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Enhanced Sensory Reinnervation of Dental Target Tissues in Rats Following Low Level Laser (LLL) Irradiation

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Abstract. Previous studies have suggested that low level laser (LLL) treatment at specific wavelengths can enhance motor and sensory function in peripheral nerves after injury. The dental pulp is innervated by unmyelinated C fibres and small myelinated Aδ fibres derived from the trigeminal ganglion, both of which are known to contain calcitonin gene related peptide (CGRP).

The aim of the present study was to examine the effects of daily LLL treatment on the sensory reinnervation of the first molar pulp 10 days subsequent to inferior alveolar nerve (IAN) axotomy, by counting the number of nerve fibre profiles immunoreactive to CGRP in rats.

Axotomy of the IAN was performed via an extraoral buccal incision. The animals ($n=11$) were divided into two groups receiving either daily LLL treatment with a GaAlAs laser ($\lambda=830$ nm), or sham laser treatment over the site of injury for 10 days postoperatively. The animals were transcardially perfused and fixed at death before excising the jaws for further fixation and demineralisation. The jaws were cryosectioned obliquely through the mesial root pulp of the first molar tooth, which was the chosen level for evaluation of reinnervation. The CGRP antigen–antibody binding sites were visualised using a standard avidin biotin peroxidase technique. Both sham and LLL treatment and the evaluation of results were conducted blind. The results were statistically analysed and presented as median with 25%–75% quartiles.

A statistically significant ($p \leq 0.0000$) greater number of CGRP immunoreactive nerve profiles were seen in the LLL-treated group (median=5, range 4–6) compared to the sham laser treated group (median=2, range 1–3), at the specified area for evaluation.

These results suggest that LLL treatment can enhance reinnervation of denervated dental tissues after IAN axotomy in the rat. The enhanced reinnervation could be due to accelerated regeneration of the axotomised nerve, to collateral reinnervation, or a combination of both these nerve responses. The mechanisms whereby these changes are effected are still unknown.

Keywords: Calcitonin gene related peptide; Dental pulp; Immunohistochemistry; Inferior alveolar nerve axotomy; Low level laser; Nerve regeneration; Photobiostimulation

INTRODUCTION

Low level laser (LLL) treatment using devices with outputs in the milliwatt range has been advocated as a therapeutic treatment for a variety of conditions including wound healing [1,2], pain relief [3] and the treatment of long-standing paraesthesias [4–6]. Previous animal studies have suggested that LLL treatment on a daily basis enhances nerve recovery subsequent to injury [7,8].

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The dental pulp is innervated by unmyelinated C-fibres, myelinated axons in the Aδ range and a few Aβ fibres, which are derived from the trigeminal ganglion. The sensory fibres are involved in a variety of protective functions [9,10]. Myelinated A-fibres and unmyelinated C-fibres represent the majority of pulpal nerves and both have been shown to contain calcitonin gene related peptide (CGRP) [11,12]. Previous studies using autoradiography and immunohistochemistry have shown that, two days after inferior alveolar nerve (IAN) axotomy, there was an almost complete sensory denervation of the first rat molar, partial denervation of the second molar

and minimal effect on the innervation of the third molar and gingiva [13,14]. Based on axonal transport [13] reinnervation of the first molar tooth was first seen 6–7 days post-axotomy. Pulpal reinnervation was shown to proceed for several weeks, and had reached an almost normal density over a 2-month period postaxotomy. Comparison of the nerve structure and function for the first molar showed that return of sensitivity correlated with reinnervation of both pulp and dentine [13].

The aim of the present study was to examine the effects of daily LLL treatment, using a GaAlAs laser (λ=830 nm), on sensory reinnervation of the first molar root pulp subsequent to IAN axotomy in rats.

METHOD

The experiments were approved by the Norwegian Experimental Animal Board (NEAB) and performed according to the recommended guidelines.

Eleven 70-day-old, female Wistar rats, were divided into two groups, LLL treated (n=6) and sham laser treated (n=5). Anaesthesia (0.25 ml/100 g body weight) was achieved with a mixture of Hypnorm and Dormicum (1:1) in sterile water (1:2).

Unilateral sectioning of the right IAN, modified according to Retief and Dreyer [15] was performed. An extraoral horizontal incision about 10 mm in length was made on the lateral aspect of the mandible, followed by blunt dissection through the masseter muscle down to the mandibular bone. A small window of bone was removed slightly inferior to the prominence palpable on the buccal aspect of the mandible, hence permitting access to the IAN immediately upon its entry to the canal. Bone removal was achieved with a slowly rotating round bur under isotonic saline rinse. The IAN was carefully elevated with a curved angulated probe and sectioned with iris scissors. The cut ends were replaced, the soft tissues were repositioned and the skin wound closed with 4–5 interrupted 4.0 vicryl sutures (Ethicon).

The laser treated group (n=6) received daily LLL treatment with a photon plus GaAlAs diode laser (Rønvig A/S, Vejle, Denmark). This consists of a hand-held delivery probe (diameter 18 mm) connected to the console by a flexible cable. This system delivers a 70 mW output in a continuous wavelength of 830 nm, that is, the near infrared spectrum. The laser gives a spot size of approximately 0.13 cm^2 and an incident power density of 538 mW/cm^2. The hand-held delivery probe was held in gentle contact with the shaved mandibular skin overlying the site of IAN axotomy, to deliver a dose of 6 J (46 J/cm^2). The sham laser-treated group (n=5) received an equivalent time of daily sham laser treatments. The contralateral uninjured side was not laser treated.

After 10 days with unilateral laser treatment, the rats were again deeply anaesthetised and transcardially perfused with isotonic saline containing 0.03% heparin, followed by Zamboni's fixative (4% paraformaldehyde and 0.2% saturated picric acid in a solution of 0.1 M phosphate buffer).

The jaws were then excised and postfixed in Zamboni's fixative for 24 h. The specimens were subsequently rinsed in phosphate-buffered saline (PBS) for 12 h and transferred to a demineralising solution of 10% EDTA and 7.5% polyvinylpyrrolidone (PVP) at 4°C [16]. The EDTA solution was changed every third day for about 18 days. After rinsing in PBS for 24 h, the specimens were saturated in 30% sucrose and stored at −80°C until cryosectioned.

Immunohistochemistry

The hemi-sectioned jaw samples of axotomised LLL treated, axotomised sham laser treated and uninjured contralateral side groups were serially sectioned at 50 μm obliquely through the mesial root of the first mandibular molar tooth (Fig. 1). To evaluate IAN nerve regeneration 10 days after axotomy, the mandible distal to the third molar was sectioned longitudinally. Sections were immunoreacted for CGRP as previously described by Fristad et al. [14]. In short, the sections were rinsed in PBS and pretreated with 0.3% H_2O_2, to block endogenous peroxidase activity, and 2% normal goat serum (Vector Laboratories, Burlinghame, CA, USA), followed by a PBS rinse. Thereafter, the specimens were incubated with polyclonal antibody to CGRP (dilution 1:7500) (Cambridge Research Biochemicals, Cambridge, UK) for 72 h.

The antigen–antibody binding sites were visualised using a standard avidin–biotin peroxidase technique (Vector Laboratories, Burlingame, CA, USA) and a nickel-enhanced 3.3-diaminobenzidine (Sigma Chemical Company, St Louis, MO, USA) procedure. The

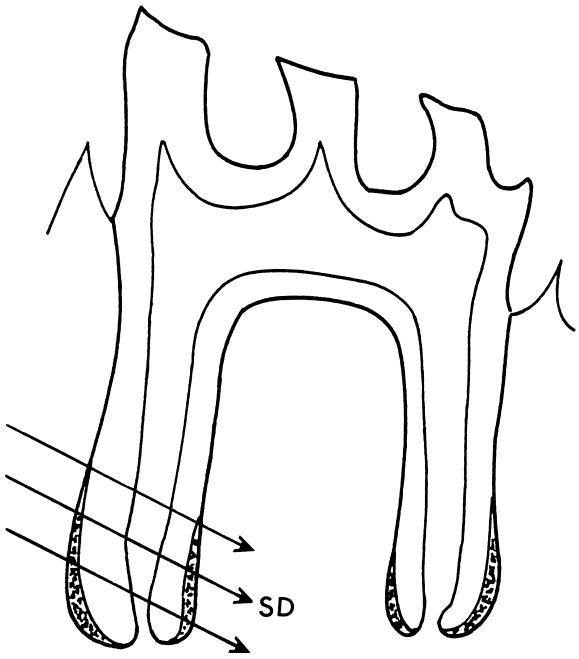

Fig. 1. Schematic drawing of a rat first mandibular molar to show the direction and orientation of the sectioning (SD) of the apical third of the mesial root pulp (M).

sections were air dried and counterstained with methylene blue/azure II in 1% sodium borate and distilled water, before embedding in Eukitt (Kindler, Freiburg, Germany).

Standard immunocontrols were routinely performed by either tissue incubation with preadsorbed primary antibody and its antigen, or by substitution of the primary or secondary antiserum with PBS. The immunocontrols were all negative with respect to immunoreactivity.

Evaluation

The apical third of the mesial root pulp of the first molar was chosen to comparatively evaluate the reinnervation of denervated dental target tissues after IAN axotomy between the LLL treated and sham laser treated groups. The sections were all encircled by dentine of tooth (Fig. 1). The sensory innervation in uninjured contralateral first molars was also evaluated at the same sectioning level in seven animals. Four 50 μm thick sections from each preparation that conformed to the above criteria were chosen by one coworker. The CGRP immunolabelled nerve profiles present in the individual preparations were counted 'blind' separately by another coworker. The number of CGRP immunoreactive nerve profiles in each of the four pulp sections were registered (Table 1). Random checks on the counting evaluation were performed. All the nerve profile counts were performed 'blind' by the same coworker. After the completion of counting, the information was pooled and sorted and the 'blind' code broken.

Statistical analysis of the nerve profile counts of the various groups was performed by the Mann Whitney test. Results are presented as median, with 25% to 75% quartiles.

RESULTS

A statistically significant ($p \leq 0.0000$) greater number of CGRP immunoreactive nerve profiles was seen in the LLL-treated group (median=5, range 4–6) compared to the sham laser-treated group (median=2, range 1–3) (Table 1), at the specified location (Fig. 1) ten days after IAN axotomy.

In addition to a significantly greater number, the CGRP immunoreactive nerve profiles seen in the LLL-treated group were generally coarser and more organised as nerve structures compared to those seen in the sham laser-treated group (Fig. 2a, b). The coarse CGRP immunoreactive nerve profiles seen in the LLL treated group at 10 days, morphologically more closely resembled the CGRP immunoreactive nerve profiles in the uninjured contralateral side of the mesial root pulp of the first molar tooth (Fig. 2a, c). However, there were still significantly ($p \leq 0.00$) more CGRP immunoreactive nerve profiles in the specified root pulp area of the uninjured side (median=10, range 9–13) compared to the LLL-treated group (median=5, range 4–6) (Fig. 2a, c).

Compared to the contralateral uninjured periodontal nerve supply (Fig. 3b), a sparse sensory reinnervation of the apical periodontal ligament was still present 10 days after IAN axotomy in the LLL-treated group (Fig. 3a). However, similar distributions of nerve profiles entering the apical foramen and the root pulp were shown for the LLL-treated and the uninjured side group (Fig. 3a, b).

In oblique IAN sections adjacent to the first mandibular molar, as well as in the IAN segment, distal to the third molar, a number of CGRP immunolabelled nerve fibres were present in both experimental groups (Fig. 4a, b) 10 days after axotomy.

Table 1. Distribution of CGRP immunoreactive nerve profile counts from four 50 μm thick sections, from the apical one-third of each mesial root pulp of the first mandibular rat molar. Low level laser (LLL) and sham laser treatment represent counts performed 10 days postaxotomy of the inferior alveolar nerve. Uninjured contralateral side represents counts from the non-injured, non-laser treated contralateral side. There was a significant difference between the number of profile counts in the LLL and sham laser treated groups $p \leq 0.0000$

Number of nerve profiles counted											
Low level laser treatment (n=6)				Sham laser treatment (n=5)				Uninjured contralateral side (n=7)			
4	3	4	4	2	1	1	0	16	12	12	10
5	5	5	5	1	1	2	3	9	9	13	9
6	5	4	4	2	2	1	1	7	9	7	12
4	7	6	6	3	3	3	4	10	14	10	10
3	5	6	6	2	4	3	3	13	16	13	9
9	6	7	6					8	8	7	10
								11	12	14	14

n=number of mesial root assessment.

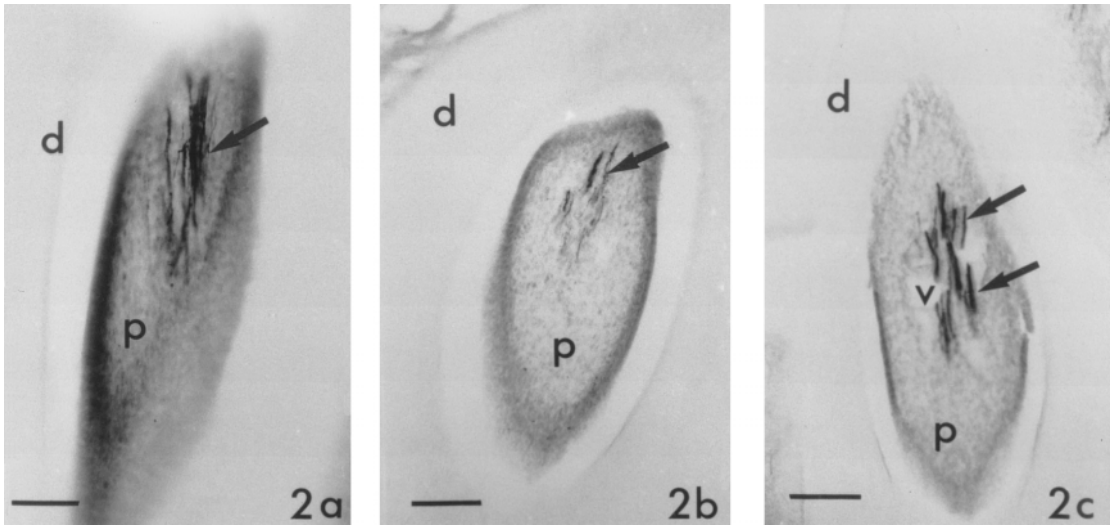

Fig. 2. Oblique sections of the root pulp (p) and dentine (d) of the apical third of the mesial root of a rat first mandibular molar immunolabelled for calcitonin gene related peptide (CGRP). (a) Section from a low level laser (LLL)-treated animal, 10 days after inferior alveolar nerve (IAN) axotomy. A number of coarse CGRP immunoreactive sensory nerve profiles (*arrow*) can be seen in the root pulp. (b) Section from a sham laser treated animal, 10 days after IAN axotomy showing a few fine, CGRP immunoreactive sensory nerve profiles (*arrow*) in the root pulp. (c) Section from a contralateral first molar of an uninjured, non-laser treated jaw side. Numerous coarse immunoreactive nerve profiles (*arrows*) are located along blood vessels (v) in the central part of the root pulp. Scale bars: 100 μm.

DISCUSSION

Previous studies have been published suggesting that LLL treatment of a specific wavelength can accelerate reinnervation in a peripheral nerve target subsequent to nerve injury [5–8]. The results of the present study seem to support these findings.

The pulp body of the mesial root of the first molar was chosen as the evaluation area, as opposed to assessing the return of CGRP immunoreactive nerve fibres to the IAN itself, for several reasons. First, the dental pulp represents a highly trophic target organ for regenerating fibres or collateral reinnervation [13]. Within the apical part of the mesial root

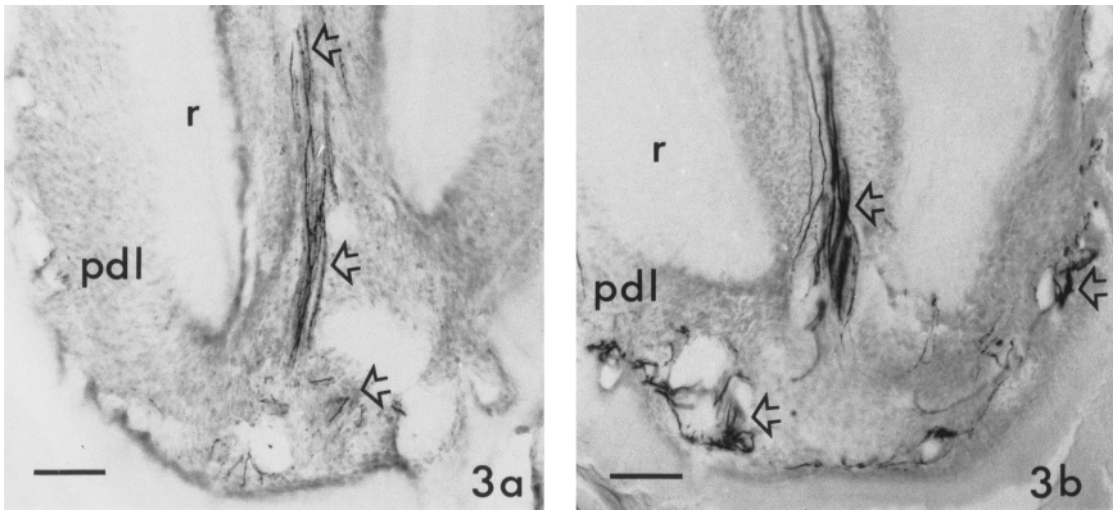

Fig. 3. Oblique sections of the apical mesial root (r) of the first mandibular molar tooth and the apical periodontium (pdl), showing sensory nerves (*open arrows*) immunolabelled for CGRP. (a) Section from an LLL-treated animal 10 days after IAN axotomy. A number of sensory nerve fibres are entering the root pulp. Compared to the uninjured tooth in (b), the apical pdl is sparsely supplied by CGRP immunolabelled fibres 10 days after IAN axotomy. (b) Section from a contralateral uninjured side. Numerous coarse and fine sensory nerve profiles are seen entering the apical foramen and root pulp. In the pdl a rich periodontal nerve plexus is seen. Scale bars: 100 μm.

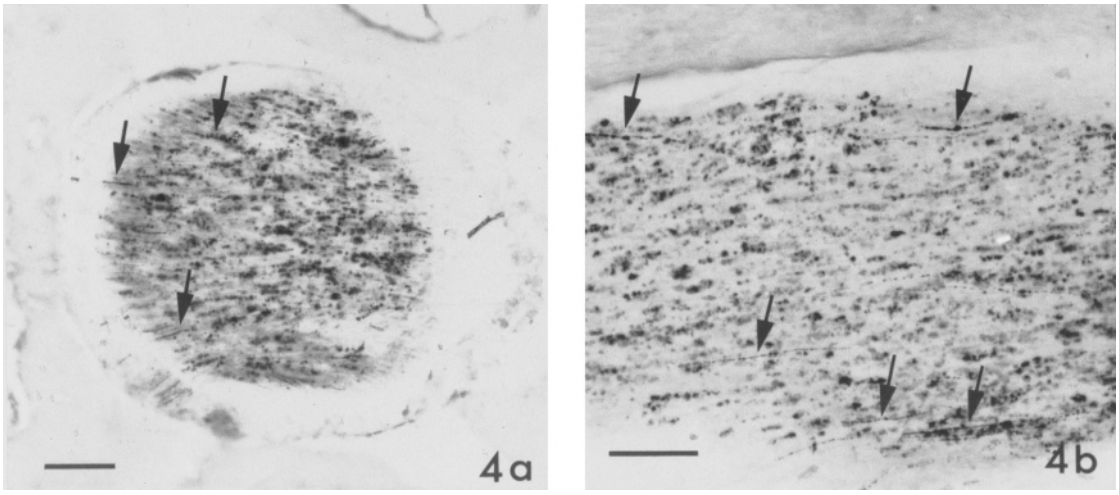

Fig. 4. Sections from the distal segment of the inferior alveolar nerve (IAN) from LLL-treated animals, 10 days after IAN axotomy. (a) An obliquely cut IAN section, adjacent to the first mandibular rat molar. Many CGRP immunolabelled thin nerve fibres (*arrows*) are seen in this part of the axotomised IAN. (b) A longitudinal section of the IAN, distal to the third mandibular molar. A number of CGRP immunoreactive fibres (*arrows*) are seen at this level of the distal IAN segment. Scale bars: 100 μm.

of the first mandibular rat molar, the axon numbers have been shown to be rather invariable [17]. Oblique, thick sections of this region allowed the immunoreactive nerve profiles present to be counted accurately. A possible collateral reinnervation via bifurcation canals may be excluded in this model, as the apical root pulp receives its nerve supply through the apical foramen.

The first mandibular molar in rats is thought to be innervated via the IAN in contrast to the second and third molars which may receive a collateral innervation mainly via the lingual nerve [13]. However, it cannot be excluded that subsequent to IAN axotomy, the root pulp of the first molar may become collaterally reinnervated by nerve branches from the lingual side perforating the alveolar bone. The return of CGRP immunoreactive fibres to the IAN adjacent to the first molar tooth six days after IAN axotomy in the same experimental rat model has been previously demonstrated [14]. In sections from two different levels of the distal IAN segment, the presence of a number of CGRP immunoreactive fibres (Fig. 4a, b), strongly indicates that reinnervating fibres

seen in the root pulp originate at least in part from the regenerating IAN.

Reinnervation of denervated tissues can be considered under two principal areas, collateral reinnervation by adjacent uninjured nerves and regeneration of the axotomised nerve. Collateral reinnervation is a growth characteristic of undamaged nerves [18]. However, all peripheral nerves exhibit regenerating abilities in response to axonal injury [19]. However, not all types of peripheral nerves exhibit the same potential for sprouting of regenerating axons after nerve injury [20]. Kinnman and Wiesenfeld-Hallin [21] suggested that C fibres, and possibly Aδ fibres, had a greater capacity to expand into denervated areas than low threshold mechanoreceptors after a peripheral rat sciatic nerve injury. The fibres innervating the dental pulp are known to consist mainly of unmyelinated C fibres and small myelinated Aδ fibres [10,11]. During IAN regeneration, these fibre types may, therefore, show a greater potential for expansion into the denervated pulp tissues. The present study has demonstrated that after LLL treatment there is an enhanced reinnervation of the first molar root pulp 10 days after axotomy. These reinnervating fibres could be derived from increased sprouting in regenerating axons from the IAN as numerous CGRP immunoreactive fibres were seen to be present at two different levels in the distal segment of the IAN 10 days post-axotomy (Fig. 4a, b). Therefore, a laser-induced enhancement of collateral re-innervation, or a combination of increased collateral reinnervation, IAN regeneration and enhanced sprouting could possibly occur. Long-standing sensory deficits in the area supplied by the IAN have shown an improvement in mechanosensory but not thermosensory perception following LLL treatment with a GaAlAs laser [5,6], which could depend on collateral reinnervation in the area of sensory deficit.

In contrast, the LLL enhanced reinnervation seen after acute nerve injury in the present study may be due to different, but unknown mechanisms. Previous workers have shown that the influence of LLL light on neural tissues is both wavelength and dosage specific [22–25]. An acute effect by LLL on neural preparations has been reported. A brief exposure to an argon laser is reported to alter the firing pattern of isolated abdominal ganglion cells [26]. Irradiation with a HeNe laser in humans has been reported to suppress certain spinal reflexes [27]. Furthermore, short irradiation pulses from a ruby laser have been reported to enhance acetylcholine release from isolated Auerbach's plexus in the guinea-pig ileum [28]. All these changes in electrophysiological activity and neurochemical signalling are reported to occur before there is any measurable increase in temperature, and the biological changes described are not reproduced by heating.

Nerve growth factor (NGF) attracts and encourages nerve growth [29,30]. Endogenously derived NGF is suggested to be an essential requirement for collateral sprouting of both myelinated and unmyelinated nociceptive fibres in the skin of the adult rat [20]. A study by Diamond et al. [20] suggested that in the presence of NGF a precocious collateral sprouting could be initiated if the neurons were primed by an excitatory impulse. Thus, if LLL enhances spontaneous firing in the proximal part of the regenerating nerve and induces priming of the trigeminal neurons, daily treatment could accelerate the regeneration process through these mechanisms.

Collateral sprouting is evoked and sustained by an increase in target NGF [31]. Nerve regeneration triggered by axotomy is regulated by numerous signals [32], and appears therefore to be less dependent solely on NGF [31]. Hence, it would seem that NGF is only one of the essential trophic or enhancing factors for regeneration of injured neurons but is an essential factor for collateral sprouting in intact neurons.

For laser light to interact with tissues it must be absorbed by chromophores within the cells. In addition, the cytochromes of the respiratory chain could, in theory, also absorb LLL light and affect metabolic processes [33]. Indeed, this principle is the basis of photodynamic therapy (PDT), whereby irradiated light is shown to be absorbed by a selectively retained photosensitive dye within the tumour cell; the absorbed energy then causes cell destruction [34]. However, other workers have found that LLL irradiation using a HeNe (630 nm) laser could result in free radical production – thought to be singlet oxygen [35]. It has been suggested that singlet oxygen may be a significant biochemical intermediate in biological processes when present in small amounts [36,37]. Friedmann et al. [38] have suggested a possible mechanism whereby cell mitosis could be stimulated by singlet oxygen. Singlet oxygen is a potent oxidising agent and could thereby stimulate redox activity in the

respiratory chain and thus promote mitosis. The specificity of laser light for producing signals causing biological changes in neuronal as well as non-neuronal cells could provide a stimulus which enhances reinnervation of target tissues.

Alternatively LLL light absorption by chromophores within the axotomised nerve fibres could result in intracellular modifications in neuropeptide production, or other growth-promoting factors which are essential for the regenerative process [32], restricted to the axotomy site or within the neural cell body, or both. Growth associated protein (GAP 43) is involved in a variety of intracellular signalling mechanisms as well as neurotransmitter release, which may be upregulated after axotomy [39]. During the regenerative process subsequent to axotomy of the IAN in the rat, a distinct peripheral upregulation of GAP 43 is already seen four days postaxotomy, thought to be of Schwann cell origin [40]. This is followed by a time-delayed upregulation of GAP 43 by the cell bodies within the trigeminal ganglion, accompanied by a concomitant upregulation of neuropeptide Y (NPY) production by the same trigeminal cell bodies [40]. Peripheral axotomy induces an extensive change in signalling within the environment at the site of injury, the affected neurons and their supporting cells, as well as signal systems in the peripheral segment and target tissues [41,42], and could be influenced by laser light.

The cells and nerves of the dental tissues are of neural crest origin [43]. A rhodopsin kinase-like protein has been demonstrated in tissues other than the retina which are of neural crest origin [44]. A vestigeal photosensitivity to specific wavelengths may therefore be present in certain tissue derivatives of neural crest origin such as the IAN.

The results of the present study support earlier findings suggesting that LLL treatment appears to enhance reinnervation of target tissues subsequent to nerve injury. Several theories are presented and discussed, but as yet no satisfactory explanation exists for how the reported effects of LLL therapy are achieved. Hence, further investigations at cellular levels are to be considered.

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