Light-emitting diode treatment reverses the effect of TTX on cytochrome oxidase in neurons

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Light close to and in the near-infrared range has documented benefits for promoting wound healing in human and animals. However, mechanisms of its action on cells are poorly understood. We hypothesized that light treatment with a light-emitting diode array at 670 nm (LED) is therapeutic in stimulating cellular events involving increases in cytochrome oxidase activity. LED was administered to cultured primary neurons whose voltage-dependent sodium channels were blocked by tetrodotoxin. The down-regulation of cytochrome oxidase activity by TTX was reverted to control levels by LED. LED alone also up-regulated enzyme activity. Thus, the results are consistent with our hypothesis that LED has a stimulating effect on cytochrome oxidase in neurons, even when they have been functionally silenced by TTX. *NeuroReport* 12:3033–3037

Key words: 670 nm; Impulse blockade; LED; Mitochondria; Near-infrared; Primary neuronal cultures; Rat visual cortical neurons; Tetrodotoxin

INTRODUCTION

Light close to and in the near infrared range has documented benefits for promoting wound healing in humans and animals [1–3]. Our recent results that used light-emitting diodes in this range have also demonstrated 2- to 5-fold increases in growth-phase-specific DNA synthesis in normal fibroblasts, muscle cells, osteoblasts and mucosal epithelial cells in tissue cultures [4]. However, the mechanisms of action of such light on cells are poorly understood.

Britton Chance's group reported that about 50% of near-infrared light is absorbed by mitochondrial chromophores such as cytochrome c oxidase [5], which is the terminal enzyme of the electron transport chain [6]. Cytochrome oxidase is an integral membrane protein and contains four redox active metal centers: the dinuclear Cu_A, Cu_B, heme a and heme a_3, all of which have absorbance in the red to near-infrared range detectable *in vivo* by near-infrared spectroscopy [7,8].

We hypothesized that the therapeutic effects of such light result from the stimulation of cellular events associated with increases in cytochrome oxidase activity. As a first step in testing our hypothesis, we subjected primary neuronal cultures to impulse blockade by tetrodotoxin (TTX) and applied light at 670 nm via a light emitting diode array (LED) to determine if it could partially or fully reverse the reduction of cytochrome oxidase activity by TTX. The reasons for using LED instead of lasers are given in the Discussion.

MATERIALS AND METHODS

Primary neuronal cultures: The general protocol for culturing neurons from postnatal rat cortex was described previously [9] and was modified from Goslin and Banker [10]. Briefly, a glial feeder layer from postnatal 2-day-old rat cortex were cultured in 60 mm dishes. After 2 weeks, when this layer was subconfluent, visual cortical neurons from 3- to 4-day-old rats were cultured on coverslips coated with poly-L-lysine. Cytosine arabinoside were added one day after plating of neurons to inhibit the replication of non-neuronal cells. Neurons were co-cultured with glial cells, but were separated from them by wax spheres.

Impulse blockade with tetrodotoxin: TTX at a final concentration of 0.4 μM was added to the culture media at different ages of culture and for varying periods of time. TTX was replenished twice a week. Controls had no TTX.

Light-emitting diode treatment: GaAlAs light emitting diode array of 670 nm wavelength (LED; bandwidth 25–30 nm at 50% power), power intensity 50 mW/cm^2 and energy density 4 J/cm^2 when applied for 1 min 20 s, was used on cultured neurons. The wavelength and parameters were previously determined to be beneficial for wound healing [4].

Test groups: Three culture age groups were used at the start of the experiments: early (5–6 days of culture), middle

(11–12 days) and late (15–16 days) groups. The purpose was to determine whether the effects observed were age-dependent. Within each age group, cultures were exposed once a day (1 min 20 s) for the last 5 of the 6 days and to LED exposure without TTX, once per day starting from the second day for 5 days. For the early group, we also tested for the effect of a single LED exposure on the last day of TTX treatment. All experiments were repeated six times.

Cytochrome oxidase histochemistry: Control and experimental cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h on ice. Cytochrome oxidase reactions were performed in an incubation medium containing 0.05% DAB and 0.03% cytochrome C in phosphate-buffered saline, pH 7.2, for 2 h [11].

Optical densitometry and statistical analysis: To analyze quantitative changes in cytochrome oxidase activity following different periods of TTX treatment and LED treatment, optical densities of reaction product were measured by means of a Zeiss Zonax MPM 03 photometer (Zeiss, Thornwood, NY) attached to a Zeiss compound microscope. Multiple, 2 μm spot-size readings were taken from the cytoplasm of each cell. Between 150 and 300 cells were measured from each group, using a ×25 objective lens. The background was subtracted by setting zero over a blank area (without cells) in each slide, and all lighting conditions, magnifications, and reference points were kept constant.

Two-tailed Student's *t*-test for paired comparisons and ANOVA for group comparisons were used to analyze differences between and among treated and untreated groups. Results are expressed as mean ± s.e.m. $p \leqslant 0.01$ was considered significant.

RESULTS

Control: Neurons cultured from postnatal rat visual cortex initially had rounded cell bodies and no processes. Within the first day of culture, neurites formed and lengthened with time, becoming bi- and multipolar neurons that resembled those *in vivo*. After the first week in culture a well-developed neuronal network was present. We have previously shown with neuron-specific neurofilament 200 antibodies that >95% of cells grown in our cultures were neurons [9].

At all time points examined, all neurons had detectable levels of cytochrome oxidase in their cell bodies and processes. However, just as sizes and shapes varied, the intensities of their cytochrome oxidase reaction product also differed. Thus, neurons could be broadly subdivided into three metabolic cell types: dark, moderate, and light according to their optical densitometric values (Fig. 1a) and as described previously [9]. The levels of cytochrome oxidase activity in neuronal processes continued to increase with time in culture.

TTX blockade: At the dosage and times applied, TTX caused a significant reduction in cytochrome oxidase activity of all neurons examined, without causing detectable changes in cell size, shape, or viability (Fig. 1b, Fig. 2a).

TTX blockade and LED treatment: In the presence of TTX, LED treatment daily for 5 days increased cytochrome oxidase activity to levels comparable to that of controls (Fig. 1c, Fig. 2a). This effect was significant for all three metabolic categories of neurons, and was highly reproducible in six repeated experiments.

LED treatment alone: In the absence of TTX, LED treatment caused a significant increase in cytochrome oxidase activity above control levels in all neurons examined (Fig. 2a).

Effect of age in culture: The effects of LED on TTX-inactivated and control neurons were comparable in the three age groups analyzed: starting ages of 5–6 days in culture (DIC), 11–12 DIC, and 15–16 DIC (Fig. 2a–c).

Single LED treatment: A single treatment of LED on neurons previously inactivated by TTX for 5 days brought about a significant increase in cytochrome oxidase activity in darkly reactive neurons, though not in moderately or lightly reactive neurons (Fig. 2c). This increase, however, did not reach control levels, as achieved by five treatments of LED. A single treatment of LED in control neurons without TTX did not result in a significant change in all neurons examined (Fig. 2d).

DISCUSSION

Low energy laser irradiation has been widely used to promote the healing of infected, ischemic, and hypoxic wounds (reviewed in [1]). The rationale is that it stimulates tissues and causes increased cellular activity during wound healing [5,12]. However, lasers have some inherent characteristics that complicate their use in a clinical setting, including limitations in wavelength capabilities and beam width, and in the size of wounds that may be treated [4]. The focal beam of laser light can damage the eye through accidental exposure, and heat produced from the laser light can also damage the tissue.

Light-emitting diode (LED) arrays developed for NASA manned space flight experiments offer an effective alternative to lasers. Unlike lasers and other LED equipment, this patented LED technology produces negligible heat, has clinically been tested to be safe, and therefore has earned FDA non-significant risk status for human trials [4]. These diodes can generate multiple wavelengths, and be assembled into large, flat arrays for the treatment of large wounds. The present study utilized such a flat array with even illumination to treat sister cultures simultaneously. The wavelength, power, and energy parameters used in the present study are based on their beneficial effects for human wound healing [4].

In vivo, light close to and in the near-infrared range is primarily absorbed by two compounds in the mammalian brain, cytochrome oxidase and hemoglobin [8,12–14]. In primary neuronal cultures, hemoglobin (both oxyhemoglobin and deoxyhemoglobin) is not present, and only cytochrome oxidase needs to be considered.

Cytochrome c oxidase (EC 1.9.3.1) is the terminal enzyme of the electron transport system of all eukaryotes, oxidizing its substrate cytochrome c and reducing molecular oxygen to water. It is an important energy-generating

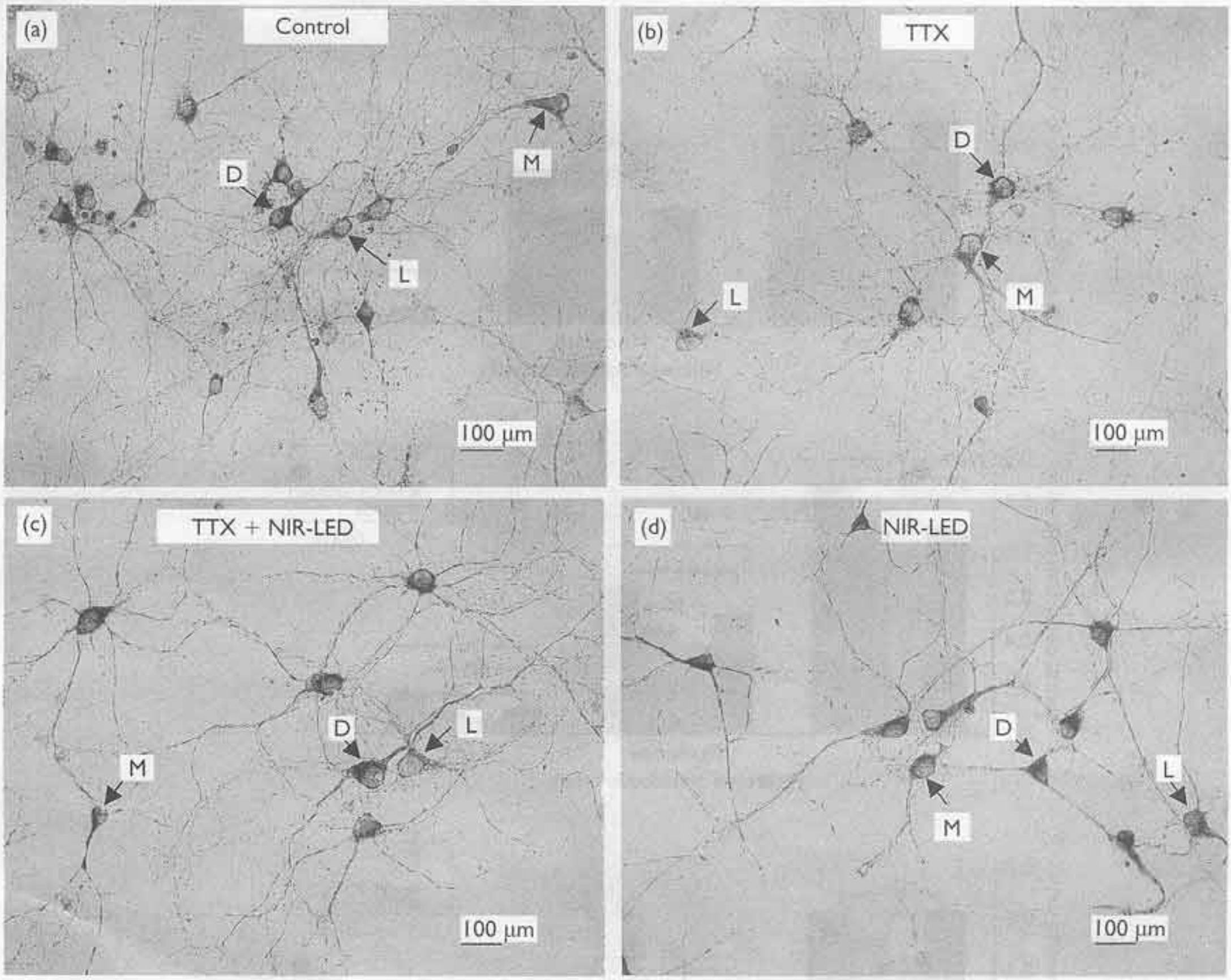


Fig. 1. Neurons from postnatal rat primary visual cortex cultured for 16 days. In control and experimental cases, neurons exhibited varying levels of cytochrome oxidase activity, and were broadly subdivided into darkly (D), moderately (M) and lightly (L) reactive categories (arrows). (**a**) Control cultures. (**b**) Neurons exposed to TTX on days 11–16 of culture. Cytochrome oxidase levels were reduced in all metabolic cell types. (**c**) Neurons exposed to TTX as in (b), but treated with LED on days 12–16 of culture. Cytochrome oxidase levels were higher than those in (b). (**d**) Neurons not exposed to TTX were treated with LED on days 12–16 of culture. Cytochrome oxidase levels were higher than those of controls in (a). See Fig. 2 for optical densitometric analyses

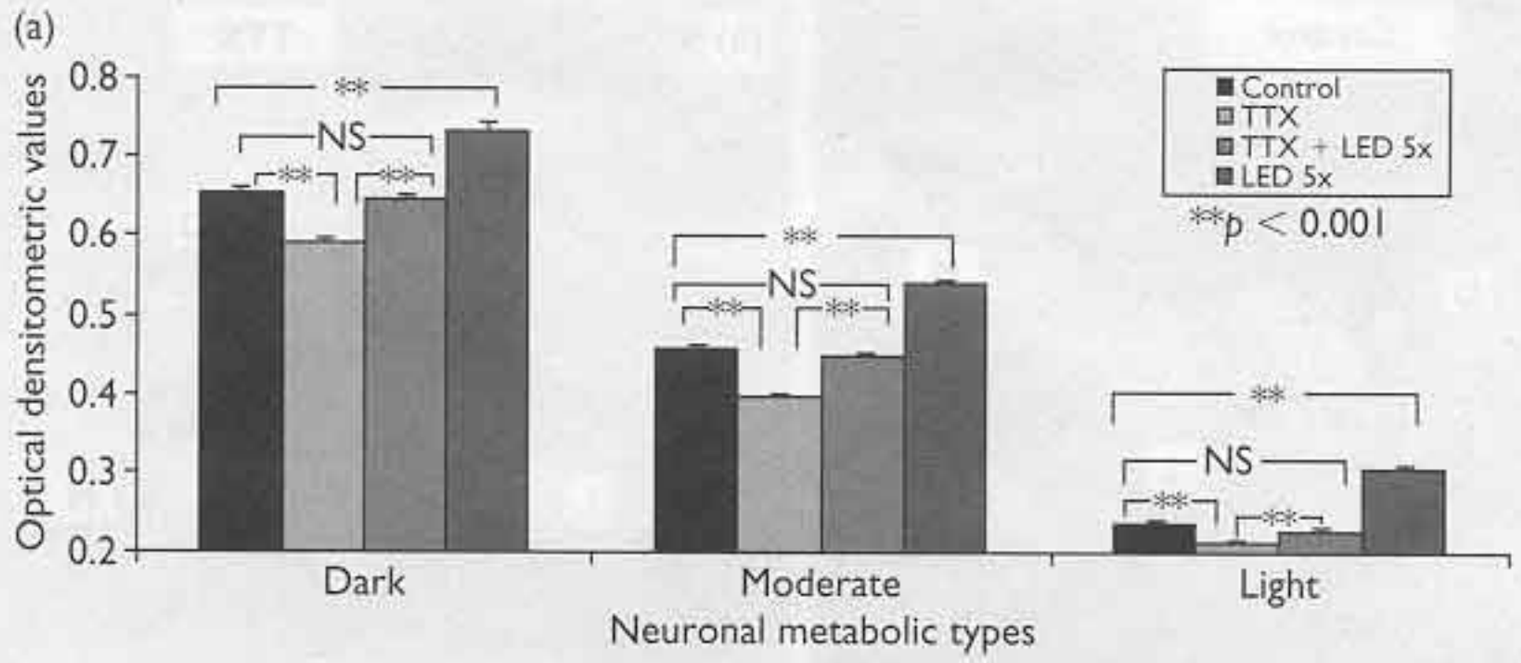
enzyme critical for the proper functioning of almost all cells, especially those of highly oxidative organs such as the brain. The level of energy metabolism in neurons is closely coupled to their functional activity, and cytochrome oxidase has proven to be a sensitive and reliable marker of neuronal activity (reviewed in [15]).

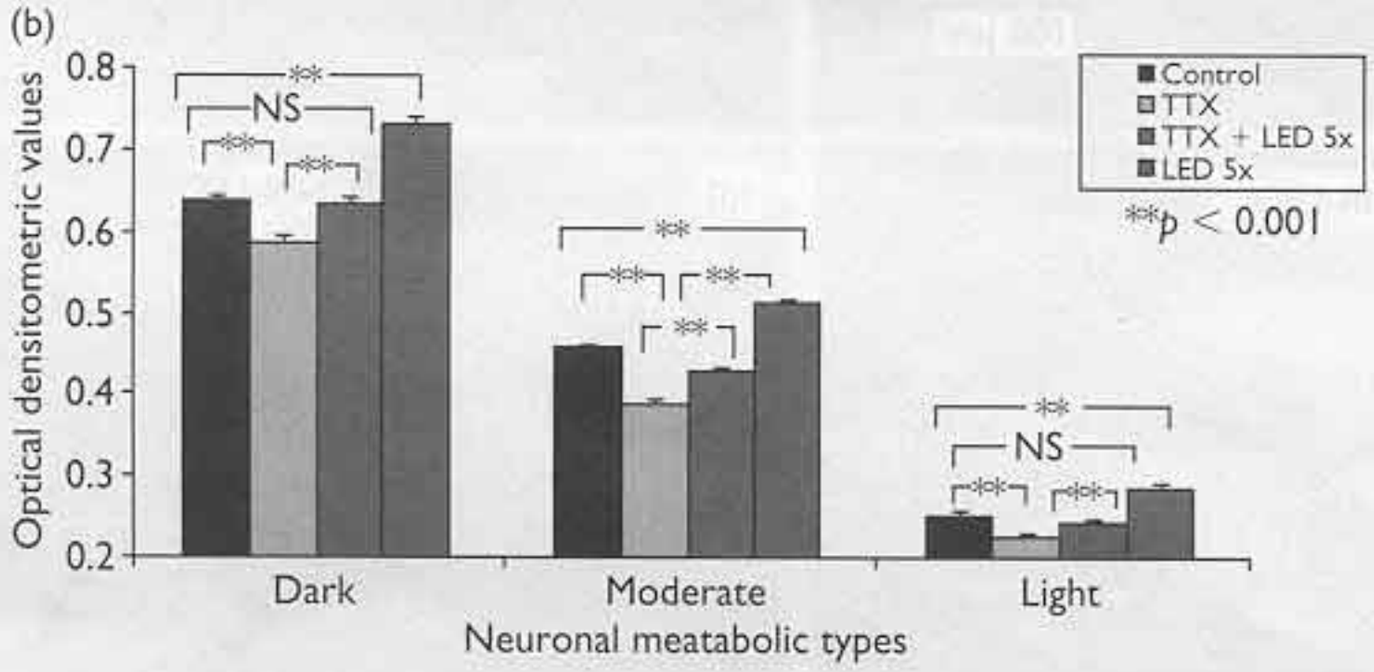
Of all the ATP-requiring functions of neurons, membrane repolarization subsequent to depolarization (both graded and action potentials) consumes the bulk of the energy [15–17]. Thus, excitatory synaptic or spontaneous activity requires membrane repolarization that is highly energy-dependent, whereas protein synthesis *per se* consumes relatively little energy [18,19]. At the ages examined in the present study, there is a progressive increase in cytochrome oxidase activity that corresponds in time to the formation of morphologically mature synapses [20,21] and physiologically recordable synaptic currents [20] in cultured visual cortical neurons.

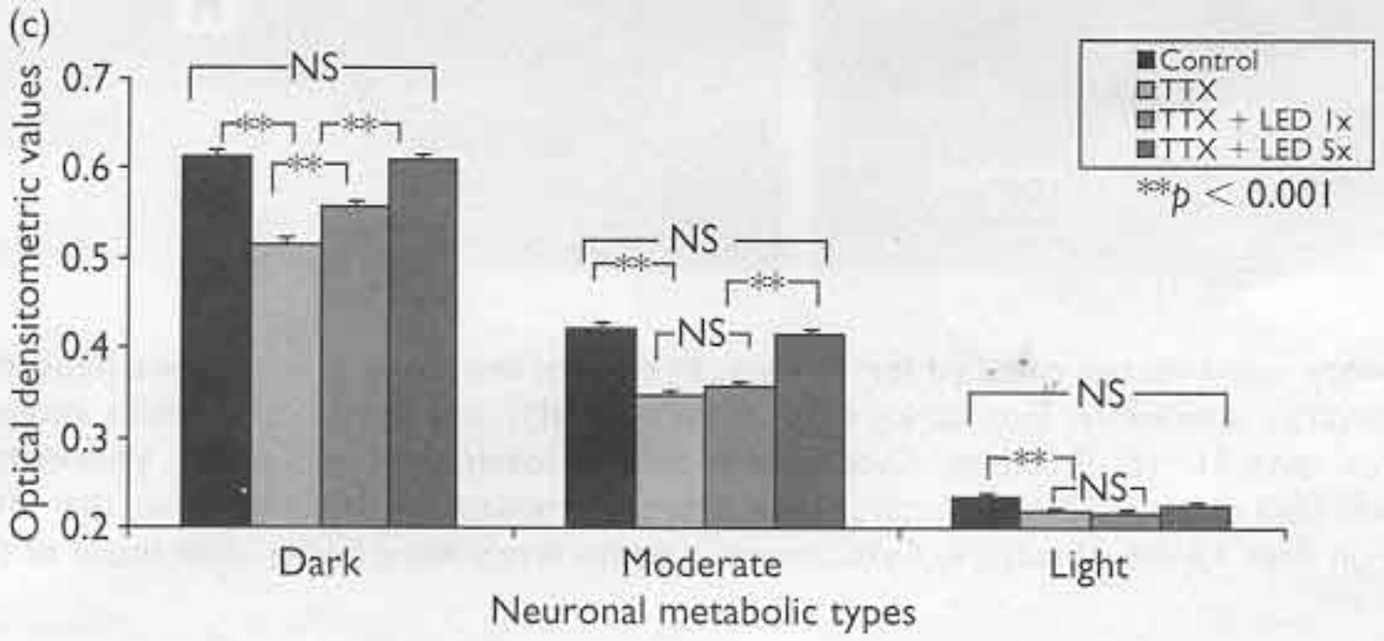
TTX blocks voltage-dependent sodium channels and prevents neurons from firing action potentials. This results in a significant reduction of cytochrome oxidase activity in not only neurons exposed to TTX, but those along the excitatory synaptic chain [9,15]. The present results are consistent with our previous findings.

LED treatment at the parameters used significantly reversed the detrimental effect of TTX on neuronal cytochrome oxidase activity. TTX blocks only action potentials but not graded potentials, the maintenance of which requires ATP generated from the oxidative pathway. The activation of cytochrome oxidase by LED is able to maintain the energy production. Neurons of all three metabolic categories (dark, moderate, and lightly reactive) benefited from this treatment. The effect is more prominent with five treatments over 5 days than one treatment on the last day of TTX exposure, indicating a cumulative event.

The fact that LED applications of only 1 min 20 s over a 24 h period activates and sustains elevated levels of cytochrome oxidase activity indicates that a cascade of events must have been initiated by the initial absorption of light by the enzyme. These events may include the activation of immediate early genes, transcription factors [22], cytochrome oxidase subunit gene expression [22,23], subunit protein synthesis, and a host of other enzymes and pathways related to increased oxidative metabolism. Future







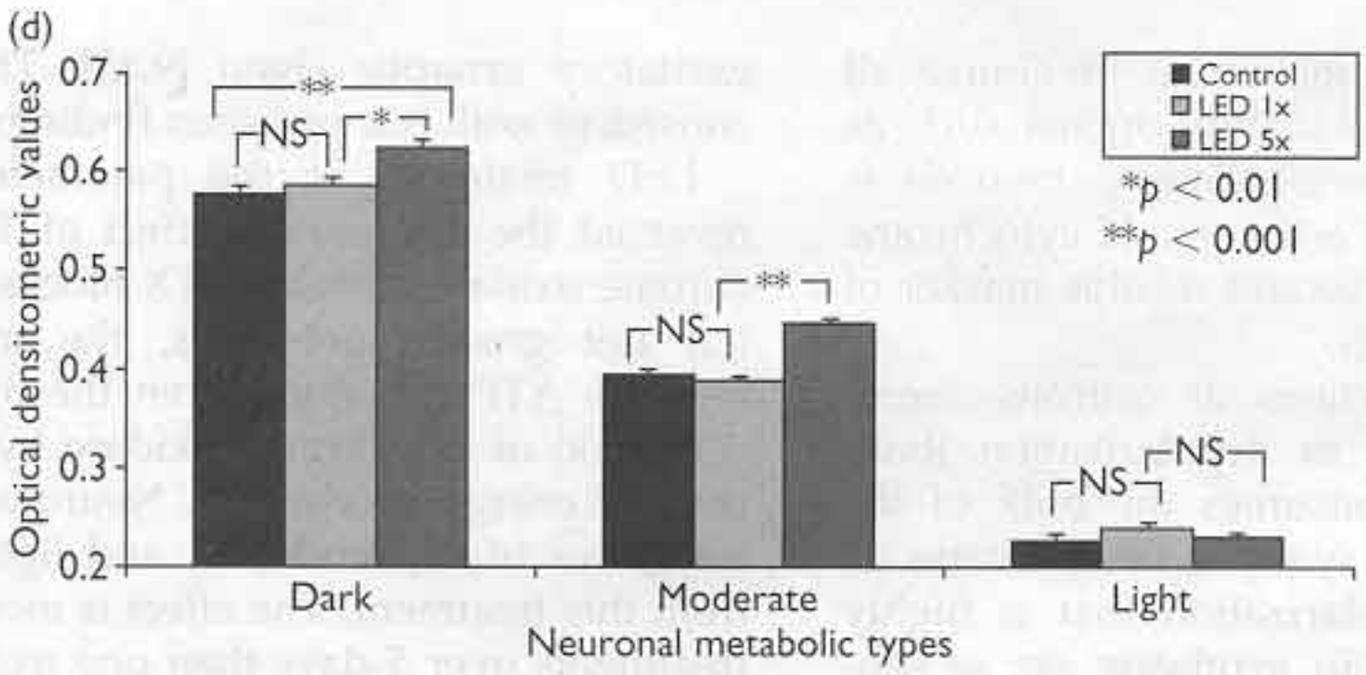


Fig. 2. Optical densitometric measurements of cytochrome oxidase reaction product in neurons of the three metabolic categories: dark, moderate, and light. (**a**) Control and treatment paradigms were the same as those in Fig. 1a–d. There was a reduction in cytochrome oxidase levels in all three metabolic cell categories after TTX treatment ($p < 0.001$) and an increase to control levels after LED exposure ($p < 0.001$). LED alone increased cytochrome oxidase levels above those of controls in all three metabolic cell categories ($p < 0.001$). (**b**) Control and treatment paradigms were comparable to those of (a), except that TTX was given between days 15 and 20 of culture, and LED was delivered between days 16 and 20 of culture. All changes were significant ($p < 0.001$). (**c**) Control and treatment paradigms were comparable to those of (a), except that TTX was given between days 5 and 10 of culture, and LED was delivered between days 6 and 10 of culture. All changes were significant ($p < 0.001$). A single treatment with LED increased cytochrome oxidase level only in the darkly reactive cell type ($p < 0.001$), but it did not reach control values. (**d**) Control and treatment paradigms were comparable to those of (c), except that TTX was not given and LED alone was delivered between the 6 and 10 days of culture. Changes were significant for the dark and moderate cell types ($p < 0.001$), but not for the light type. A single exposure to LED did not bring about any significant change over controls.

studies will be directed at probing these molecular mechanisms underlying the activation of cytochrome oxidase in neurons by LED treatment.

CONCLUSION

Light close to and in the near infrared range effectively promotes wound healing in humans. The recent development of LED arrays has added benefits of lower cost, virtual absence of heat, and larger arrays for large wound treatment. The fact that cytochrome oxidase is a key photoacceptor in the red to near infrared range points to the importance of examining this enzyme in biological systems. The present study indicates that LED reverses the detrimental effects of impulse blockade on cytochrome oxidase activity in primary neurons. Moreover, it up-regulates enzyme activity of normal neurons not exposed to TTX. The prolonged effect of a brief LED treatment implies that it induces a cascade of events leading to the stimulation of gene expression, protein synthesis, and oxidative metabolism.

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