

Differential Involvement of Conotoxin-Sensitive Mechanisms in Neurogenic Vasodilatation Responses: Effects of Age

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During aging there is a decline in sensory nerve function that is associated with reduced neurogenic inflammation and poor wound repair. The cellular mechanism(s) responsible for this decline in function with age is not well understood. We previously reported that sensory nerves in aged rats release sensory neuropeptides preferentially in response to low-frequency (5 Hz) as compared with higher-frequency (15 Hz) antidromic electrical stimulation, and that low-frequency transcutaneous electrical nerve stimulation accelerates wound healing. The present study investigates possible mechanisms for this preferential response. Using laser Doppler techniques, we have measured changes in blood flow in the base of vacuum-induced blisters induced in the rat hind footpad of young and old animals in response to low-frequency (5 Hz) or high-frequency (15 Hz) electrical stimulation (20 V, 2 ms for 1 minute) of the sciatic nerve. The relative contributions of the sensory neuropeptides, substance P and calcitonin gene-related peptide (CGRP), and of *N*-type voltage-gated calcium channels to the vascular responses were assessed by using the specific receptor antagonists RP67580, which is 2-(1-imino-2-(2-methoxyphenyl)ethyl)-7,7-diphenyl-4-perhydroisoindolone-(3aR, 7aR); CGRP₈₋₃₇; and ω -conotoxin GVIA (*Conus geographus*), respectively. The results showed a greater involvement of substance P at high-frequency electrical stimulation and of CGRP at low-frequency stimulation. Our finding that ω -conotoxin-sensitive *N*-type calcium channel function was preserved with age and was only involved in the vascular response to low-frequency electrical stimulation could explain our previous report demonstrating beneficial effects of low-frequency transcutaneous electrical nerve stimulation to wound repair in aged animals. The current results have important practical implications for improving tissue repair in the aged.

NEUROGENIC vascular responses to antidromic electrical stimulation (ES) of the saphenous and sciatic nerves have been used extensively as measures of the axon-reflex phenomenon and sensory nerve activity (1–4). The activation of primary afferent sensory nerves causes the release of sensory neuropeptides into the effector site by means of an axon-reflex mechanism. ES of the peripheral end of “cut” sciatic nerve can mimic this event of neurogenic inflammation. ES causes the influx of calcium ions (Ca⁺⁺) through voltage-operated Ca⁺⁺ channels (VOCCs) in neural tissue, leading to transmitter release from peripheral terminals. The entry of Ca⁺⁺ is regulated by multiple types of Ca⁺⁺ channels and neurotransmitter receptors.

Studies in both animals and humans have shown that aging causes a significant decrease in the efferent functions of sensory nerves (4–6). This is due, in part, to decreases in neuropeptide synthesis, content, and release that contribute to the overall decrease in neurogenic inflammation in old rats (4). As a consequence of this decline in sensory nerve activity, there is an associated delay in wound repair in animals and in the healing of leg ulcers in humans (5,7). One

report (7) demonstrated that the exogenous application of sensory neuropeptides could significantly enhance the rate of cutaneous healing in old rats, hence supporting the notion that dysfunction of these nerves may be a contributing factor to the reported delay in wound healing with advancing age (5,8,9).

Omega-conotoxin (ω -CTX), a polypeptide toxin, has been a useful pharmacological tool with potent and selective inhibitory effects on *N*-type VOCCs, which play an important role in transmitter release in the peripheral nervous system. Reports have shown that ω -CTX blocks the release of sensory neuropeptides from chick and rat sensory nerves (10–13). Furthermore, studies have revealed that ω -CTX preferentially inhibits or attenuates sensory neurotransmission at low-frequency stimulation compared with higher frequencies (10,14–16). In contrast, Rane and colleagues (17) have reported that substance P (SP) release from sensory nerves can be inhibited by blocking *L*-type calcium channels. From these observations, it seems likely that different calcium channels may selectively and/or differentially release neurotransmitters.

Because of their role in mediating transmitter release in neural tissues, *N*-type Ca^{++} channels have attracted much attention. This attention has been directed toward responses in normal young animals, with little or no interest directed toward the effects of aging. This comes as a surprise, considering that disruption of neuronal Ca^{++} homeostasis and influx mechanisms in the aging process are well documented (18–22). An early study found the pattern of ω -CTX binding in the central nervous system to be similar in young and old rats (23). This study was followed by subsequent reports indicating that synaptic transmission through ω -CTX-sensitive channels does not change with age (24,25). These previous studies focused on neurotransmission in the autonomic nervous system. Possible age-related changes in the release of neuropeptides following activation of ω -CTX-sensitive channels in sensory nerves has not yet been examined. Hence, investigating age-related changes in ω -CTX-sensitive channels in sensory nerves may further our understanding of the observed dysfunction in sensory nerve activity of old animals and humans, and could have important implications for improving tissue repair with age.

Clinically, ES has been used to improve cutaneous wound healing. However, most of the ES management strategies utilized in various clinical conditions lack experimental support or physiological rationale. This is important, particularly in view of a recent report from our laboratory in which we showed that aged sensory nerves respond preferentially to low-frequency (5 Hz) antidromic ES compared with a higher-frequency (15 Hz) one (26). In addition, we have recently shown that low-frequency (5 Hz) transcutaneous electrical nerve stimulation (TENS) improves peripheral sensory nerve activation with subsequent acceleration of wound healing (27). Therefore, this current study was designed to investigate the mechanisms underlying differential responses of aged sensory nerves to different frequencies of ES and possible involvement of ω -CTX-sensitive VOCCs in these responses. In addition, the relative contribution of the neuropeptides calcitonin gene-related peptide (CGRP) and SP to different frequencies of ES was also investigated by using the specific receptor antagonists CGRP₈₋₃₇ and RP67580, respectively (see *Drugs and Materials* below for the chemical composition of RP67580).

METHODS

Young (3 months) and old (24 months) outbred male Sprague-Dawley rats were used. Anesthesia was induced intraperitoneally with sodium pentobarbitone (60 mg/kg) and was maintained by supplementary injections throughout the 2-hour duration of the experiment. Body temperature was maintained at 37°C. Animals were sacrificed by barbiturate overdose at the completion of experiments. All experimental procedures were approved by the Royal Melbourne Hospital Research Foundation Animal Ethics Committee and adhered to International Association for the Study of Pain guidelines.

Blister Induction

A blister was induced on the hind paw of the anesthetized rat, using a vacuum pressure of -40 kPa. This induction period lasted 30 minutes in young rats and 60 minutes in old

rats. The surface epidermis was then removed and a perspex chamber (with inlet and outlet ports) was secured over the blister base. An initial 20-minute equilibration with Ringer's solution (NaCl, 9.0 g; KCl, 0.42 g; CaCl_2 , 0.48 g; NaHCO_3 , 0.2 g in 1000 ml of dH_2O) was allowed before each experiment, during which time a stable baseline was established. Ringer's solution and/or receptor antagonists were perfused over the blister surface continuously during the experiment and maintained at 4 ml/h, as in previous experiments (28,29).

Antidromic Stimulation of Sensory Nerve

The sciatic nerve at the midthigh region was carefully exposed and cut. This is a commonly used procedure in which the distal portion of the cut nerve is stimulated to activate a local neuroeffector function that mimics a neurogenic inflammatory response without activating the afferent pain-conducting pathway. The distal portion of the cut nerve was placed over platinum electrodes and immersed in a warm oil pool formed from the skin flaps of the wound. The electrodes were fixed in such a position that electrical leakage to adjacent nerve and tissue structures was minimized. Activation of the sensory fibers was achieved with a Grass stimulator at 20 V, 2 ms for a 1-minute duration at either a 15-Hz (high) or 5-Hz (low) frequency (different groups of rats were used to examine the effect of each frequency tested). These parameters have been previously used to stimulate efferent C-fiber responses (1,2,26,30) to evoke an immediate increase in local blood flow. The time elapsed between nerve section and stimulation was approximately 30 minutes, during which baseline blood flow was established and drug perfusion was carried out prior to stimulation.

Administration of Sensory Neuropeptide Receptor Antagonists and ω -CTX (GVIA)

The receptor antagonists for CGRP and SP (CGRP₈₋₃₇ and RP67580, respectively) were dissolved in Ringer's solution and perfused over the blister base (at concentrations ranging from 0.1 μM to 100 μM) 10 minutes prior to and throughout the stimulation and poststimulation periods. Omega-conotoxin GVIA (*Conus geographus*; ω -CTX GVIA) was dissolved in Ringer's solution and perfused over the blister base, at a concentration of 0.1 μM , similar to that used in other studies involving sensory nerves (31–32), 30 minutes prior to and throughout the stimulation and poststimulation periods. Equal perfusion time of Ringer's solution (vehicle) was used for control experiments. Sodium nitroprusside (SNP; 100 μM), a direct-acting smooth muscle vasodilator, was perfused at the completion of the experiment and used to control for variations in smooth muscle reactivity.

Measurement of Cutaneous Blood Flow

A laser Doppler flowmeter probe was positioned vertically over the exposed blister in the hind paw by means of the perspex chamber. 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 electrical stimulation of the cut sciatic nerve were continuously displayed

on a chart recorder. Raw data were evaluated by calculating the area under the stimulation-evoked response curve (in square centimeters) for a poststimulation period of 20 minutes. All measurements were made relative to a stable baseline obtained prior to nerve stimulation. Results are expressed as a percentage of the area (in square centimeters) of the response obtained in the control group of rats. Sodium nitroprusside, a direct-acting smooth muscle vasodilator, was perfused in all rats for 10 minutes after baselines were reestablished following the stimulation period, and the baselines did not differ between control rats and any of the other acute treatments. The overall duration of the experiment did not exceed 2 hours.

Drugs and Materials

Omega-conotoxin GVIA was purchased from Peninsula Laboratories (Belmont, CA). The receptor antagonist CGRP₈₋₃₇ was obtained from Auspep (Melbourne, Australia). RP67580, 2-(1-imino-2-(2-methoxyphenyl)ethyl)-7,7-diphenyl-4-perhydroisoindolone-(3aR,7aR), was a gift from Rhone Poulenc Rorer chemicals (Paris, France). These were all dissolved in Ringer's solution. Pentobarbitone sodium (Nembutal) was obtained from Boehringer Ingelheim (Ararmon, Australia).

Statistical Analysis

Differences in group vasodilatation responses were assessed by using a simple factorial analysis of variance (ANOVA), and this was followed by post hoc independent Student's *t* tests (significance level of .05). The latter statistical tests were used for the analysis of vascular responses and basal blood flow between the acute treatments applied to the rats.

RESULTS

Effect of Different Frequencies of ES on Sensory Nerve Responses

High-frequency ES of the sciatic nerve in young rats resulted in a biphasic response: an initial transient drop in blood flow followed by a short-acting vasodilatation response (Figure 1A), with an area under the curve (AUC) of 17.2 ± 1.0 cm². Stimulation of the sciatic nerve at 5 Hz resulted in an immediate increase of skin microvasculature blood flow in young rats. The profile consisted of a maintained vasodilatation response (compared with that obtained at high frequency), lasting 15–20 minutes after cessation of stimulation (Figure 1B). In young rats, the magnitude of the AUC measurement for low frequency ES was 18.8 ± 1.7 cm² and was not different from that obtained at high frequency.

Effect of CGRP and SP Receptor Antagonists on Sensory Nerve Responses

The difference in the response profile to low and high frequencies of ES prompted us to investigate the relative or quantitative contributions of the sensory neuropeptides CGRP and SP to those responses. This was achieved by perfusing the CGRP and SP receptor antagonists, CGRP₈₋₃₇ and

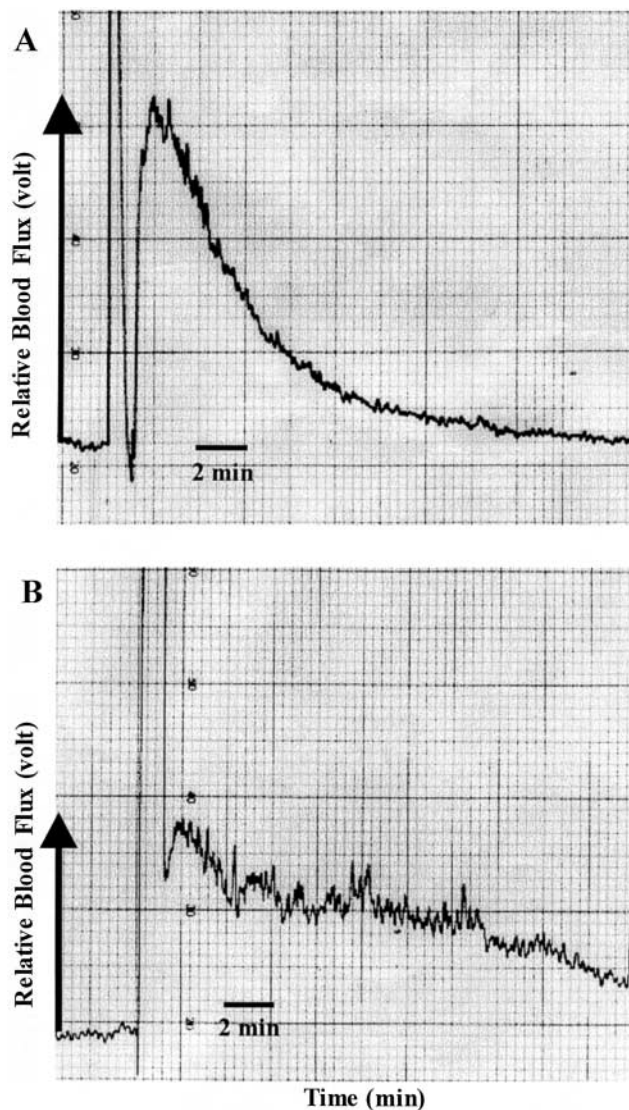


Figure 1. Digitized scan of a typical record of a vasodilatation response to **A**, high-frequency (15 Hz), and **B**, low-frequency (5 Hz), electrical stimulation of the sciatic nerve in young rats (with other parameters being 20 V, 2 ms for 1 minute). At 15 Hz, an initial transient vasoconstriction is observed, followed by a short-acting vasodilatation. At 5 Hz, an immediate and maintained increase in local blood flow is observed, following stimulation. Upward arrows indicate the direction of vasodilatation; vertical lines indicate points of stimulation (and represent associated stimulus artifacts).

RP67580, respectively, over the blister base prior to sciatic nerve stimulation.

Basal blood flow (BBF) measurements in young rats prior to ES did not reveal any significant differences caused by perfusion of the receptor antagonists CGRP₈₋₃₇ and RP67580 (control perfusion, 2.9 ± 0.2 cm; RP67580 perfusion, 3.2 ± 0.3 cm; CGRP₈₋₃₇ perfusion; 2.6 ± 0.2 cm). In addition, perfusion of the receptor antagonists CGRP₈₋₃₇ and RP67580 did not have significant effects on SNP-induced vascular responses (24.1 ± 2.9 cm² and 27.0 ± 5.1 cm², respectively). SNP, a direct smooth muscle vasodilator, was

used as an indicator for smooth muscle reactivity (and thereby, an internal control for our experiments).

At low-frequency ES, perfusion of CGRP₈₋₃₇ reduced the vascular response in a dose-dependent manner. At 0.1 μM, there was no apparent difference in the vascular response compared with control levels, with an AUC of 17.5 ± 3.1 cm². The CGRP receptor antagonist at 1-μM concentration resulted in a significant decrease in the vascular response to nerve stimulation (AUC, 10.8 ± 1.8 cm²; 42.5% decrease, Figure 2A) compared to control responses. At 10 μM of CGRP₈₋₃₇, there was a further decrease with an AUC of 4.2 ± 0.9 cm², which was a decrease of 78% compared to controls.

Perfusion of the SP receptor antagonist RP67580 at low-frequency ES also caused a dose-dependent decrease in the subsequent vasodilatation response. Higher concentrations, however (relative to concentrations of CGRP₈₋₃₇), were required for this effect. RP67580 at 0.1 μM and 1.0 μM did not alter the vascular response (AUCs of 20.1 ± 1.6 cm² and 18.9 ± 2.2 cm², respectively). At 10 μM of RP67580 we observed a 34% decrease (AUC, 12.5 ± 2.7 cm²), and following 100-μM perfusion, the vascular response was

significantly reduced by 63% (AUC, 6.9 ± 1.7 cm²; Figure 2A).

Utilizing the submaximal concentrations as best determined from the above experiment, we then examined the quantitative contribution of these sensory neuropeptides to vascular responses obtained to high-frequency (15 Hz) ES. Perfusion of 1.0 μM of CGRP₈₋₃₇ prior to and during 15-Hz ES resulted in a significant decrease in the vascular response with an AUC of 9.3 ± 1.3 cm² (45% decrease compared with control stimulations; Figure 2B). Perfusion of RP67580 at a concentration of 10 μM also caused a significant attenuation of the vascular response following high-frequency ES (AUC, 4.9 ± 0.3 cm², corresponding to a 72% decrease; Figure 2B).

Effect of Age on BBF and on Smooth Muscle Reactivity

BBF was found to be significantly higher in old rats (3.9 ± 0.4 cm) compared with young rats (2.9 ± 0.2 cm; see Table 1). This may be due to the prolonged period required for blister induction in old rats compared with that of young rats (60 and 30 minutes, respectively). The vasodilator responses to SNP were not significantly different between young (25.3 ± 2.3 cm²) and old (28.5 ± 2.3 cm²) rats.

Effect of Age on Sensory Nerve Responses

High-frequency ES of the sciatic nerve in old rats resulted in a significantly reduced vascular response (AUC, 10.5 ± 1.3 cm²), a 40% decrease, compared with that of young controls (AUC, 17.2 ± 1.0 cm²; Figure 3).

Stimulation of the sciatic nerve at 5 Hz in old rats resulted in a vascular response that was similar in both profile and magnitude (AUC, 17.9 ± 1.5 cm²) to that of young rats (AUC, 18.8 ± 1.7 cm²) and was significantly greater than the response of aged rats at high-frequency stimulation (Figure 3).

Effect of ω-CTX GVIA Perfusion on Sensory Nerve Responses in Young and Old Rats

A selective inhibitor of the N-type voltage sensitive calcium channels (VSCC), ω-CTX GVIA, was used to examine the contribution of these channels to the vascular responses. BBF, measured prior to nerve stimulation, was significantly increased during ω-CTX GVIA perfusion in young rats relative to their controls (see Table 1). Although old rats exhibited a large increase in BBF during ω-CTX GVIA application, this did not attain significance. The administration of ω-CTX was without effect on the vasodilator responses to SNP in young (22.8 ± 2.0 cm²) and in old (33.0 ± 4.8 cm²) rats.

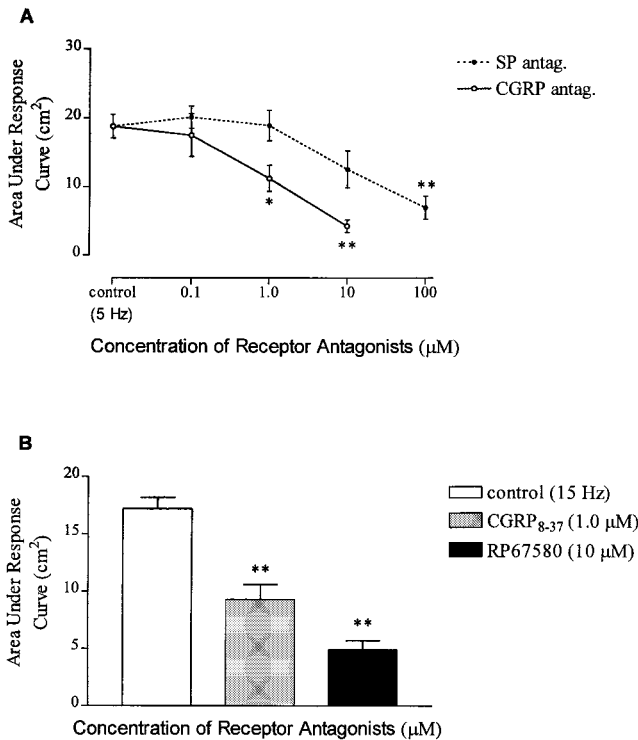


Figure 2. Effect of CGRP₈₋₃₇ and RP67580 (calcitonin gene-related peptide [CGRP] and substance P [SP] receptor antagonists, respectively) on neurogenic vasodilatation responses following **A**, low-frequency, and **B**, high-frequency, sciatic nerve stimulation in young rats (20 V, 2 ms for 1 minute at 5 or 15 Hz, respectively). The antagonists were perfused 10 minutes prior to electrical stimulation (ES). Results are expressed as mean ± SEM of the area under the response curve (in square centimeters; n = 5–16). The effect of submaximal concentrations of CGRP₈₋₃₇ and RP67580 determined from data in **A** were used in **B**. Results are expressed as mean ± SEM of the area under the response curve (in square centimeters; n = 5–11). The asterisks denote significant difference (*p ≤ .05; **p ≤ .001) relative to control.

Table 1. Effect of ω-Conotoxin GVIA Perfusion on Basal Blood Flow

Perfusion	Young	Old
control	2.9 ± 0.2	3.9 ± 0.4*
ω-conotoxin	4.3 ± 0.5*	5.3 ± 0.8

Note: Basal blood flow is measured in centimeter height, above zero reference level; GVIA = *conus geographus*.

*Significant difference relative to control data (p ≤ .05).

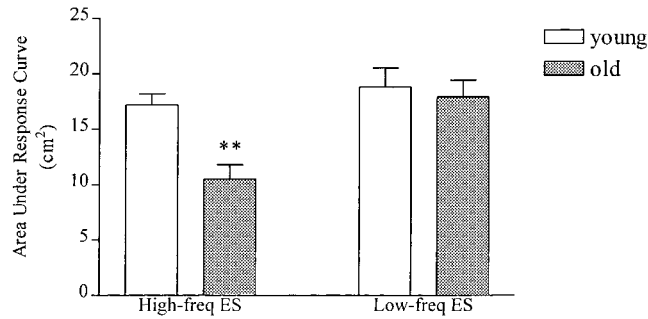


Figure 3. Effects of aging on neurogenic vasodilator responses following high- and low-frequency sciatic nerve stimulation. Electrical stimulation (ES) parameters were 20 V, 2 ms for 1 minute at high or low frequency (15 or 5 Hz, respectively). Results are expressed as mean \pm SEM of the area under the response curve (in square centimeters; $n = 5$ –16). The asterisks denote significant difference (** $p \leq .001$) relative to young control and to old low-frequency groups. No significant difference was observed between the two young groups or between the young and old groups at low frequency.

High-frequency ES in young rats during continuous perfusion of ω -CTX GVIA ($0.1 \mu\text{M}$) over the blister base resulted in a small but insignificant decrease in the subsequent vascular response (AUC, $16.4 \pm 2.1 \text{ cm}^2$) compared with that of young controls (AUC, $17.2 \pm 1.0 \text{ cm}^2$). When this was examined in older rats, we also observed no significant change in the blood flow response following 15-Hz stimulation (AUC, $12.8 \pm 2.2 \text{ cm}^2$; Figure 4A) compared with that of old controls (AUC, $10.5 \pm 1.3 \text{ cm}^2$). At low-frequency (5 Hz) ES, perfusion of ω -CTX induced a significant reduction in the vasodilator response in young rats by 53% (AUC, $8.9 \pm 1.4 \text{ cm}^2$) compared to control perfusions (AUC, $18.8 \pm 1.7 \text{ cm}^2$). In older rats, ω -CTX GVIA perfusion in combination with 5-Hz stimulation also resulted in a significant decrease of 42% in the vascular response with an AUC of $10.4 \pm 1.8 \text{ cm}^2$ (Figure 4B) compared to old controls (AUC, $17.9 \pm 1.5 \text{ cm}^2$).

DISCUSSION

Sensory Nerve Responses to Different Frequencies of ES

Sensory nerve-induced neurogenic inflammation has an important role in the initiation of tissue repair. Delayed wound healing is a major clinical problem associated with aging, and we previously provided evidence that the delay in wound repair correlates with a decline in sensory nerve activity (4,7). To optimize the efferent response of sensory nerves with age, we need to understand the changes in the inflammatory process and its modulation under different conditions of sensory nerve activation.

The ES parameters used in this study were previously shown to activate sensory nerves (1,2,4,26,30). Different response profiles were obtained following high- and low-frequency ES in young control rats. These differences might suggest possible quantitative and/or qualitative differential contribution of sensory peptides and/or differential modulation of neuropeptide release. As a way to clarify this issue, the relative contribution of the sensory neuropeptides SP and CGRP to these responses was investigated in young rats.

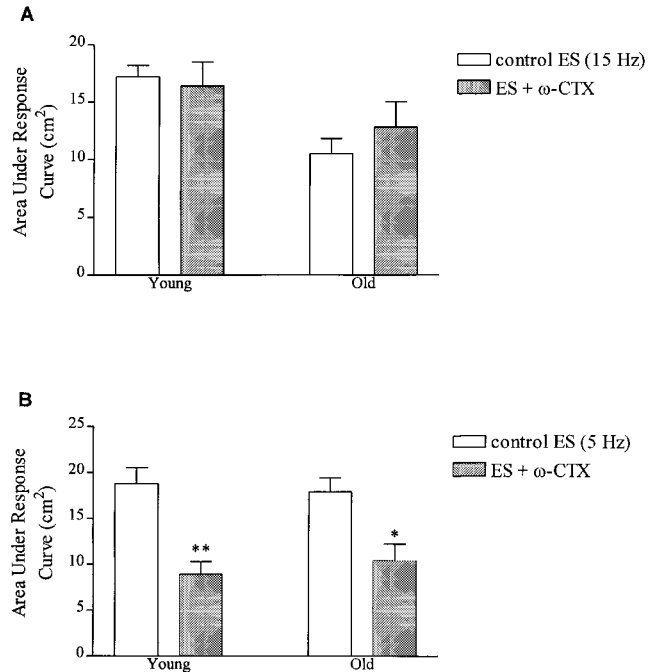


Figure 4. Effect of ω -conotoxin (CTX) GVIA ($0.1 \mu\text{M}$) on neurogenic vasodilator responses to **A**, high-frequency, and **B**, low-frequency, sciatic nerve stimulation (20 V, 2 ms for 1 minutes at 15 or 5 Hz, respectively). The N -type Ca^{++} channel inhibitor was perfused 10 minutes prior to electrical stimulation (ES). Results are expressed as mean \pm SEM of the area under the response curve (in square centimeters; $n = 4$ –5). The asterisks denote significant difference (* $p \leq .05$, ** $p \leq .001$) relative to control data. No significant effect for ω -CTX GVIA was observed at high-frequency ES.

The CGRP receptor antagonist (CGRP₈₋₃₇) caused a dose-dependent decrease in the blood flux response to 5-Hz nerve stimulation, with $1 \mu\text{M}$ and $10 \mu\text{M}$ inducing approximately 42.5% and 77% inhibition, respectively. This is in accord with the previous results of Escott and Brain (33) obtained with rat saphenous nerve and trigeminal ganglion stimulation (34) and confirms the role of CGRP in neurogenic vasodilatation. Similar results using CGRP₈₋₃₇ concentrations of 1–10 μM were also obtained by Kurosawa and colleagues (35) by looking at meningeal blood flow following ES at 5–10 Hz (see Figure 2A).

At the same frequency of ES, the SP receptor antagonist was found to be less effective in inhibiting the vasodilator response. Perfusion at $10 \mu\text{M}$ caused a 34% decrease in the subsequent vascular response, compared with 77% obtained with CGRP₈₋₃₇ at a similar concentration. Increasing the concentration of RP67580 by tenfold only resulted in a 63% inhibition, still neither as potent nor effective as CGRP₈₋₃₇ at lower strengths. Hence, our data using specific receptor antagonists suggest that the vascular response to low-frequency ES involves a greater release of CGRP.

With the use of submaximal concentrations of the peptide receptor antagonists ($1 \mu\text{M}$ and $10 \mu\text{M}$ for CGRP₈₋₃₇ and RP67580, respectively), the relative contribution patterns of the two neuropeptides to the vascular response was altered with high-frequency ES. RP67580 was very effective at inhibiting the vascular response at this frequency with a 72%

decrease. The CGRP receptor antagonist, in contrast, resulted only in a 45% inhibition (Figure 2B). These results suggest that SP has a relatively greater contribution to the vascular response at higher frequencies of nerve stimulation.

The difference in the relative contribution of SP and CGRP to the vascular responses obtained at high and low frequencies of ES may explain the different vascular response profiles obtained for these frequencies. Although CGRP appears to have an equal contribution at the two frequencies examined, it has been reported that SP can terminate an existing CGRP-induced vasodilator response (7,36). This would suggest that with higher frequency stimulation, where there is a greater role for SP, we can expect a shorter or "interrupted" vasodilator response to CGRP, whereas the response obtained for low frequency would be more prolonged or "ongoing." This statement indeed appears to correlate well with the actual response profiles observed (see Figures 1A and 1B).

Effect of Age on BBF, Smooth Muscle Reactivity, and Sensory Nerve Responses

In the current study, BBF was found to be significantly higher in old rats compared with young. This may be due to the prolonged period required for blister induction in old rats compared with young (60 and 30 minutes, respectively). It was previously reported that the aged stratum corneum provides qualitatively inferior barrier activity (37). Hence, the attenuation of transudation processes in older skin is most likely responsible for the delay in blister formation. The longer exposure period of the skin in the old rat hind limb during the induction process may result in a greater increase in mediator release at the blister site, and thus contribute to the higher BBF observed in these animals (see Table 1).

Perfusion of the nitrovasodilator SNP showed no change in smooth muscle reactivity with age or with drug perfusion. This suggests that any differences observed in sensory nerve responses between young and old animals cannot be attributed to alterations in vascular smooth muscle reactivity. Although many investigators have reported that aging is associated with marked structural and functional changes in the vascular system—for a review, see Dohi and colleagues (38)—in vivo studies in rats and humans demonstrate that the responses induced by SNP are unaffected by aging (4,39,40).

The vascular response to high-frequency ES in old rats was significantly reduced compared with that of young rats (Figure 3). This finding follows the general trend reported by Khalil and colleagues (4) in which older animals show diminished sensory nerve responses to antidromic stimulation. In that report, the authors used prolonged 15-Hz stimulation to show a significantly delayed and smaller vascular response compared to the young (other parameters being 10 V, 0.5 ms for 30 minutes). The latter study, when taken together with the current data using high-frequency ES in old rats, suggests that the decline in aged sensory nerve function can be highlighted when utilizing high-frequency ES of these nerves. The reported defects in neuropeptide release mechanisms associated with aging (4) may partly account

for the observed reduction in vasodilator responses of old rats in response to high-frequency nerve stimulation.

At a lower frequency of stimulation, however, there was no difference in the subsequent vascular response as measured in the blister base of young and old rats (Figure 3). This observation might contradict the general notion that sensory nerve activity is impaired with age. It is possible, however, that there is a redundancy in aged sensory nerves that becomes apparent at this frequency of stimulation, and that allows the aged sensory nerve to produce an equivalent response to young sensory nerves.

In an attempt to understand the mechanisms underlying age-related differential responses at high and low frequencies of ES, we investigated the role of ω -CTX-sensitive channels in these responses. These experiments arose from reports that showed that ω -CTX-sensitive channels are predominantly active at low frequencies of activation (10,14–16).

In this study, it was found that ω -CTX GVIA significantly inhibited the vascular response of young and old rats at low frequency, but was without effect at high-frequency ES. Hence, ω -CTX-sensitive VOCCs appear to play a role in releasing sensory neuropeptides from peripheral nerve terminal endings at low frequency in our model. We further suggest that the involvement of these channels remains intact with age, thus contributing to the near-normal response obtained in older rats following low-frequency ES. The observations in these 24-month-old rats parallel those of Dooley and colleagues (23), who demonstrated no change in ω -CTX GVIA binding sites in the central nervous system of 18-month-old rats.

As in young rats, ω -CTX GVIA perfusion at higher-frequency ES in old rats did not alter the vascular response. This indicates that neuropeptide release during high-frequency nerve stimulation occurs via mechanisms that do not involve ω -CTX-sensitive VOCCs, and it further supports the earlier reports of predominant activation of *N*-type calcium channels at low-frequency ES (10,14–16). A role for *L*-type calcium channels in this instance could be a possibility, considering the report by Rane and colleagues (17) demonstrating SP release from sensory nerves can be inhibited by blocking *L*-type calcium channels. This proposition is further supported by our data demonstrating SP has a greater contribution to the sensory nerve-evoked vasodilator response at high-frequency ES (see Figure 2B).

It was observed that perfusion of ω -CTX GVIA in young rats significantly increased BBF prior to ES, whereas there was a strong trend for an increase in old rats. It should be mentioned that VOCCs have also been characterized in sympathetic neurones (41–45). It is reasonable to postulate that blockade of the *N*-type Ca^{++} channels could cause inhibition of noradrenaline (NA) release from sympathetic nerves (which is the major determinant of vascular tone), leading to this increase in local blood flow. That the increase in BBF in old rats did not reach statistical significance could be related to a reduction in sympathetic control of vascular tone with age (46). This then raises the question that ω -CTX GVIA might also inhibit NA release from sympathetic fibers upon nerve stimulation. This is highly unlikely considering that such an action would have resulted in an enhancement of the vascular responses rather than the

inhibition observed following ω -CTX GVIA perfusion. We have recently demonstrated that sympathetic fibers play an important role in modulating sensory nerve function and that blockade of sympathetic neurotransmitter action or release increases neurogenic vasodilator responses under similar experimental conditions (26).

Conclusions

In conclusion, the current results demonstrate that there is a differential qualitative and quantitative contribution of sensory neuropeptides at the two frequencies examined, with SP and CGRP playing more important roles at high and low frequencies of ES, respectively. The data demonstrate that aged sensory nerves preferentially respond to low-frequency ES. The results also suggest that ω -CTX-sensitive channels are involved in peptide release at low-frequency ES but not at high-frequency ES. That the function of N-type VOCCS is preserved with age and that these channels play a large role in peptide release at low frequency might explain the ability of the aged sensory nerve to generate a near-normal response at a low level of activation. This study provides a physiological rationale with an understanding of the cellular and molecular basis for the previously demonstrated beneficial effects of low-frequency electrical stimulation to wound repair in aged animals. The current results therefore could have important clinical and practical implications for improving tissue repair in the aged.

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REFERENCES

- Lembeck F, Holzer P. Substance P as a neurogenic mediator of antidromic vasodilatation and neurogenic plasma extravasation. *Naunyn-Schmiedeberg's Arch Pharmacol.* 1979;310:175–183.
- White DM, Helme RD. Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. *Brain Res.* 1985;336:27–31.
- Brain SD, Hughes SR, Cambridge H, O'Driscoll G. The contribution of calcitonin gene-related peptide (CGRP) in neurogenic vasodilator responses. *Agents Actions.* 1993;38:C19–C21.
- Khalil Z, Ralevic V, Bassirat M, Dusting GJ, Helme RD. Effect of ageing on sensory nerve function in rat skin. *Brain Res.* 1994;641:265–272.
- Ardron ME, Helme RD, McKernan S. Microvascular skin responses in elderly people with varicose leg ulcers. *Age Ageing.* 1991;20(2):124–128.
- Helme RD, McKernan S. Neurogenic flare responses following topical application of capsaicin in humans. *Ann Neurol.* 1985;18:505–509.
- Khalil Z, Helme RD. Sensory peptides as neuromodulators of wound healing in aged rats. *J Gerontol Biol Sci.* 1996;51A:B354–B361.
- Eaglstein W. Wound healing and ageing. *Clin Geriatr Med.* 1989;5:183–188.
- Gerstein AD, Phillips TJ, Rogers GS, Gilchrist BA. Wound healing and ageing. *Dermatol Clin.* 1993;11(4):749–757.
- Maggi CA, Patacchini R, Santicioli P, et al. The effect of omega conotoxin GVIA, a peptide modulator of N-type voltage-sensitive calcium channels, on motor responses produced by activation of efferent and sensory nerves in mammalian smooth muscle. *Naunyn-Schmiedeberg's Arch Pharmacol.* 1988;338:107–113.
- Aosaki T, Kasai H. Characterization of two kinds of high-voltage-activated Ca-channel currents in chick sensory neurons. Differential sensitivity to dihydropyridines and omega-conotoxin GVIA. *Pflugers Arch.* 1989;414(2):150–156.
- Maggi CA, Tramontana M, Cecconi R, Santicioli P. Neurochemical evidence for the involvement of N-type calcium channels in transmitter secretion from peripheral endings of sensory nerves in guinea pigs. *Neurosci Lett.* 1990;114(2):203–206.
- Hong KW, Kim CD, Rhim BY, Lee WS. Effect of omega-conotoxin GVIA and omega-agatoxin IVA on the capsaicin-sensitive calcitonin gene-related peptide release and autoregulatory vasodilation in rat pial arteries. *J Cereb Blood Flow Metab.* 1999;19(1):53–60.
- Boeckxstaens GE, De Man JG, Pelckmans PA, Cromheeke KM, Herman AG, Van Maercke YM. Ca²⁺ dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves. *Br J Pharmacol.* 1993;110(4):1329–1334.
- Zygmunt PM, Zygmunt PK, Hogestatt ED, Andersson KE. Effects of omega-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor. *Br J Pharmacol.* 1993;110(4):1285–1290.
- Tran S, Boot JR. Differential effects of voltage-dependent Ca²⁺ channels on low and high frequency mediated neurotransmission in guinea-pig ileum and rat vas deferens. *Europ J Pharmacol.* 1997;335(1):31–36.
- Rane SG, Holz GG, Dunlap K. Dihydropyridine inhibition of neuronal calcium current and substance P release. *Pflugers Arch.* 1987;409(4–5):361–366.
- Roberts J, Mortimer ML, Ryan PJ, Johnson MD, Tumer N. Role of calcium in adrenergic neurochemical transmission in the aging heart. *J Pharmacol Exp Ther.* 1990;253(3):957–964.
- Kirischuk S, Pronchuk N, Verkhatsky A. Measurements of intracellular calcium in sensory neurons of adult and old rats. *Neuroscience.* 1992;50(4):947–951.
- Hartmann H, Eckert A, Muller WE. Disturbances of the neuronal calcium homeostasis in the aging nervous system. *Life Sci.* 1994;55(25–26):2011–2018.
- Verkhatsky A, Shmigol A, Kirischuk S, Pronchuk N, Kostyuk P. Age-dependent changes in calcium currents and calcium homeostasis in mammalian neurons. *Ann N Y Acad Sci.* 1994;747:365–381.
- Duckles SP, Tsai H, Buchholz JN. Evidence for decline in intracellular calcium buffering in adrenergic nerves of aged rats. *Life Sci.* 1996;58(22):2029–2035.
- Dooley DJ, Lickert M, Lupp A, Osswald H. Distribution of [¹²⁵I]omega-conotoxin GVIA and [³H]isradipine binding sites in the central nervous system of rats of different ages. *Neurosci Lett.* 1988;93(2–3):318–323.
- Friedman DJ, Duckles SP. Effect of calcium channel blockers on norepinephrine release and modulation by prejunctional D2 dopamine receptors. *Life Sci.* 1994;54(21):1545–1557.
- Hall SC, Griffith WH. Calcium channel types mediating synaptic transmission during aging. *Brain Res.* 1997;745:339–342.
- Merhi M, Helme RD, Khalil Z. Age-related changes in sympathetic modulation of sensory nerves. *Inflamm Res.* 1998;41:239–244.
- Khalil Z, Merhi M. Effects of ageing on neurogenic vasodilator responses evoked by transcutaneous electrical nerve stimulation: relevance to wound healing. *J Gerontol Biol Sci.* 2000;55A:B257–B263.
- Ralevic V, Khalil Z, Dusting GJ, Helme RD. Nitric oxide and sensory nerves are involved in the vasodilator response to acetylcholine but not calcitonin gene-related peptide in rat skin microvasculature. *Br J Pharmacol.* 1992;106:650–655.
- Khalil Z, Chen H, Helme RD. Mechanisms underlying the vascular activity of β -amyloid protein fragment (β A_{25–35}) at the level of skin microvasculature. *Brain Res.* 1996;736:206–216.
- Merhi M, Dusting GJ, Khalil Z. CGRP and nitric oxide of neuronal origin modulate neurogenic vasodilatation in rat skin microvasculature. *Br J Pharmacol.* 1998;23:863–868.
- Evans AR, Nicol GD, Vasko MR. Differential regulation of evoked peptide release by voltage-sensitive calcium channels in rat sensory neurons. *Brain Res.* 1996;712(2):265–273.
- Kopp UC, Cicha MZ. PGE₂ increases substance P release from renal pelvic sensory nerves via activation of N-type calcium channels. *Am J Physiol.* 1999;276:R124–R128.
- Escott JK, Brain SD. Effect of a calcitonin gene-related peptide antag-

- onist (CGRP₈₋₃₇) on skin vasodilatation and oedema induced by stimulation of the rat saphenous nerve. *Br J Pharmacol.* 1993;110:772-776.
34. Escott JK, Beattie DT, Connor HE, Brain SD. Trigeminal ganglion stimulation increases facial blood flow in the rat: a major role for calcitonin gene-related peptide. *Brain Res.* 1995;669:93-99.
 35. Kurosawa M, Messlinger K, Pawlak M, Schmidt RF. Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: mediation by calcitonin gene-related peptide. *Br J Pharmacol.* 1995;114(7):1397-1402.
 36. Brain SD, Williams TJ. Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature.* 1988;335(6185):73-75.
 37. Grove GL. Age-related differences in healing of superficial skin wounds in humans. *Arch Dermatol Res.* 1982;272(3-4):381-385.
 38. Dohi Y, Thiel MA, Buhler FR, Luscher TF. Activation of endothelial L-arginine pathway in resistance arteries. Effect of age and hypertension. *Hypertension.* 1990;16(2):170-179.
 39. Hajdu MA, McElmurry RT, Heistad DD, Baumbach GL. Effects of aging on cerebral vascular responses to serotonin in rats. *Am J Physiol.* 1993;264:H2136-H2140.
 40. Tominaga M, Fujii K, Abe I, Takata Y, Kobayashi K, Fujishima M. Hypertension and ageing impair acetylcholine-induced vasodilation in rats. *J Hypertens.* 1994;12(3):259-268.
 41. Boehm S, Huck S. Inhibition of N-type calcium channels: the only mechanism by which presynaptic alpha 2-autoreceptors control sympathetic transmitter release. *Eur J Neurosci.* 1996;8(9):1924-1931.
 42. Brain KL, Bennett MR. Calcium in sympathetic varicosities of mouse vas deferens during facilitation, augmentation and autoinhibition. *J Physiol (Lond).* 1997;502(Pt 3):521-536.
 43. Yahagi N, Akiyama T, Yamazaki T. Effects of omega-conotoxin GVIA on cardiac sympathetic nerve function. *J Auton Nerv Syst.* 1998;68(1-2):43-48.
 44. Nedergaard OA. Effect of omega-conotoxin GVIA on noradrenaline release from postganglionic sympathetic neurones in rabbit aorta. *Pharmacol Toxicol.* 2000;86(1):30-35.
 45. Yang XP, Chiba S. Effects of omega-conotoxin GVIA and diltiazem on double peaked vasoconstrictor responses to periarterial electric nerve stimulation in isolated canine splenic artery. *Br J Pharmacol.* 2000;129(1):47-52.
 46. Khalil Z, LeVasseur S, Merhi M, Helme RD. Sympathetic modulation of sensory nerve activity with age: human and rodent skin models. *Clin Exp Pharmacol Physiol.* 1997;24(11):883-886.

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