Eeffects of Aging on Neurogenic Vasodilator Responses Evoked by Transcutaneous Electrical Nerve Stimulation: Relevance to Wound Healing

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We have previously shown an age-related decline in the modulation of skin vascular reactivity by sensory nerves that correlates with a decline in wound repair efficacy. This study was designed to examine the possibility that improving the functional ability of aged sensory nerves using noninvasive transcutaneous electrical nerve stimulation (TENS) could also accelerate tissue repair. TENS of the sciatic nerve, combined with measuring blood flow responses in the rat hind-footpad using laser Doppler flowmetry, was used to establish the vascular effects. Following TENS (using parameters 20V, 5 Hz for 1 min), similar increases in vascular responses were obtained in both young (13.2 ± 0.9 cm²) and old rats (11.6 ± 2.3 cm²). In contrast, capsaicin-pretreated rats showed markedly diminished responses. Sympathetic fibers did not appear to modulate these sensory nerve responses. In the second part, a thermal wound was induced (using a CO₂ laser) in the interscapular region of old rats (under anesthesia). In the active treatment group, TENS was applied twice daily for the initial 5 days, and the sham group received inactive TENS. Using the healing endpoint as the time when full wound contraction occurred, the active group required 14.7 ± 0.2 days for complete healing, a significant improvement over the sham group (21.8 ± 0.3 days). We contend that low-frequency TENS can improve the vascular response of old rats. In addition, wound healing in aged rats can be accelerated by peripheral activation of sensory nerves at low-frequency electrical stimulation parameters.

WOUND repair is a dynamic and highly complex process that is dependent on a number of general factors including nutritional status and concurrent morbidity, as well as specific factors that affect the microenvironment at cellular and neurovascular levels. An intact nociceptor system of primary afferent sensory nerves is an essential prerequisite for successful wound healing (1), as are sensory neuropeptides (2–5). This latter notion is supported by a number of reports which show that calcitonin gene-related peptide (CGRP) and substance P (SP) have growth-promoting properties (5–8). This is made possible by the fact that sensory neuropeptides can have a trophic influence in wound healing via their vasodilator properties (9–12), and the ability to stimulate the proliferation of endothelial cells (7), arterial smooth muscle cells, and skin fibroblasts (6). A recent study by Richards and colleagues (1) has documented that sensory peptides are important for the chemotactic activity of monocytes, macrophages, and T-lymphocytes in skin wounds, and the subsequent availability of cytokines that are essential for healing.

However, it is known that neurogenic inflammatory reactions are attenuated with age (13). As a result, there is an associated impairment in wound healing with age, as demonstrated from both animal and human studies (5,14–17). With this in mind, Khalil and Helme (5) reported that exogenous administration of the sensory neuropeptides in an experimental wound model accelerated the rate of healing in old rats.

Over the years, many wound healing treatment interventions—including debridement/irrigation, dressings, pressure-relieving devices, and ultrasound—have been applied with mixed results (18). An intervention commonly used to treat wounds is electrical stimulation (ES); and when applied superficially and noninvasively, it is referred to as transcutaneous electrical nerve stimulation (TENS). Electrical stimulation has been shown to augment healing of chronic wounds in human subjects and induced wounds in animal models (19,20). More specifically, it has been shown to significantly increase the rate of wound epithelialization (19) and contraction (21,22). ES treatments have also been reported to increase the survival rate of musculocutaneous and ischemic flaps by increasing cutaneous blood flow (9,23).

Although ES has been used to facilitate wound healing (24–26), it is infrequently used for healing management, due to ambiguity centered on the stimulation parameters and the types of wounds receiving maximal benefit from this adjunct treatment. It should be noted that the ES management strategies developed in a variety of clinical conditions lack physiological rationale or experimental support. In addition, no previous attempts were directed toward understanding the confounding effect of age on the healing process, and no specific treatment was designed with this thought in mind. We have recently shown that the aged sensory nerve responds preferentially at low frequencies of invasive antidromic ES (5 Hz) compared to higher frequencies (15 Hz) (27), resulting in neurogenic vasodilator responses that are similar to those of young animals.

It is also known that stimulation parameters that activate small unmyelinated sensory nerves will also activate the sympathetic efferents that are of similar diameter (28). Neuropeptides of sympathetic origin including noradrena-
line (NA) and neuropeptide Y (NPY) strongly influence blood flow in skin microcirculation (27,29–31) and modulate the action of sensory nerves at both pre- and post-terminal sites (32–37). These are important interactions and must be considered when examining ES responses of sensory nerves.

Based on the above reports and our recent observations, the aim of this study was to establish noninvasive conditions of activation under which the aged sensory nerve will preferentially respond. The focus is on the use of this noninvasive TENS in stimulating the peripheral sensory nerve fibers to modulate the local microvascular response and to develop a treatment protocol to accelerate healing that is specifically designed to accommodate the physiological changes in sensory nerves with age.

**METHODS**

Outbred male Sprague-Dawley rats with an average weight of 250–350 g for 3-month-old control and capsaicin-pretreated, and an average weight of 600–700 g for 24-month-old control and capsaicin-pretreated animals were used. Anesthesia was induced with pentobarbital sodium (60 mg/kg) and maintained by supplementary injections. Mean arterial blood pressure (MAP) was monitored using a COBE pressure transducer (JRAK-Biosignals, Melbourne, Australia) attached by a catheter to the right carotid artery. The left jugular vein was cannulated for intravenous administration of phentolamine or saline solutions. Body temperature was maintained at 37°C. At the completion of the experiment, the animals were killed by anesthetic overdose.

**Neonatal capsaicin-pretreatment.**—A group of neonatal rats were pretreated with a single subcutaneous injection of capsaicin (50 mg/kg) on the second day of life. This treatment has been shown to selectively destroy a large proportion of unmyelinated sensory C-fibers (38–40). Efficacy of this treatment was assessed at 3 months of age by applying a drop of capsaicin (0.1%) to the eye and recording the subsequent number of eyewipes. Rats were considered capsaicin-denervated if the number of eyewipes were less than 25% of controls. Those that were not sensory denervated were sacrificed by anesthetic overdose. Experiments were performed on animals aged 3 and 24 months.

**Transcutaneous electrical nerve stimulation and measurement of cutaneous blood flow.**—The right sciatic nerve in the mid-thigh region was stimulated with a Grass S48 stimulator using parameters of 20 V, 2 ms, 5 Hz (low frequency) for 1 min. This was carried out using the noninvasive technique of TENS. For this protocol, the rats were shaved on the lateral side of the thigh and the medial side of the calf of the right hindlimb. Electrode gel was then applied to the shaved area. The stimulating TENS electrode (3 cm²) was placed over the proximal part of the sciatic nerve, and the other electrode was positioned on the calf on the medial side above the ankle. Skin blood flow in the right footpad was measured using a laser Doppler flowmeter probe inserted vertically through a port in the perspex chamber above the footpad skin. The flux output of the laser Doppler monitor is a function of the concentration and the velocity of the red blood cells moving in the tissue penetrated by the laser light. The changes in relative blood flow (as determined by changes in red cell flux) following TENS of the sciatic nerve were continuously displayed on a chart recorder. Raw data were evaluated by calculating the area under the stimulation-evoked response curve (AUC, in cm²) for a poststimulation period of 20 min. All measurements were made relative to a stable baseline obtained prior to nerve stimulation. Results are expressed as mean ± SEM. Statistical analyses were performed using two-way analysis of variance (ANOVA) and/or independent samples t tests, and a p value of <.05 was considered significant.

**Effect of phentolamine and guanethidine on TENS responses.**—To assess the contribution of sympathetic efferents to the vascular response obtained by electrical stimulation, the nonselective α-adrenoceptor antagonist phentolamine and the sympathetic neurotransmitter blocker guanethidine were used in this study. Young rats were treated with phentolamine (3 mg/kg i.v., prepared in saline) or guanethidine (50 mg/kg i.p., prepared in double-distilled, de-ionized water) 20 and 60 min, respectively, prior to TENS of the sciatic nerve. These concentrations have previously been used in our laboratory (27,41). The resultant vascular responses were analyzed using a two-way ANOVA followed by post hoc independent t tests. Mean arterial blood pressure was monitored prior to and following administration of the drugs. Potential differences in blood pressure responses were analyzed using independent and/or paired samples t tests.

**Thermal injury model.**—A standardized and reproducible thermal skin wound model that permits monitoring of wound healing over time (5) was used. The lesion was induced 24 hours after the fur on the interscapular region had been removed. Rats were anesthetized, and a thermal burn was induced using a CO₂ laser. This circular wound was formed via a 0.5 sec pulse of 25 watts from the laser (stimulation duration and power, respectively), with the beam diameter set at 10 mm. Animals were examined daily, and measurements of the open wound were taken every day after the burn for 6 consecutive days and every 48 hours thereafter until complete wound closure/re-epithelialization had occurred. The area of burn (maximum diameter of the wound) was traced under a Zoom Stereo microscope for accuracy and then measured with a digital planimeter. Tracing and measurements are performed by two independent investigators who have no knowledge of the treatment groups or regimes. The healing endpoint is the time when full wound contraction has occurred.

**Thermal wound injury and TENS.**—For the wound healing experiments, the skin nerves in the vicinity of the wound were stimulated transcutaneously (noninvasive) with a Grass S48 stimulator using a pair of silicon rubber/carbon electrodes (1.5 cm²) at stimulation parameters 20 V, 2 ms, 5 Hz for 1 min. These parameters were previously shown to induce antidromic vasodilatation (27,39). This should ensure an adequate density of current transmitted through the skin to activate the peripheral terminals of sensory nerves in the vicinity of the wound. Rats were lightly anesthetized (20
mg/kg) and the two electrodes were placed on the skin surface on either side of the wound, stimulated twice daily for 5 days; this period of treatment relates to the documented delay in wound healing in aged rats (5). In another group of rats, the electrodes were placed on either side of the wound but were not connected to the Grass stimulator (sham treatment). We allowed the wound to reach full contraction to assess the efficacy of the TENS treatment regime in accelerating wound healing/closure. Statistical analyses of the different times for wound closure were performed using independent t tests.

Drugs and materials.—Pentobarbitone sodium (Nembutal) was obtained from Boehringer Ingelheim (Artamon, Australia), and phentolamine (Regitine) from Ciba-Geigy Australia Ltd. These drugs were prepared in 0.9% saline. Guanethidine monosulphate was purchased from Sigma Chemical Company (St. Louis, MO). This drug was initially dissolved in double-distilled, de-ionized water and then prepared in 0.9% saline. Capsaicin was from Fluka Chemika (Buchs, Switzerland), and prepared in saline with 10% ethanol and 10% Tween 80 (for neonatal pretreatment).

Results
In our recent report (27), it was determined that aged rats preferentially respond to antidromic ES at low frequency (5 Hz) (as opposed to higher frequency, 15 Hz). Therefore, the following experiments were performed using the same low-frequency parameters 5 Hz, 20 V, 2 ms for 1 min.

Blood flow responses induced by TENS.—TENS of the sciatic nerve in young control rats resulted in an immediate increase in blood flow in the microvasculature of the hind foot (area under curve [AUC]: 13.2 ± 0.9 cm²). The profile was similar to that obtained using antidromic stimulation, but of a smaller magnitude (27). TENS also resulted in a vasodilatation response in old rats that was comparable to that observed with young (AUC: 11.6 ± 2.3 cm²; 12% decrease compared to young control; Figure 1). The experiments were also performed on capsaicin-pretreated rats. This group was used to determine the contribution of sensory nerves to the vascular responses obtained. A group of old capsaicin rats was also utilized to examine the combined effects of aging and neonatal capsaicin pretreatment on the activity of sensory nerves. Capsaicin-pretreated rats of both ages showed an attenuated blood flow response following TENS (AUC: 4.2 ± 0.6 cm² and 5.0 ± 0.3 cm² for young capsaicin and old capsaicin, respectively; Figure 1).

To examine any possible differences in basal blood flow (BBF) measured prior to TENS between the young control and the other group of rats, a two-way ANOVA was applied, using age and rat type (i.e., normal or capsaicin-pretreated) as the two factors. From the analysis, there was a significant difference between the age groups [F(1,25) = 6.49, p < .05]. Further analysis determined this difference to occur between the young capsaicin and old capsaicin rats (see Table 1).

Effect of phentolamine and guanethidine on blood pressure.—Following the intravenous administration of phentolamine, the MAP dropped markedly in the young animals. Although the depressor response was immediate, a rapid recovery was shortly in effect: the MAP stabilized to 105 ± 5 mmHg by the start of the stimulation period, and these values remained stable until the completion of the experiment (Figure 2).

Unlike phentolamine, guanethidine is a sympathetic nerve blocker; that is, it prevents the release of neurotransmitters from these fibers. Intraperitoneal injection of 50 mg/kg guanethidine in young rats caused an immediate drop in blood pressure (from 127 ± 5 to 97 ± 4 mmHg). This was followed by a rapid and greater recovery than that observed for phentolamine, reaching almost basal levels at 20 min post-administration (124 ± 4 mmHg). The blood pressure remained stable at this level until the completion of the experiment.

Effect of phentolamine or guanethidine treatment on TENS-induced blood flow responses.—To examine the effects of α-adrenergic blockade on peripheral vascular responses induced by TENS in young rats, phentolamine was administered intravenously 20 min prior to nerve stimulation. Compared to control responses (AUC: 13.2 ± 0.9 cm²), phentolamine did not appear to influence the vasodilator response induced by TENS (AUC: 12.6 ± 2.3 cm²; Figure 3).

The effects of sympathetic nerve blockade on TENS-induced blood flow responses were also examined using guanethidine (50 mg/kg i.v., 60 min prior to stimulation). Similar to the observations with phentolamine, pretreatment with guanethidine in young rats did not alter the TENS-induced blood flow response with an AUC of 14.5 ± 3.2 cm².

### Table 1. Basal Blood Flow in Intact Skin (cm Height Above Zero Reference Level)

<table>
<thead>
<tr>
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<th>Young Control</th>
<th>Old Control</th>
<th>Young Capsaicin</th>
<th>Old Capsaicin</th>
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<tr>
<td>Reference Level</td>
<td>1.7 ± 0.3 cm</td>
<td>1.2 ± 0.4 cm</td>
<td>1.9 ± 0.2 cm*</td>
<td>1.0 ± 0.2 cm*</td>
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*p < .05, significant difference between the two groups.
compared to control responses of 15.5 ± 1.3 cm² (Figure 3). These results were confirmed using a two-way ANOVA (using the experimental group [phentolamine or guanethidine] versus the condition group [vehicle or drug]) in which no interaction or group effects were noted.

To determine whether or not application of these drugs affected BBF, a two-way ANOVA was again employed. Similar to the above findings, there were no significant interaction or between-subjects effects. Hence, phentolamine and guanethidine were without effect on BBF in treated rats (see Table 2).

Examination of the effect of phentolamine and guanethidine treatment in old rats with TENS was not performed because there was no apparent change in the vasodilator response following these treatments in young rats, combined with earlier reports that phentolamine administration in older rats at low frequency was also without effect (27).

### Wound healing experiments: effects of TENS

A thermal wound was induced in the interscapular region on the back of old rats. To assess whether TENS can influence the rate of healing, we compared two groups with thermal wounds: both groups were subjected to TENS (twice daily for the initial 5 days). The treatment group received active TENS and the control group received sham TENS (i.e., electrodes were placed on each side of the wound but not connected to the Grass stimulator). In the sham treated group, the size of the thermal wound was observed to increase progressively until day 4, after which the wound size started to fall. Complete wound closure was achieved by 21.8 ± 0.3 days (Figure 4). When active TENS was applied to the wound site (over the initial 5 days), the size of the wound increased only until day 2 and then started to heal; complete wound closure in this group was achieved by 14.7 ± 0.2 days (Figure 4). At days 8 and onward, the TENS group showed significantly faster rates of healing as evidenced by wound size (Figure 4). The reduced time of the “end-healing point” in the TENS group was also determined to be statistically different from the time required for the control group (*p < .05*).

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**Figure 2.** Effect of phentolamine (3 mg/kg i.v.) and guanethidine (50 mg/kg i.p.) on mean arterial blood pressure in young rats, measured before stimulation period. Values are mean ± SEM (n = 7–10). Both drugs were administered at time = 0 min, TENS of sciatic nerve at time = 20 and 60 min (for phentolamine- and guanethidine-treated animals, respectively).

**Figure 3.** Effect of phentolamine and guanethidine on neurogenic vasodilator responses evoked by low-frequency TENS of sciatic nerve (20 V, 2 ms for 1 min at 5 Hz) in young rats. Phentolamine was administered 20 min prior to TENS at 3 mg/kg i.v., and guanethidine was administered at 50 mg/kg i.p. 60 min prior to ES. Results are expressed as mean ± SEM of the area under the response curve (in cm²) (n = 7–10). Statistical analyses revealed no significant differences between the groups.

**Figure 4.** Application of low-frequency TENS to a thermal wound model in aged rats. Values are expressed as average size of wound (cm²) ± SEM. Open circle with broken lines represents TENS active treatment group, and solid circle with unbroken lines represents sham TENS treatment group (n = 6). The rate of healing was significantly faster in the TENS active group (days to healing average = 14.7 ± 0.2 days) compared to the sham group (days to healing average = 21.8 ± 0.3 days), and denoted by the asterisks (*p < .05; **p < .001**).

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**Table 2.** Effect of Phentolamine and Guanethidine on Basal Blood Flow in Intact Skin of Young Rats (cm Height Above Zero Reference Level)

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<thead>
<tr>
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<th>No Treatment (control)</th>
<th>Drug Treatment</th>
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<tr>
<td>Phentolamine</td>
<td>1.7 ± 0.3 cm</td>
<td>1.5 ± 0.3 cm</td>
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<tr>
<td>Guanethidine</td>
<td>2.1 ± 0.3 cm</td>
<td>2.0 ± 0.2 cm</td>
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*Note: There were no significant differences across any of the groups.*
To investigate this interaction under noninvasive conditions, ten associated with coactivation of sympathetic efferents. As mentioned previously, activation of sensory nerves is of sensory nerves and promote tissue healing. In the context of this study, which has focused on the application of sensory nerves upon antidromic stimulation (and thereby, central mechanisms) is beyond the review, see (42)]. Further discussion of TENS in pain alleviation (and thereby, central mechanisms) is beyond the context of this study, which has focused on the application of noninvasive TENS to activate the local effector function of sensory nerves and promote tissue healing.

Noninvasive TENS and local blood flow.—We have previously determined that antidromic electrical nerve stimulation techniques induce an increase in local blood flow via an action on sensory nerves. We have provided evidence that sensory nerves in old rats are capable of responding to low-frequency antidromic ES by releasing sensory peptides with a subsequent increase in local blood flow (27). These same parameters were then examined using TENS, a noninvasive, simple technique to administer.

Blood flow measured in the hind footpad of young rats was found to increase following TENS of the skin nerve fibers, along the anatomical path of the sciatic nerve. The response profile was similar to that obtained using invasive antidromic nerve stimulation (27).

A similar response using TENS was also obtained in old rats, supporting the proposition that aged sensory nerves preferentially respond to low frequencies of activation. This is a promising result in that the use of the noninvasive TENS can be effective in improving the local vascular “effector” response of sensory nerves in older rats.

Responses in capsaicin-pretreated rats was found to be significantly attenuated, regardless of age, indicating that capsaicin-sensitive sensory fibers are primarily responsible for the observed vasodilator response obtained in control rats. The results obtained also demonstrate that the stimulation parameters employed throughout this study (20 V, 2 ms, 5 Hz for 1 min) predominately activate these sensory nerves.

Low-frequency TENS and sympathetic modulation.—As mentioned previously, activation of sensory nerves is often associated with coactivation of sympathetic efferents. To investigate this interaction under noninvasive conditions, the nonselective α-adrenoceptor antagonist phenolamine or the sympathetic blocker guanethidine was administered to a group of young rats. Each caused a marked drop in MAP, followed by a rapid recovery with the MAP stabilizing at levels above 100 mmHg. This effect is similar to our earlier report (27).

On the other hand, basal blood flow in young rats was not altered following treatment with phenolamine and guanethidine. A possible explanation for this observation may be that NA and/or other sympathetic neurotransmitters are not involved in the control of skin microvasculature under intact physiological conditions. These drugs also had no effect on the vascular response induced by TENS of the sciatic nerve. This is an interesting result considering that sympathetic efferents play an important role in modulating the sensory nerve responses upon antidromic stimulation. Because the changes in blood pressure responses following administration of phenolamine or guanethidine are indicative of the effectiveness of these treatments, it is possible to postulate that when the sciatic nerve is activated under “intact” nerve conditions (i.e., noninvasive), sympathetic efferents do not release sufficient neurotransmitters to have a significant modulatory effect.

Another possibility could be that intact nerve stimulation leads to an inhibition of sympathetic-mediated peripheral vasoconstriction. Scudds and colleagues (43) reported that local vasodilatation induced by low-frequency TENS (4 Hz) on the hand may be attributed to a simultaneous inhibition of the cutaneous sympathetic efferent fibers.

We have already established that at low-frequency parameters, sympathetic modulation of sensory nerve function was limited in young rats and absent in old rats (27). This, together with the current results demonstrating no effect of phenolamine or guanethidine on vascular responses to noninvasive low-frequency ES, raises the possibility that sympathetic modulation of sensory nerve activity at low-frequency parameters is limited under invasive conditions and absent under noninvasive conditions of stimulation.

Low frequency TENS and tissue healing.—We have established that the noninvasive TENS technique is capable of generating a localized vascular response via activation of the peripheral terminals of sensory nerves. It has also been reported that successful tissue repair is dependent upon an adequate supply of blood to the site of injury. Using a standardized and reproducible thermal skin wound model, we examined the effect of low-frequency TENS on the time required for complete wound closure. TENS was applied twice daily for 5 days via skin surface electrodes placed on either side of the wound. This protocol was chosen because it was recently shown that old animals show a delay of approximately 5 days before wound contraction is initiated (5). It was anticipated that stimulating the sensory nerves in old rats during this lag period would provide the most advantage. In the old control group, in which the thermal injury was allowed to heal without active intervention, we observed a similar 5-day lag period before the wound size started to fall; complete healing was achieved by 21.8 ± 0.3 days. In the treatment group, a similar size lesion was observed over the first two days compared to the old control.
group; however, from this stage onward, the TENS treatment group showed an accelerated rate of healing, with complete wound closure achieved by 14.7 ± 0.2 days. This method of treatment achieved results similar to those obtained by Khalil and Helme (5), who injected the sensory neuropeptides at sites around the wound. The time required for complete wound closure is similar to that of a group of young rats, where a similar wound required 14.9 ± 0.2 days to heal naturally (5). These results collectively provide more evidence for the significant role of sensory nerves in the processes of tissue repair. Activation of the peripheral terminals of the aged sensory nerve can enhance the local efferent response, and this can have important implications for conditions associated with a dysfunction of such nerve fibers. Our results with the TENS treatment are promising, considering that accelerated wound healing was achieved using the endogenous stores of the sensory neuropeptides, released via noninvasive external stimuli.

It should be noted that low firing frequencies in nociceptors are sufficient to evoke flare (afferent response) (44), and yet human microneurographic studies have demonstrated that these low frequencies do not evoke a conscious perception of pain in the subjects (afferent response) (45). On the basis of these observations, it has been proposed that low rates of activation of nociceptors result in a localized tissue response, whereas more intense stimulation of nociceptors triggers central nervous system mediated responses including the perception of pain (46). Hence, the parameters employed above to improve tissue healing are sufficient to induce the required efferent response without a concomitant afferent response (feeling of “pain”).

Conclusion.—We have presented data to suggest that aged sensory nerves are capable of inducing a microvascular response similar to young rats at low-frequency, noninvasive TENS (5 Hz). Under these intact conditions, the function of sensory nerves does not appear to be modulated by sympathetic fibers. This finding was then successfully translated into a treatment protocol to accelerate wound repair in old rats who achieved wound closure within a time frame similar to that of young rats. Although individual clinical conditions have different wound healing requirements, we contend that the information provided in this report could be used to improve the local effector functions of aged sensory nerves, which are an important prerequisite for successful tissue repair.

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