with the published data that 700 nm light penetrates soft tissue and blood vessels to depths of several millimeters without damage to intervening cells and structures\textsuperscript{1–4,15}.

\section*{DISCUSSION}

The experiments reported here explore the feasibility of dye-targeting selective subpopulations of neurons in the dorsal root ganglia (DRG). Figure 2 illustrates a time-lapse experiment of selective photothermolysis (SP) in DRG neurons. The figure shows control cells (not labeled with WGA-PB) that received exposure to a high laser dose (600 nm, 50 J/cm\textsuperscript{2}). Propidium iodide (PI, 0.05 mg/ml) was added to the medium prior to laser exposure. Plates A and B were recorded immediately after laser exposure, and C and D 28 h later. Cultures were prepared as described in the text. A: phase appearance of the field immediately after laser exposure. Note the overall health of the DRG neurons. B: the phase light has been turned off and the field is exposed to the fluorescent light source. The overall absence of any positive PI cells indicates that the neurons pictured in A are healthy, at least to the extent of having an intact membrane. C: phase appearance of the field 28 h after laser exposure. Black circles indicate DRG neurons that are traced onto a sheet of clear acetate. Note that the neuron in the lower right hand corner of the field has moved closer to the other neurons during the course of the experiment. The overall health of the DRG neurons is still quite good, as judged by phase-bright appearance. D: fluorescence appearance of the same field, 28 h after laser exposure. The clear acetate sheet was centered on the phase appearance of the field in C, phase light turned off, and the field exposed to the fluorescent light source. White arrows indicate glial cells that have deteriorated over time such that they have become PI positive. If these cells were neurons (i.e., black circles) they would have received a fluorescent score of +2, indicating distinct cytoplasmic fluorescence. Note that all the neurons (black circles) have remained PI negative over this same time period. Because images were recorded by the video system at the rate of 30 frames/s, it was possible to focus up and down and record all focal planes in a matter of seconds. The video recording is analyzed frame-by-frame, and cells scored for their brightest fluorescence appearance in any focal plane.
cells using exogenous chromophores. Procion blue (PB) was selected as an appropriate chromophore for these experiments because of desirable spectral, non-toxic and reactive properties. Its peak absorption occurs at wavelengths where hemoglobin and melanin absorb relatively weakly. Conjugates of WGA-FLC with PB (WGA-FLC-PB) were used to:

1. provide evidence that lectin molecules subjected to the chemical reactions necessary for carbodiimide derivatization would still be taken up and transported selectively within neurons; and
2. to allow documentation of selective cellular accumulation of the conjugates.

Selective transport of the class of glycoproteins

Fig. 3. Sample video images of selective photothermolysis (SP) time lapse experiment, ×250. This figure illustrates a field of experimental cells labeled by exposure to WGA-PB and exposed to a high laser dose (600 nm, 50 J/cm²). Propidium iodide (PI, 0.05 mg/ml) was added to the medium prior to laser exposure. A: phase appearance of DRG neurons immediately after laser exposure. Note the phase bright appearance of the neurons. White arrows indicate two neurons which are followed in all 4 plates, and which receive a fluorescent score of +3 in panel D. B: fluorescence appearance of the same field at the same time point. The complete absence of any PI fluorescence of the circled neurons indicates that the neurons pictured in A are healthy, at least to the extent of having an intact membrane. This indicates no dramatic immediate damage to these neurons. Some non-neuronal cells have been indicated on the acetate sheet by black arrows. These non-neuronal cells are PI positive and thus indicate that the PI was working. C: phase appearance of the same field 28 h after laser exposure. White arrows indicate two of the neurons which have been traced onto clear acetate, and which received in D a fluorescent score of +3, indicating bright nuclear fluorescence. D: fluorescence appearance of the same field, 28 h after laser exposure. The clear acetate sheet has been left on the monitor to indicate location of the circled neurons. The particular focal plane illustrated shows bright nuclear fluorescence for the two neurons indicated by the white arrows. These neurons received a fluorescence score of 3. In another focal plane, some of the other neurons displayed weak cytoplasmic fluorescence and received a fluorescence score of +1. This example illustrates the need to score a field in all focal planes.
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Fig. 4. Means and standard errors of the mean (S.E.M.) are graphed for experimental and control groups at various times following laser exposure. The filled squares represent experimental cells labeled in vitro with WGA-PB as described in the text and subsequently exposed to 600 nm laser light, 50 J/cm². W represents control cells labeled with WGA-PB, but receiving no laser exposure. H represents control cells not labeled with WGA-PB, and subsequently exposed to a 'high laser dose', 600 nm, 50 J/cm². C represents control cells which were neither labeled by WGA-PB nor exposed to laser illumination. Data were analyzed statistically with analysis of variance using a general linear model. Differences between means were analyzed using the Student-Neuman-Keuls test. Non-parametric analysis was carried out using analysis of variance of ranked scores. Differences between groups were compared using the Kruskal-Wallis test. Non-parametric analysis yielded the same results as parametric analysis. For simplification, only parametric statistics are reported. Fluorescence scores for PB-labeled cells in fields exposed to high doses of laser energy (50 J/cm²) increased early and rapidly. As early as 9 h following laser exposure the average score for the laser experimental group (0.51 ± 0.07, mean ± S.E.M.) is significantly higher (P < 0.05) than any of the control groups. The difference continues to increase up to the longest time point studied.

known as lectins by neuronal subpopulations is dependent on active carbohydrate-binding sites on lectin molecules5,6,12. Affinity chromatography demonstrated that these active sites were unchanged following our conjugation procedures. Mouse dorsal root ganglia neurons have been previously shown to selectively endocytose, transport and accumulate lectins in vivo, and our results in vitro are in agreement with such selective uptake and transport6 (Fig. 1).

Because not all DRG neurons take up wheat germ agglutinin4, we did not expect all neurons within our chosen fields to be labeled by the PB-WGA. The use of WGA-FLC-PB would have allowed us to identify which neurons had taken up the conjugate. However, we chose not to use WGA-FLC-PB because any damage due to fluorescent light absorption by the fluorescein would confound our results. Therefore, we had no a priori knowledge of which neurons in our chosen fields were actually labeled with the procion blue. In addition, one would expect a graded uptake of PB-WGA such that not all neurons that take up the conjugate would do so to the same extent. We chose to follow every presumptive neuron in our chosen fields. The fields thus contained a continuum of unlabeled to labeled cells.

Our scores for the experimental fields are therefore quite conservative, since some scored cells actually contained little or no procion blue. In other words, the real difference between experimental and control groups is probably much greater than our results suggest.

Selection of long wavelengths of laser light in the range of 650–850 nm allows penetration depths of several millimeters through mammalian tissue. On the other hand, wavelengths suitable for excitation of most fluorescent dyes (i.e. < 500 nm) penetrate mammalian tissues very poorly, if at all15,16. Using the endogenous pigments hemoglobin and melanin the selectivity of damage to microvessels and melanosomes has been demonstrated, at depths of several millimeters1–4. In these experiments the overlying epidermis and dermis sustained no observable damage.

This previous work with endogenously pigmented tissues predict that exquisitely specific damage could be obtained with selective photothermolysis utilizing exogenous chromophores selectively delivered to target cells. Such selective delivery should be possible by any number of commonly used neuroanatomical tracing methodologies8. With an appropriate concentration of exogenous chromophore within or on a given class of cells embedded within a 3 dimensional matrix of other tissues, it is theoretically expected that the cells of interest can be killed, leaving all other cell types intact.

With SP, there is no need to optically focus the laser energy to target cells other than to insure that sufficient energy arrives within the area surrounding the cells. As long as there is an adequate concentration of exogenous dye absorbing at the wavelength of the laser in use, and as long as the wavelength is one which is not absorbed by normal tissue, selective damage to labeled cells is not only practical, but prov-
en to be possible. This can be accomplished by adjusting either the amount of label in cells or the dose of laser energy. The mechanism of damage due to SP is not presently clear, from this study or from previous research within the field of photomedicine. Theoretical and thermodynamic considerations suggest that the damage is secondary to thermal effects rather than complex photochemical reactions or singlet oxygen formation. Selective photothermolysis will provide an experimental tool for neurobiologists in particular, and it will be of general use to the entire biomedical field. Potential therapeutic applications of SP targeted via exogenous chromophores include situations in which it is desirable to damage selectively pathological cells embedded within normal tissue.

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