RESEARCH REPORT

830 nm laser irradiation induces varicosity formation, reduces mitochondrial membrane potential and blocks fast axonal flow in small and medium diameter rat dorsal root ganglion neurons: implications for the analgesic effects of 830 nm laser

Roberta T. Chow[1,2], Monique A. David[2], and Patricia J. Armati[3]

[1]Castle Hill Medical Centre, Private Medical Practice; [2]Discipline of Medicine and; [3]Nerve Research Foundation, The University of Sydney, Sydney, Australia

Abstract We report the formation of 830 nm (cw) laser-induced, reversible axonal varicosities, using immunostaining with β-tubulin, in small and medium diameter, TRPV-1 positive, cultured rat DRG neurons. Laser also induced a progressive and statistically significant decrease ($p < 0.005$) in MMP in mitochondria in and between static axonal varicosities. In cell bodies of the neuron, the decrease in MMP was also statistically significant ($p < 0.05$), but the decrease occurred more slowly. Importantly we also report for the first time that 830 nm (cw) laser blocked fast axonal flow, imaged in real time using confocal laser microscopy and JC-1 as mitotracker.

Control neurons in parallel cultures remained unaffected with no varicosity formation and no change in MMP. Mitochondrial movement was continuous and measured along the axons at a rate of 0.8 μm/s (range 0.5–2 μm/s), consistent with fast axonal flow. Photoacceptors in the mitochondrial membrane absorb laser and mediate the transduction of laser energy into electrochemical changes, initiating a secondary cascade of intracellular events. In neurons, this results in a decrease in MMP with a concurrent decrease in available ATP required for nerve function, including maintenance of microtubules and molecular motors, dyneins and kinesins, responsible for fast axonal flow. Laser-induced neural blockade is a consequence of such changes and provide a mechanism for a neural basis of laser-induced pain relief. The repeated application of laser in a clinical setting modulates nociception and reduces pain. The application of laser therapy for chronic pain may provide a non-drug alternative for the management of chronic pain.

Key words: 830 nm, axonal varicosities, fast axonal flow, mitochondrial membrane potential

Introduction

Low-level laser therapy (LLLT) is the clinical use of laser for the treatment of medical conditions at power densities not associated with macroscopic thermal effects, in contrast to thermally mediated surgical applications. Efficacy of LLLT in painful clinical conditions has been established by several recent systematic reviews and meta-analyses [level 1 evidence, according to the Australian Government, NHMRC *(1999)*]. This level of evidence relates to chronic neck pain *(Chow and Barnsley, 2005)*, tendonitis *(Bjordal et al., 2001)*, chronic joint disorders *(Bjordal et al., 2003)*, and chronic pain *(Enwemeka et al., 2004)*.

Address correspondence to: Dr. Roberta T. Chow, MB BS (Hons), FRACGP, FAMAC, MApplSci (Med Acu), Nerve Research Foundation, Room 475, Blackburn Building, D06, The University of Sydney, NSW 2006, Australia. E-mail: rtchow@bigpond.net.au

Blackwell Publishing

Randomised controlled trials (RCTs) provide level II evidence for the efficacy of laser therapy in chronic low back pain *(Umegaki et al., 1989; Soriano and Rios, 1998; Basford et al., 1999)*. In other reviews of laser therapy for painful conditions such as rheumatoid arthritis *(Brosseau et al., 2005)* and musculoskeletal pain *(Gam et al., 1993; de Bie et al., 1998)*, the evidence is equivocal. Such variability in outcomes may be due to the multiplicity of parameters used, including wavelengths, energy, and power densities, with differing frequencies of application *(Chow and Barnsley, 2005)*.

For clinical use, wavelength is generally recognised as one of the most important parameters *(Tunér and Hode, 2002)*. In the treatment of painful conditions, both visible (e.g., $\lambda = 632.8$, 670 nm) and infrared (e.g., $\lambda = 780$, 810–830, 904 nm) wavelengths have been used *(Beckerman et al., 1992)*. In some conditions, infrared wavelengths have been confirmed to be more effective than visible wavelengths, such as in the modulation of neck pain *(Chow and Barnsley, 2005)*. Furthermore, there is mounting evidence that the narrow wavelength spectrum from 810 to 830 nm is effective in a range of painful clinical conditions *(Palmgren et al., 1989; Umegaki et al., 1989; Toya et al., 1994; Fukuuchi et al., 1998; Ozdemir et al., 2001)*. In this wavelength range, we showed statistically significant, clinically relevant improvement in chronic neck pain using 830 nm laser *(Chow et al., 2004; 2006)*.

A number of reports suggest that neural mechanisms are the basis for laser-induced pain relief. These include increased production of serotonin *(Walker, 1983)*, increase in β-endorphin synthesis *(Laakso et al., 1994)*, increased synaptic activity of acetylcholine esterase *(Navratil and Dylevsky, 1997)*, and, at high energy densities (EDs), inhibition of Na^+-K^+-ATPase, responsible for maintaining the resting potential of nerves *(Kudoh et al., 1990)*. Following on from our clinical studies *(Chow et al., 2004; 2006)*, we therefore sought to test the hypothesis that 830 nm laser acts to modulate pain via direct neural inhibition.

In support of inhibitory mechanisms are a number of studies that demonstrate that 830 nm, continuous wave (cw), laser slows nerve conduction velocity and increases latencies in median *(Baxter et al., 1994)* and sural nerves *(Cambier et al., 2000)*. This has led to the proposal that conduction block underpins 830 nm laser-mediated analgesic effects *(Baxter et al., 1994; Kasai et al., 1996; Navratil and Dylevsky, 1997; Cambier et al., 2000)*. Consistent with this is the finding that 830 nm (cw) laser irradiation specifically suppresses nerve conduction in small diameter, thinly myelinated Aδ and unmyelinated C fibres following electrical stimulation of nerves *in vivo (Tsuchiya et al., 1993; 1994; Wakabayashi et al., 1993)*. It is these nociceptors that respond to noxious heat, mechanical, and chemical stimuli and can be identified by the presence of transient receptor potential vanilloid type-1 (TRPV-1) receptors on cell bodies *(Tominaga et al., 1998)*.

To further explore laser-induced inhibitory neural mechanisms at a neuronal level, we investigated the effects of 830 nm (cw) laser irradiation on those domains of nerve function which are critically dependent on mitochondrial function and ATP production because mitochondria are known to be the primary site of laser energy absorption and transduction. We therefore chose to observe effects on microtubule arrays, fast axonal flow (FAF), and mitochondrial membrane potential (MMP), which are functionally interdependent, relying on normal mitochondrial activity and production of ATP, essential to normal nerve function and action potential propagation. We therefore tested the hypothesis that 830 nm (cw) laser irradiation induces such changes by a direct effect on nociceptors in the peripheral nervous system (PNS) and propose that neural inhibition is the basis for the pain modulating effects of laser irradiation.

The laboratory study concentrates on the effect of 830 nm (cw) laser at EDs shown to be effective in laser-induced modulation of neck pain in our clinical trials. Based on this, we focussed on the effect of a single 30 s, 830 nm (cw) laser exposure, while examining EDs and irradiation times around the clinically relevant exposure time of 30 s, in a rat dorsal root ganglion (DRG) culture model. We also defined the subclasses of neurons that were affected and used real-time confocal microscopy and video imaging of living neurons in real time to define 830 nm (cw) laser effects on (1) the morphology of axons; (2) distribution of mitochondria; and (3) changes in MMP and FAF.

Materials and Methods

Preparation of DRG cultures

The study was approved and carried out in accordance with the guidelines of the Animal Ethics Committee, Sydney University # L04/12-2002/1/3669. One- to three-day-old Sprague-Dawley rat DRG were removed aseptically and placed in Hanks calcium- and magnesium-free saline plus 0.05% collagenase and 0.25% trypsin, incubated for 25 min at 37°C, centrifuged at 700 *g* for 4 min and the pellet resuspended in culture medium composed of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.6% additional glucose. Centrifugation was repeated and the pellet resuspended in medium, plated onto collagen-coated glass coverslips in 24-well culture plates, in alternate

wells and maintained at 37°C in 5% CO_2-humidified environment for 4 days. Well plates were designated as experimental or control.

Laser irradiation

The laser device used for irradiation of the cells was a Spectramedics 830 nm, 1 W, cw, diode laser, (parameters of irradiation are outlined in Table 1). To ensure that the EDs delivered to the cultures were equivalent to those used in our clinical studies *(Chow et al., 2003; 2004; 2006)*, the laser output was measured at the Department of Laser Physics, Macquarie University, Australia, using a Trimedyn Optical Power Meter, Model: 210/100, with a 22 mm aperture of detection. These measurements determined that a distance of 4.5 cm between the laser aperture of the handpiece and the surface of the coverslip on which cultures were growing would deliver the appropriate EDs.

The laser handpiece was mounted vertically in a retort stand in a class II laminar flow cabinet. All artificial light sources in the laboratory were switched off. To ensure that laser irradiation did not cause a confounding rise in the temperature of the culture medium, a thermocouple was inserted into replicate wells of culture medium, which was irradiated for 120 s and the temperature of the culture medium measured before and after irradiation. No temperature change occurred after 30 s, with a rise of 0.1°C only at 120 s. This is within the range defined for LLLT *(Kert and Rose, 1989)*. For each experiment, laser irradiation was delivered under sterile conditions to replicate cultures ($n = 2$) for 5 s [total energy (TE) of 1.5 J at ED: 1.4 J/cm^2], 30 s (TE: 9 J at ED: 8.3 J/cm^2), 60 s (TE: 18 J at ED: 16.7 J/cm^2) or 120 s (TE: 36 J at ED: 33.3 J/cm^2). Each experiment was repeated twice. Control cultures were handled in the same way as experimental cultures but without laser irradiation.

Immunohistochemistry

Experimental cultures were fixed at 1, 4, and 24 h post-laser irradiation in chilled acetone at room temperature for 20 min. Control cultures were fixed at corresponding times.

Replicate experimental and control cultures were immunostained with (1) monoclonal anti-β-tubulin isotype 111 (Sigma), specific for neurons; or (2) the nociceptor receptor VR-1 antibody, now known as TRPV-1 *(Tominaga et al., 1998)*. Replicate cultures incubated for 1 h at room temperature with anti-β-tubulin were washed three times in phosphate-buffered saline (PBS), incubated in anti-mouse fluoro-isothiocyanate (FITC; Amersham), diluted 1 : 100 in trizyme buffered saline (TBS) for 1 h at room temperature and washed as above. Replicate cultures incubated overnight with goat anti-VR-1 polyclonal antibody (1 : 100; Santa Cruz) primary antibody were washed three times in PBS buffer, incubated in rabbit anti-goat FITC, diluted in TBS for 1 h at room temperature, then washed as before. Replicate cultures were treated in the same manner with the omission of primary antibody to ensure there was no non-specific staining. Anti-fade solution (Vectashield, Vector Laboratories) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) diluted to 2 μg/ml were added to the cultures and the coverslips sealed onto microscope slides.

Table 1. Parameters of laser device for 830 nm irradiation of rat dorsal root ganglion neurons in culture.

Diode	Gallium aluminum arsenide (GaAlAs)
Power	1 W delivering 400 mW at 4.5 cm above coverslips in well plate
Mode	Continuous wave
Spot size	1.4 cm^2
Power density	300 mW/cm^2

Identification and measurement of neuronal subsets

β-tubulin-positive cells were identified as neurons. These cells also had characteristic neuronal morphology with distinct cell bodies and extended axons forming a neuronal network. TRPV-1 positive cells exhibited the same neuronal morphology. Non-neuronal cells (fibroblasts and Schwann cells) were identified by their morphology and were neither β-tubulin nor TRPV-1 positive with fibroblasts characterized by their irregular, flattened shape and Schwann cells by their primarily filiform shape.

Images were collected by a blinded observer from three to five fields of view from each replicate of the experimental and control coverslips. Images from each source (FITC, DAPI, and Differential Interface Contrast) were collected by CCD (SensiCam) using a Nikon E800 microscope and merged using Adobe Photoshop 5.0. A minimum of 100 cell body diameters from each of the replicate experimental and control cultures was measured from the collected images.

Measurement of MMP

To measure MMP in living cultures, each coverslip in a parallel set of experimental and control cultures was incubated just prior to observation in 3 μM of the mitotracker JC-1, (Md. Probes Inc.) in PBS for 15 min at 37°C, washed with PBS, then mounted in a chamber filled with physiologic solution *(Dedov and Roufogalis, 1998)*.

Each experimental culture was at room temperature in a darkened room, irradiated for 30 s, and imaged by inverted confocal scanning microscopy using Leica TCS SP2 (Leica Microsystem Heidelberg

GMBH) with neutral density filters of 10%, a 488 nm argon laser, and a pinhole of 1 mm. JC-1 was imaged as red fluorescent emission (570 nm) indicating high MMP, while green fluorescent emission (530 nm) indicated low MMP. Background fluorescence was subtracted from the ratios of fluorescent intensity of the red and green channels. 3D reconstructions of J-aggregates, representing mitochondria, were performed using "VoxBlast" NT Version 1.3.2 software (Image Analysis Facility, University of Iowa).

The ratio of red to green fluorescence was measured every 5 min for 30 min, from each of 10 randomly selected fields of view from each culture, and recorded independently in (1) axons and (2) cell bodies, in replicate laser-irradiated ($n = 3$) and control cultures ($n = 2$).

Statistical analysis

Data were analysed by a one-way ANOVA with a level of significance set at $p < 0.05$.

Measurement of FAF by video imaging

In a further set of experiments, living experimental cultures irradiated with laser as above ($n = 3$) and control, non-laser irradiated cultures ($n = 2$) were video imaged in real time with images collected at 1-min intervals for 10 min. The rate of FAF was calculated by measurement of the distance travelled by randomly selected mitochondrial clusters over a 10-min observation period.

Results

Identification of neuronal subsets

Morphology of β-tubulin-positive neurons showed that 86.9% of cell bodies were 10–30 μm in diameter, before and following laser irradiation. This is consistent with their being Aδ and C fibre classes of small and medium diameter nociceptors *(Light and Perl, 1984)* (Fig. 1). These neurons were also TRPV-1 positive.

Laser irradiation induced axonal varicosities in DRG neurons

Single laser exposures of 5, 30, 60, or 120 s of DRG neurons induced intensely β-tubulin III-positive axonal varicosities at all exposure times (Fig. 2). At 120 s, some axonal processes partially detached from the substratum showing disruption of adhesion molecules. There was no cell body detachment. Non-laser irradiated control cultures showed no detectable morphological changes with axonal processes remaining firmly attached to the substratum, which also confirmed that the argon laser of the microscope was not a factor in the experiments. JC-1 staining, discussed in detail later, also showed that the axonal varicosities contained clusters of mitochondria (Fig. 3). Importantly, following laser irradiation, varicosities had resolved by 24 h (Fig. 4). Axonal processes that had shown some partial detachment had reattached.

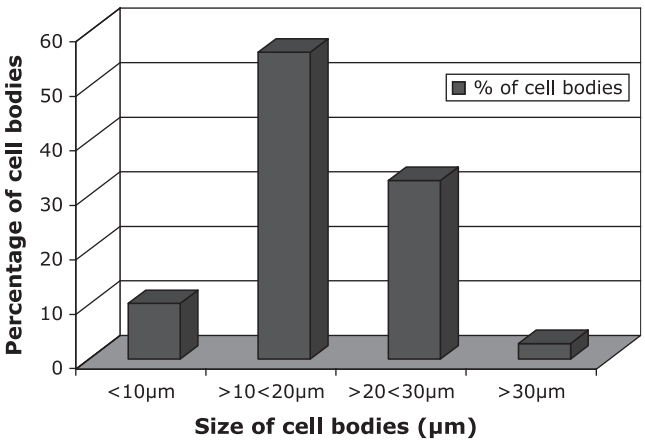

Figure 1. Histogram of cell body measurements of subsets of rat dorsal root ganglion neurons showing 86.9% of the cells are within the cell size range of Aδ and C nociceptors.

Real-time confocal imaging of living DRG neurons

Confocal imaging of living laser-irradiated cultures showed that mitochondria within the varicosities were static over the 10-min observation time (Fig. 5A). This was of great interest as, in contrast, control neurons showed no varicosities, with mitochondria continuing to move along the axons over the 10-min period of observation at a rate of 0.8 μm/s. This rate is consistent with FAF because FAF has a range of 0.5–2 μm/s *(Hirokawa et al., 1991)* (Fig. 5B).

MMP decreased in laser-irradiated small and medium diameter neurons resulting in block of FAF

Five minutes after laser irradiation, there was a statistically significant decrease in MMP ($p < 0.002$) within the axons, including mitochondrial clusters within the axonal varicosities (Fig. 6A). This decrease continued over the 30-min period of observation ($p < 0.005$). MMP of mitochondria in the cell bodies did not decline significantly for 20 min after which there was a significant decrease ($p < 0.05$) (Fig. 6B).

Discussion

We report for the first time that 830 nm (cw) laser irradiation of rat DRG cultures at all EDs used induced the formation of static axonal varicosities in small and

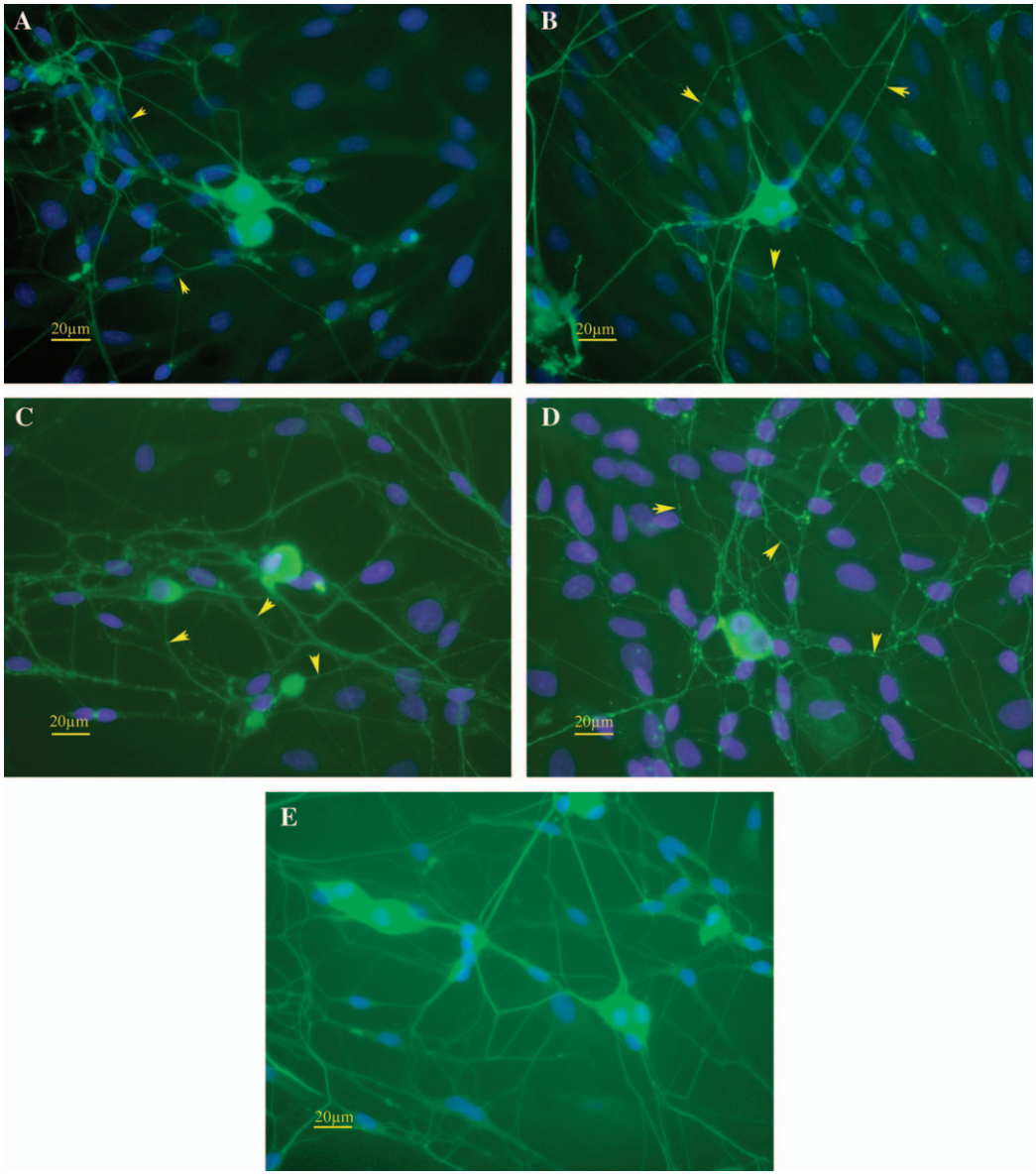

Figure 2. Representative photomicrographs of β-tubulin-positive neurons 4 h after 830 nm (continuous wave) laser irradiation of (A) 5 s, (B) 30 s, (C) 60 s, and (D) 120 s. Note axonal varicosities (➢) and (E) control non-laser irradiated neurons.

medium diameter, TRPV-1 positive neurons, which resolved after 24 h. Laser irradiation also induced a progressive and statistically significant decrease in MMP in and between the axonal varicosities where the mitochondria were seen as clusters. The MMP decrease in the cell bodies was also statistically significant but occurred more slowly. Most interestingly, 830 nm laser irradiation blocked FAF.

The changes occurred in the TRPV-1 positive subset of neurons, consistent with their being nociceptors, i.e., the Aδ and C fibre neurons *(Rang et al., 1991; Gold et al., 1996; Kress and Reeh, 1996; Julius and Basbaum, 2001; Mandadi, 2001)*. The importance of TRPV-1, which responds to noxious stimuli such as capsaicin, high temperatures (>43°C) and acidity (pH < 6.5), is highlighted by the elegant studies of Caterina *et al. (2000)* in TRPV-1 knockout mice, where the mice exhibited little or no response to noxious thermal stimuli. Moreover, the mice failed to exhibit pain behaviour when the skin of their hind paws was injected with capsaicin, sensitivity to which is a hallmark of unmyelinated, small-diameter nociceptors.

In other studies, when 830 nm laser irradiation (40 mW, cw) was applied proximal to the site of electrical stimulation of rat saphenous nerve, there was selective inhibition of the slowest component of the action potential, again indicative of selective Aδ and C fibre inhibition *(Tsuchiya et al., 1993; 1994)*. In

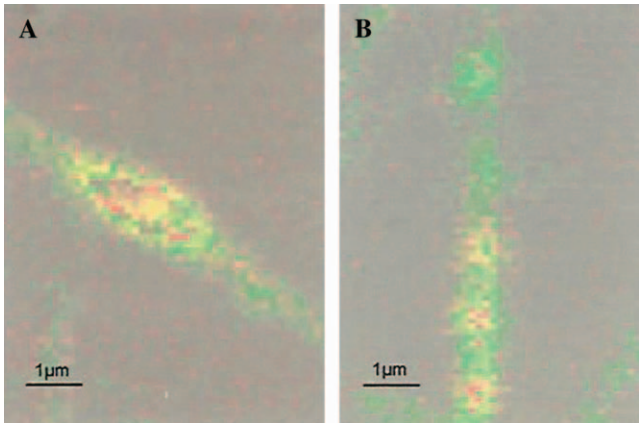

Figure 3. Representative pixelated images of JC-1 stained mitochondria seen as red/green fluorescence (a) clustered within an axonal varicosity (➢) after 30 s, 830 nm (continuous wave) laser irradiation and (b) in a control non-lasered axon.

a parallel study, rats treated with capsaicin at birth and devoid of Aδ and C fibres subjected to the same protocol had action potentials with no slow component but no change in the conduction of large, myelinated fibres *(Tsuchiya et al., 1993)*. In yet another study, 830 nm laser irradiation (120 s, 350 mW, cw), applied to the rat incisor and the tooth pulp electrically stimulated, suppressed only the slowest component, i.e., C fibres *(Wakabayashi et al., 1993)*.

Such studies are directly relevant to our hypothesis that 830 nm laser irradiation acts on nociceptor-specific neurons with varicosity formation as a major morphological feature. Varicosity formation has previously been reported in only one other study of 830 nm laser irradiation, where mouse DRG neurons had varicosities restricted to neurons that were substance P-positive, another characteristic of nociceptors *(Chen et al., 1993)*. In these studies, the neurites ceased outgrowth following irradiation but recommenced after 5 h. This is relevant to our finding that varicosities had resolved by 24 h, showing that the neurons were undamaged. Consistent with these findings are those of Park *et al. (1996)* who applied N-methyl-D-aspartic acid (NMDA) to mouse neocortex neurons, stimulating NMDA receptors and inducing varicosities, which resolved 2 h after stimulation ceased. The authors propose that reversible varicosity formation is a physiologically adaptive response to non-toxic stimuli.

In other non-laser studies, varicosities were observed when substance P was applied to rat DRG neurons *(Tanelian and Markin, 1997)* and following application of high concentrations of substance P to central nervous system (CNS) neurons, varicosities were found in dendrites *(Mantyh et al., 1995a)*. Mantyh *et al.* considered that the varicosities were a response to internalization of substance P bound to its receptor and depressed the neuronal response to nociceptive stimuli. They also suggest that small-diameter fibres are selectively more sensitive to varicosity formation.

Of direct relevance to our findings are ultrastructural studies of the local anaesthetics lidocaine and procaine, which demonstrated by transmission electron microscopy that these agents caused cytoskeletal changes including disruption of the microtubules and associated structures *(Poste et al., 1975; Nicolson et al., 1976)*. As well, the microtubule destabilizing agent, colchicine, used for the treatment of gout *(Ahern et al., 1987)*, affects some of its therapeutic benefit by reversible disruption of microtubule structure. These studies are consistent with pain relief by neuronal-specific changes such as those induced in our study by 830 nm laser irradiation.

The varicosities that we report here are intensely β-tubulin positive, indicative of microtubule disruption. This varicosity formation has important functional implications as high-energy mitochondria are carried by FAF along microtubules to provide ATP for maintenance, generation, and restoration of the axon potential. FAF is also essential for retrograde transport of low-energy, ATP-depleted mitochondria back to the cell body. Microtubule disruption would therefore block ATP supply also essential for the delivery of components of the synaptic vesicles, in particular synaptophysin, which is required for neurotransmission *(Nakata et al., 1998)*.

The disorganization of the microtubule infrastructure for FAF has additional implications for disruption to transport of ATP. Our finding of a significant decrease in MMP, a surrogate measure for ATP, after laser irradiation of live neurons, reflects decreased ATP availability which, in turn, will decrease ATPase activity *(Goldstein and Yang, 2000)*. ATPases include kinesins and dyneins, the molecular motors for organelle transport and Na^{+}-K^{+}-ATPases. As 830 nm irradiation of rat saphenous nerve at high EDs inhibits Na^{+}-K^{+}-ATPases *(Kudoh et al., 1990)*, this would be consistent with our findings of decreased MMP and ATP with a resultant conduction block. It would also be expected that decreased MMP could affect Ca^{2+}ATPase activity, which regulates intracellular calcium *(Budd and Nicholls, 1996; Liu and Barth, 2004)*, essential for neurotransmission, further compromising neurotransmission.

With regard to conduction block, varicosity formation reported in the studies discussed above led Tanelian and Markin to formulate a biomathematical model to quantify changes in electrophysiological parameters associated with such morphological alterations *(Tanelian*

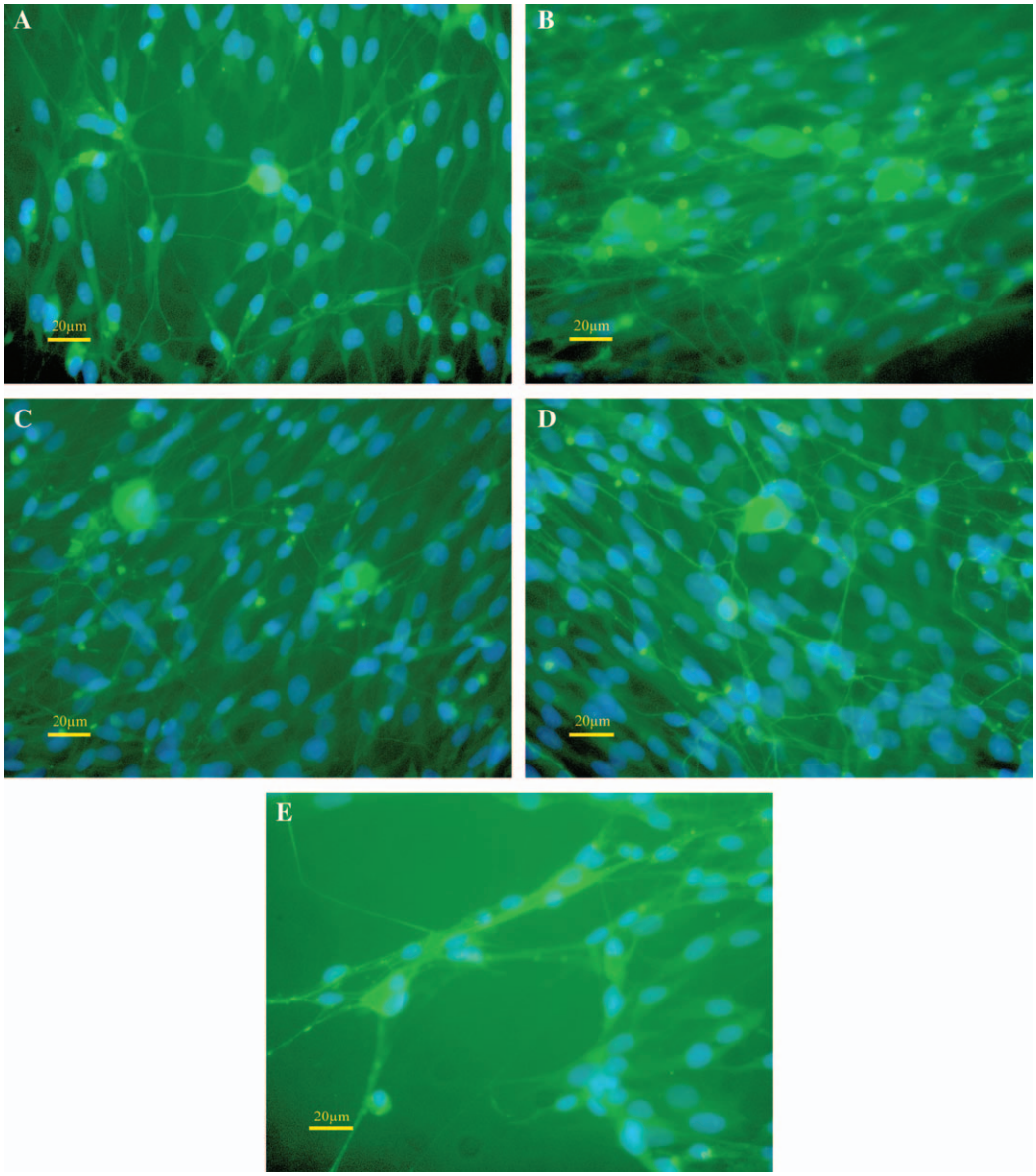

Figure 4. Representative photomicrographs of β-tubulin-positive neurons 24 h after 830 nm (continuous wave) laser irradiation of (A) 5 s, (B) 30 s, (C) 60 s, and (D) 120 s, and (E) control non-laser irradiated neurons.

and Markin, 1997). Their model proposes that the biophysical change seen as varicosities would result in slowing of nerve conduction in the affected neurons, thereby directly affecting information transfer within the nervous system.

The resolution of varicosities, found in our study after 24 h, indicates that any such laser-induced microtubule disruption, blockade of fast axonal transport, conduction, and neurotransmission block would be reversible and therefore temporary.

While this is the first report of an 830 nm (cw) laser irradiation effect on mitochondria and MMP of DRG neurons, one other study does report the effect on MMP of 780 nm (200 mW, ED: 2 J/cm^2) in the keratinocyte HaCaT cell line *(Gavish et al., 2004)*. This showed a significant increase in MMP during the first hour of observation, in contrast to our findings, and a decrease after 3 h. Studies using visible wavelengths on MMP of non-neuronal cells consistently show an increase in MMP, similar to the initial period of observation by Gavish *et al. (Passarella et al., 1994; Vacca et al., 1994; 1997; Alexandratou et al., 2002)*. Such differences may be related to the higher EDs (8.3 J/cm^2) used in our study, four times greater than that used by Gavish *et al.* This is an important point for the model we propose because the ED delivered to the neurons used in our laboratory studies were carefully measured to be equivalent to that we used

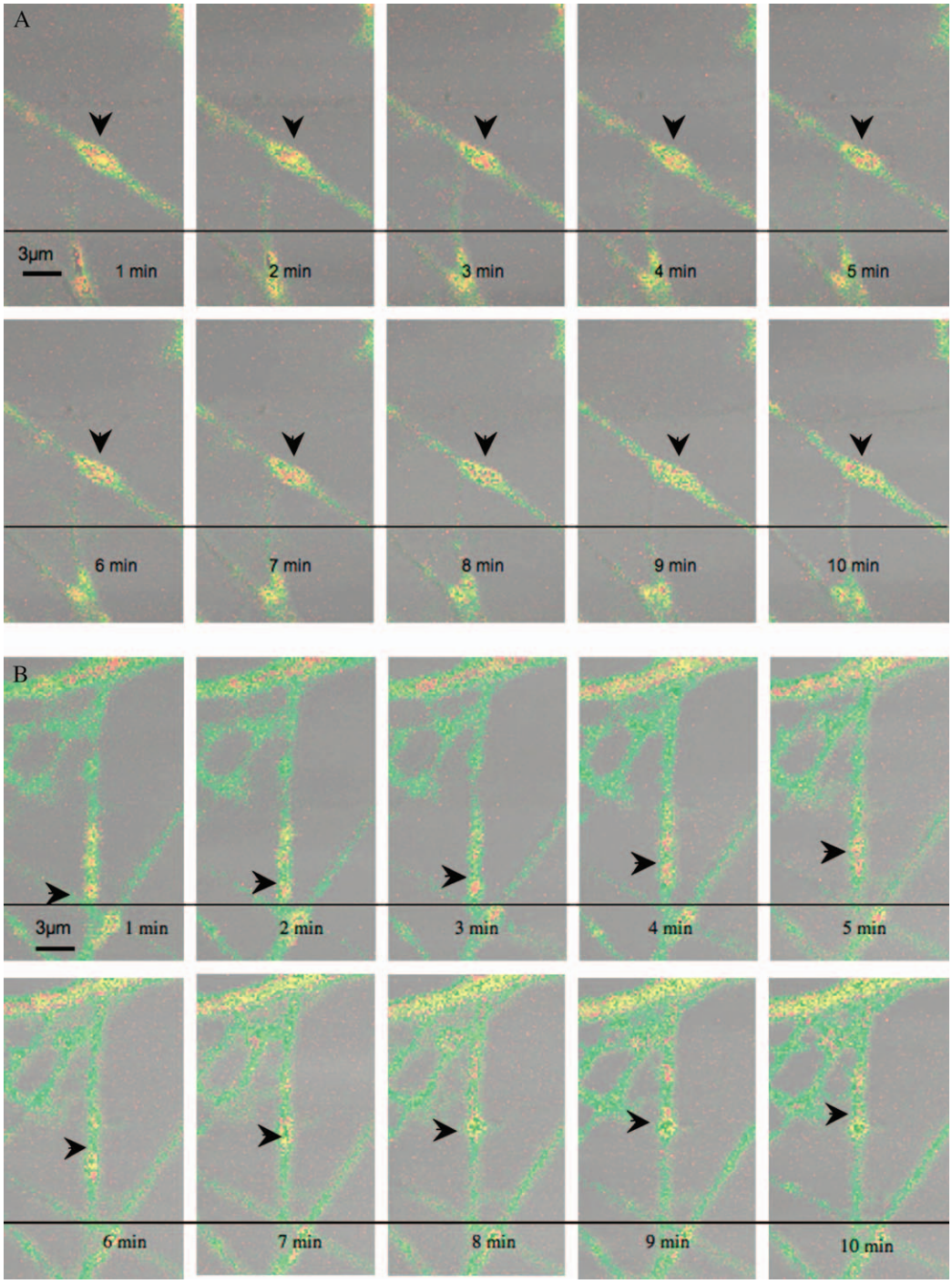

Figure 5. (A) Real-time confocal images of a JC-1 stained axon after 30 s of 830 nm (continuous wave) laser irradiation showing a static varicosity (➧) indicating blockade of fast axonal flow (FAF) and a decrease in MMP over 10 min of observation. (B) Real-time confocal images of JC-1 stained, non-laser irradiated, control axons showing movement of mitochondria (➤) at 0.8 μm/s, consistent with FAF and no decrease in MMP over 10 min of observation.

in the clinic for long-term reduction of chronic neck pain *(Chow et al., 2004; 2006)*.

Our data, showing varicosity formation and decreased MMP at all the EDs used, potentially reflect only the inhibitory phase of the Arndt-Schultz Law *(Ohshiro, 1990)*, a biphasic phenomenon where stimulation of biological activity occurs at low EDs and inhibition at high EDs. While this response is often observed in studies of visible laser irradiation on non-neuronal cells in culture *(Lam et al., 1986; Bolton*

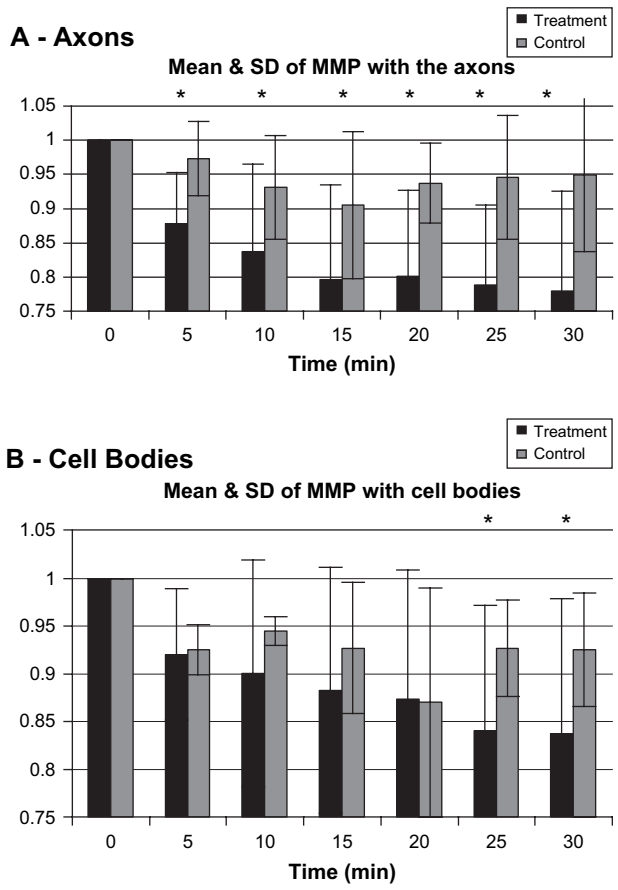

Figure 6. Histogram showing changes in mitochondrial membrane potential levels in (A) axons and (B) cell bodies after 30 s, 830 nm (continuous wave) laser irradiation (black), and control, non-laser irradiated neurons (gray)

et al., 1990; Sommer et al., 2001; Pereira et al., 2002), it does not occur universally *(Ozawa et al., 1997; Pogrel et al., 1997; Luger et al., 1998)*. Moreover, it may well be that ED is not the only relevant factor but that the morphology of sensory neurons with their single axons up to 1 m in length may be important. It is only in neurons that there is a distinct cell body where protein synthesis occurs and high energy, ATP-rich mitochondria are generated. We have supportive evidence for this from other studies related to cell morphology. An unpublished study on NTera 2 cells showed that H_2O_2-induced oxidative stress resulted in varicosity formation in those cells with a neuritic morphology, whereas NTera 2 cells with the more epithelial-like morphology did not *(David, 2002)*. This is supported by studies of primary human brain tissue in culture where neurites also became varicose under the same experimental protocol *(Roediger and Armati, 2003)*.

Thus, the 830 nm (cw) reversible, laser-induced blockade of FAF and decreased MMP we consider to be related to unique neuronal morphology, particularly as sensory neurons have a single axon and no dendrites, as well as the incident EDs.

We provide for the first time, a direct mechanism by which 830 nm laser irradiation exerts pain relieving effects, namely via PNS, nociceptor-specific inhibition. We propose that this is directly relevant to our LLLT clinical trial data *(Chow et al., 2006)* leading to the pertinent question: How does 830 nm laser irradiation initiate such analgesia? The First Law of Photochemistry states that laser energy must be absorbed to exert its effect *(Smith, 1999)*. In mitochondria, photoacceptors within the membranes absorb light, including 830 nm laser *(Karu, 1999)*, inducing conformational change in enzymes *(Friedmann and Lubart, 1995)* and increasing reactive oxygen species, such as singlet oxygen *(Lubart et al., 2000)*, which can regulate signal transduction *(Lubart et al., 2006)*. Cytochrome c oxidase has been identified by its absorption spectrum as a primary photoacceptor for 830 nm laser irradiation *(Karu et al., 2005)*. Thus, cytochrome c oxidase and possibly other photoacceptors within neuronal mitochondrial membranes provide a primary site for 830 nm laser energy absorption and transduction.

We further propose that absorption of laser energy and its transduction into electrochemical or electrophysical events triggers a secondary cascade of cell-specific events as suggested by Karu *(Karu, 1999)* and that this is represented by changes in neuronal architecture seen as varicosity formation, mitochondrial clustering, and microtubule disarray. These morphological changes reflect functional events such as the statistically significant decrease in MMP and blockade of FAF. We further propose that this leads to conduction block and failure of neurotransmission from PNS nociceptors to CNS neurons. Tanelian and Markin, in developing their mathematical model of the functional, biophysical changes of varicosity formation causing slowing of action potential propagation, propose that the morphological changes may be the basis for several forms of counter-irritation–induced analgesia such as transcutaneous electrical nerve stimulation, peripheral efferent nerve stimulation, and spinal cord and deep brain stimulation *(Tanelian and Markin, 1997)*. We would add LLLT to this list. More importantly for long-term pain modulation, Mantyh *et al. (1995a; 1995b)* suggest that morphological reorganisation might alter the integrative properties of neurons and many constitute "an important mechanism of neural plasticity," which is the basis for adaptive change in the CNS, including memory, in response to altered nociceptive input, again consistent with our proposed mechanism of effect.

As laser irradiation was applied to skin surface in our clinical trials, it is important to consider how this relates to the laboratory studies. Criticism could be

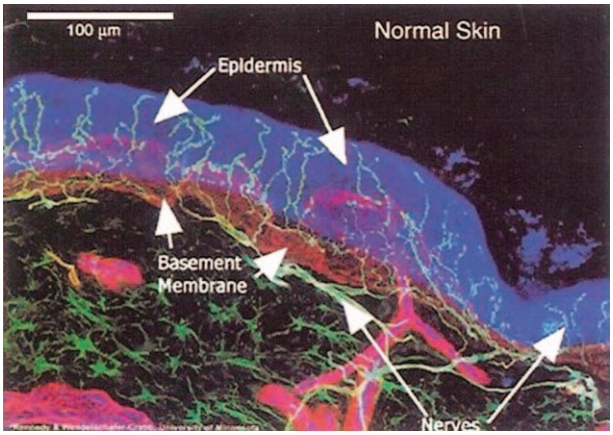

Figure 7. Cross-section of skin and subcutaneous tissues showing peripheral afferent terminals in the epidermis. Reprinted from Kennedy et al. (2005), with permission from Elsevier.

made that our neuron-specific model is based on irradiation of the whole neuron and that EDs were excessive for DRG neurons in culture. However, our laboratory studies show that the axon is more affected than the cell body, the neurons are not irrevocably damaged, and the varicosities resolve. Also, comparison of our study with other studies of 830 nm laser irradiation of cultured human foreskin keratinocytes and lung fibroblasts *(Pogrel et al., 1997)*, human periodontal cells *(Ozawa et al., 1997)*, and a number of cell lines such as the clonal osteoblastic cell line (RCJ) *(Luger et al., 1998)* show that the EDs used in these studies were of the same order of magnitude. These cells exhibited a variety of effects with no biphasic response, and there was no cell death or damage even at the higher EDs.

In our clinical studies, laser irradiation was delivered to skin surface overlying tender points. Aδ and C fibre terminals course through the epidermis, including the superficial layers of keratinocytes *(Kennedy et al., 2005)* (Fig. 7). Thus, they would be directly affected by the incident laser beam, with minimal light flux attenuation because there is also backscatter associated with such irradiation. Moreover, as 830 nm laser penetrates up to 5 cm *(Gursoy and Bradley, 1996)*, there would also be an effect on the deeper dermis and underlying muscle *(Nicolau et al., 2004)*, tendons *(Bjordal et al., 2001)*, and lymphatic tissues *(Carati et al., 2003)*, also involved in clinical pain modulation.

Although we delivered only a single laser exposure in contrast to the clinical course of 10–14 treatments over 7 weeks *(Chow et al., 2004; 2006)*, there is, however, a single RCT showing that a single treatment with 830 nm laser relieved chronic neck pain for up to 24 h, suggesting an immediate clinical effect *(Toya et al., 1994)*. How does our model relate to the clinical trials showing long-term benefit of repeated laser treatments? We propose that laser induces acute and chronic pain relief via a reversible blockade of FAF and mitochondrial transport, with a resultant decreased MMP, reduction in ATP availability, conduction block and neurotransmission failure of Aδ and C fibre nociceptors, temporary in a single treatment but long term when delivered repetitively as in our clinical trials. We further propose that this would have a flow-on effect to the CNS by inhibition of second order neurons and modulation of ascending and descending pain-associated pathways and depression of long-term potentiation as proposed by Klein *et al. (2004)*.

As 830 nm laser therapy offers a non-invasive, non-pharmacological therapy for the treatment of pain with an absence of adverse events, further defining of the mechanism is essential, however, we present a plausible platform on which to base further study. The cost benefit of 830 nm (cw) laser therapy has substantial implications for health budgets in the reduction of ongoing, costly medication with serious adverse side effects, for the current "epidemic" of chronic pain *(Cousins, 1997)*. This alone should encourage exploration of optimal protocols for other painful conditions.

References

Ahern M, Reid C, Gordon T, McCredie M, Brooks P., Jones M (1987). Does colchicine work? The results of the first controlled study in acute gout. Aust N Z J Med 17:301–304.

Alexandratou E, Yova D, Handris P, Kletsas D, Loukas S (2002). Human fibroblast alterations induced by low power laser irradiation at the single cell level using confocal microscopy. Photochem Photobiol Sci 1:547–552.

Basford J, Sheffield C, Harmsen W (1999). Laser therapy: a randomised, controlled trial of the effects of low-intensity Nd:YAG laser irradiation on musculoskeletal back pain. Arch Phys Med Rehabil 80:647–652.

Baxter GC, Walsh DM, Allen JM, Lowe AS, Bell AJ (1994). Effects of low intensity infrared laser irradiation upon conduction in the human median nerve in vivo. Exp Physiol 79:227–234.

Beckerman H, de Bie R, Bouter L, De Cuyper H, Oostendorp R (1992). The efficacy of laser therapy for musculoskeletal and skin disorders: a criteria-based meta-analysis of randomised clinical trials. Phys Ther 72:483–491.

Bjordal J, Couppe C, Chow R, Tuner J, Ljunggren A (2003). A systematic review of low level laser therapy with location-specific doses for pain from chronic joint disorders. Aust J Physiother 49:107–116.

Bjordal J, Couppe C, Ljunggren A (2001). Low-level laser therapy for tendinopathy: evidence of a dose-response pattern. Phys Ther Rev 6:91–99.

Bolton P, Young S, Dyson M (1990). Macrophage responsiveness to light therapy. A dose response study. Laser Ther 2:101–106.

Brosseau L, Gam A, Harman K, Morin M, Robinson V, Shea B, Tugwell P, Wells G, de Bie R (2005). Low level laser therapy (Classes I, II and III) for treating rheumatoid arthritis [Systematic Review]. Cochrane Musculoskeletal Group Cochrane Database of Systematic Reviews 2005:2.

Budd SL, Nicholls DG (1996). A re-evaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis. J Neurochem 66:403–411.

Cambier D, Blom K, Witvrouw E, Ollevier G, De Muynck M, Vanderstraeten G (2000). The influence of low intensity infrared laser irradiation on conduction characteristics of peripheral nerve: a randomised, controlled, double-blind study on the sural nerve. Lasers Med Sci 15:195–200.

Carati CJ, Anderson SN, Gannon BJ, Piller NB (2003). Treatment of postmastectomy lymphedema with low-level laser therapy: a double-blind, placebo-controlled trial. Cancer 98:1114–1122.

Caterina M, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313.

Chen M, Shimada K, Fujita K, Ishii J, Hirata T, Fujisawa H (1993). Neurite elongation from cultured dorsal root ganglia is inhibited by Ga-Al-As diode laser irradiation. Las Life Sci 5:237–242.

Chow RT, Barnsley L (2005). A systematic review of the literature of low-level laser therapy (LLLT) in the management of neck pain. Lasers Surg Med 37:46–52.

Chow RT, Heller GZ, Barnsley L (2006). The effect of 300 mW, 830 nm laser on chronic neck pain: a double-blind, randomized, placebo-controlled study. Pain 124:201–210.

Chow RT, Barnsley LB, Heller GZ, Siddall PJ (2003). Efficacy of 300 mW, 830 nm laser in the treatment of chronic neck pain: a survey in a general practice setting. J Musculoskel Pain 11:13–21.

Chow RT, Barnsley LB, Heller GZ, Siddall PJ (2004). A pilot study of low-power laser therapy in the management of chronic neck pain. J Musculoskel Pain 12:71–81.

Cousins MJ (1997). Pain – a hidden epidemic. Available at: http://www.medserv.com.anzca/infocentres/nadayspainepid.htm. Accessed

David M (2002). The Effects of Oxidative Stress on a Human Neuronal Cell Line – A Model for Neurodegenerative Diseases. [Honors thesis]. University of Sydney, Sydney.

de Bie R, de Vet H, Lenssen T, van der Wildenberg F, Koostra G, Knipschild P (1998). Efficacy of 904 nm laser therapy in the management of musculoskeletal disorders: a systematic review. Phys Ther Rev 3:59–72.

Dedov V, Roufogalis B (1998). Rat dorsal root ganglia neurons express different capsaicin-evoked Ca^{2+} transients and permeabilities to Mn^{2+}. Neurosci Lett 248:151–154.

Enwemeka CS, Parker JC, Dowdy DC, Harkness EE, Sanford LE, Woodruff LD (2004). The efficacy of low-power lasers in tissue repair and pain control: a meta-analysis study. Photomed Laser Surg 22:323–329.

Friedmann H, Lubart R (1995). Competition between activating and inhibitory processes in photobiology. In: Proceedings of Effects of Low-Power Light on Biological Systems. Young AR, Karu TI (Eds). SPIE – The International Society for Optical Engineering, Barcelona, Spain, pp 60–64.

Fukuuchi A, Suzuki H, Inoue K (1998). Double-blind trial of low reactive-level laser therapy in the treatment of chronic pain. Laser Ther 10:59–64.

Gam A, Thorsen H, Lonneberg F (1993). The effect of low-level laser therapy on musculoskeletal pain: a meta-analysis. Pain 52:63–66.

Gavish L, Asher Y, Becker Y, Kleinman Y (2004). Low-level laser irradiation stimulates mitochondrial membrane potential and disperses subnuclear promyelocytic leukemia protein. Lasers Surg Med 35:369–376.

Gold MS, Dastmalchi S, Levine JD (1996). Co-expression of nociceptor properties in dorsal root ganglion neurons from the adult rat in vitro. Neuroscience 71:265–275.

Goldstein LS, Yang Z (2000). Microtubule-based transport systems in neurons: the role of kinesins and dyneins. Annu Rev Neurosci 23:39–71.

Gursoy B, Bradley P (1996). Penetration studies of low intensity laser therapy (LILT) wavelengths. Laser Ther 8:18.

Hirokawa N, Sato-Yoshitake R, Kobayashi N, Pfister KK, Bloom GS (1991). Kinesin associates with anterogradely transported membranous organelles in vivo. J Cell Biol 114:295–302.

Julius D, Basbaum A (2001). Molecular mechanisms of nociception. Nature 413:203–210.

Karu T (1999). Primary and secondary mechanisms of action of visible to near-IR radiation in cells. J Photochem Photobiol B 49:1–17.

Karu TI, Pyatibrat LV, Kolyakov SF, Afanasyeva NI (2005). Absorption measurements of a cell monolayer relevant to phototherapy: reduction of cytochrome c oxidase under near IR radiation. J Photochem Photobiol B 81:98–106.

Kasai S, Kono T, Yasuhiro Y, Kotani H, Sakamoto T, Mito M (1996). Effect of low-power laser irradiation on impulse conduction in anaesthetized rabbits. J Clin Laser Med Surg 14:107–109.

Kennedy W, Wendelschafter-Crabb G, Polydefikis M, McArthur J (2005). Pathology and quantitation of cutaneous innervation. In: Peripheral Neuropathy. Dyck P, Thomas P (Eds). W.B. Saunders, Philadelphia, p 873.

Kert J, Rose L (1989). Clinical Laser Therapy – Low Level Laser Therapy. Scandinavian Medical Laser Technology, Copenhagen, p 5.

Klein T, Magerl W, Hopf H-C, Sandkühler J, Treede R-D (2004). Perceptual correlates of nociceptive long-term potentiation and long-term depression in humans. J Neurosci 24:964–971.

Kress M, Reeh PW (1996). More sensory competence for nociceptive neurons in culture. Proc Natl Acad Sci U S A 93:14995–14997.

Kudoh C, Inomata K, Okajima K, Ohshiro T (1990). Low-level laser therapy pain attenuation mechanisms. Laser Ther 2:3–6.

Laakso E, Cramond T, Richardson C, Galligan J (1994). Plasma ACTH and beta-endorphin levels in response to low level laser therapy (LLLT) for myofascial trigger points. Laser Ther 6:133–142.

Lam TS, Abergel RP, Meeker CA, Castel JC, Dwyer RM, Uitto J (1986). Laser stimulation of collagen synthesis in human skin fibroblasts. Lasers Life Sci 1:61–77.

Light AR, Perl ER (1984). Peripheral sensory systems. In: Peripheral Neuropathy. Dyck PJ, Thomas P, Lambert EH, Bunge R (Eds). W.B. Saunders Company, Philadelphia, pp 210–230.

Liu M, Barth A (2004). Phosphorylation of the sarcoplasmic reticulum Ca^{2+}-ATPase from ATP and ATP analogues

studied by infrared spectroscopy. J Biol Chem 279:49902–49909.
Lubart R, Friedmann H, Lavie R (2000). Photostimulation as a function of different wavelengths. Laser Ther 12:38–41.
Lubart R, Lavi R, Friedmann H, Rochkind S (2006). Photochemistry and photobiology of light absorption by living cells. Photomed Laser Surg 24:179–185.
Luger EJ, Wollman Y, Rochkind S, Dekel S, Ouaknine G, Chernihovsky T, Iaina A (1998). The effect of low-level laser irradiation on bone cell culture. Laser Ther 10:55–58.
Mandadi S (2001). Vanilloid receptors enhance capsaicin-induced dissipation of mitochondrial membrane potential in rat dorsal root ganglion neurons. [Honors thesis]. University of Sydney, Sydney, pp 1–89.
Mantyh PW, Allen AJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE (1995a). Rapid endocytosis of a G protein-coupled receptor: substance P-evoked internalisation of its receptor in the rat striatum in vivo. Proc Natl Acad Sci U S A 92:2622–2626.
Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE, Simone DA (1995b). Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268:1629–1632.
Nakata T, Terada S, Hirokawa N (1998). Visualization of the dynamics of synaptic vesicle and plasma membrane proteins in living axons. J Cell Biol 140:659–674.
Navratil L, Dylevsky I (1997). Mechanisms of the analgesic effect of therapeutic lasers in vivo. Laser Ther 9:33–39.
NHMRC (1999). How to Review the Evidence: Systematic Identification and Review of the Scientific Literature. Australian Government Publishing Service, Canberra, pp 20–21.
Nicolau R, Martinez M, Rigau J, Tomas J (2004). Neurotransmitter release changes induced by low power 830 nm diode laser irradiation on the neuromuscular junction. Lasers Surg Med 35:236–241.
Nicolson GL, Smith JR, Poste G (1976). Effects of local anaesthetics on cell morphology and membrane-associated cytoskeletal organization in BALB/3T3. J Cell Biol 68:395–402.
Ohshiro T (1990). An introduction to LLLT. In: Progress in Laser Therapy. Ohshiro T, Calderhead R (Eds). John Wiley & Sons, Chichester, England, pp 36–40.
Ozawa Y, Simizu N, Abiko Y (1997). Low-energy diode laser irradiation reduced plasminogen activator activity in human periodontal ligament cells. Lasers Surg Med 21: 456–463.
Ozdemir F, Birtane M, Kokino S (2001). The clinical efficacy of low-power laser therapy on pain and function in cervical osteoarthritis. Clin Rheumatol 20:181–184.
Palmgren N, Jensen GF, Kaae K, Windelin M, Colov HC (1989). Low-power laser therapy in rheumatoid arthritis. Lasers Med Sci 4:193–196.
Park JS, Bateman MC, Goldberg MP (1996). Rapid alterations in dendrite morphology during sublethal hypoxia or glutamate receptor activation. Neurobiol Dis 3:215–227.
Passarella S, Atlante A, Ostuni A, Pastore D, Quagliariello E (1994). Light-biological system interaction: mitochondria and isolated enzymes as light targets. Trends Photochem Photobiol 3:307–317.
Pereira AN, Eduardo CdP, Matson E (2002). Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg Med 31:263–267.
Pogrel MA, Wei CJ, Zhang K (1997). Effects of low-energy gallium-aluminum-arsenide laser irradiation on cultured fibroblasts and keratinocytes. Lasers Surg Med 20: 426–432.
Poste G, Papahadjopoulos D, Nicholson G (1975). Local anaesthetics affect transmembrane cytoskeletal control of mobility and distribution of cell surface receptors. Proc Natl Acad Sci U S A 72:4430–4434.
Rang HP, Bevan S, Dray A (1991). Chemical activation of nociceptive peripheral neurones. Br Med Bull 47:534–548.
Roediger B, Armati PJ (2003). Oxidative stress induces axonal beading in cultured human brain tissue. Neurobiol Dis 13:222–229.
Smith K (1999). The first law of photochemistry and lasers. Laser Ther 11:162–163.
Sommer AP, Pinheiro ALB, Mester AR, Franke RP, Whelan HT (2001). Biostimulatory windows in low-intensity laser activation: lasers, scanners and NASA's light-emitting diode array system. J Clin Laser Med Surg 19:29–33.
Soriano F, Rios R (1998). Gallium arsenide laser treatment of chronic low back pain: a prospective, randomized and double blind study. Laser Ther 10:175–180.
Tanelian DL, Markin VS (1997). Biophysical and functional consequences of receptor-mediated nerve fiber transformation. Biophys J 72:1092–1108.
Tominaga M, Caterina M, Malmberg A, Rosen T, Gilbert H, Skinner K, Raumann B, Basbaum A, Julius D (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531–543.
Toya S, Motegi M, Inomata K, Ohshiro T, Maeda T (1994). Report on a computer-randomised double blind clinical trial to determine the effectiveness of the GaAlAs (830 nm) diode laser for pain attenuation in selected pain groups. Laser Ther 6:143–148.
Tsuchiya D, Kawatani M, Takeshige C (1994). Laser irradiation abates neuronal responses to nociceptive stimulation of rat-paw skin. Brain Res Bull 43:369–374.
Tsuchiya D, Kawatani M, Takeshige C, Sato T, Matsumoto I (1993). Diode laser irradiation selectively diminishes slow component of axonal volleys to dorsal roots from the saphenous nerve in the rat. Neurosci Lett 161:65–68.
Tunér J, Hode L (2002). What parameters to use. In: Low Level Laser Therapy – Clinical Practice and Scientific Background. Prima Books AB, Grangesberg, Sweden, pp 68–90.
Umegaki S, Tanaka Y, Hisakai M, Koshimoto H (1989). Effectiveness of low-power laser therapy on low-back pain – double-blind comparative study to evaluate the analgesic effect of low power laser therapy on low back pain. Clin Rep 23:2838–2846.
Vacca R, Morro L, Petragallo V, Greco M (1997). The irradiation of hepatocytes with HeNe laser causes an increase of cytosolic free calcium concentration and an increase in cell membrane potential, correlated with it, both increases taking place in an oscillatory manner. Biochem Mol Biol Int 43:1005–1014.
Vacca RA, Marra E, Quagliariello E, Greco M (1994). Increase of both transcription and translation activities following separate irradiation of the in vitro system components with HeNe laser. Biochem Biophys Res Commun 203:991–997.
Wakabayashi H, Hamba M, Matsumoto K, Tachibana H (1993). Effect of irradiation by semiconductor laser on responses evoked in trigeminal caudal neurons by tooth pulp stimulation. Lasers Surg Med 13:605–610.
Walker J (1983). Relief from chronic pain by low power irradiation. Neurosci Lett 43:339–344.