RECOVERY OF NOCICEPTIVE FUNCTION IN THE DENERVATED SKIN: LONGITUDINAL STUDIES USING SCANNING LASER DOPPLER IMAGING AND VASCULAR LABELING

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II. **Lakatos Sz**; Jancsó G; Horváth Á; Dobos I; Sántha P (2020) Longitudinal Study of Functional Reinnervation of the Denervated Skin by Collateral Sprouting of Peptidergic Nociceptive Nerves Utilizing Laser Doppler Imaging; Frontiers in Physiology (1664–042X): 11 Paper 439. 10. p., 2020,
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Sántha P; **Lakatos Sz**; Horváth Á; Segesdi D; Jancsó G, New method for longitudinal examination of the cutaneous sensory reinnervation and its regulation in the denervated rat skin, Hungarian Physiological Society (MÉT), Szeged, Hungary, pp 124-125 Paper P5.11., May 25–30, 2015

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Sántha P; **Lakatos Sz**; Horváth Á; Jancsó G, Collateral sprouting of nociceptive afferents is inhibited by spared axons in the chemodenervated rat skin, 15th Annual Conference of the Hungarian Neuroscience Society, Budapest, Hungary, January 22–23, 2015

LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
DRG	dorsal root ganglion
FRAP	fluoride-resistant acid phosphatase
GDNF	glial-derived neurotrophic factor
IGF	insulin-like growth factor
LDI	laser Doppler imaging
NGF	nerve growth factor
NK1	neurokinin 1
NKA	neurokinin A
NPY	neuropeptide-Y
NT	neurotrophin
PBS	phosphate buffered saline
PGP9.5	protein gene product 9.5
PU	perfusion unit
SOM	somatostatin
SP	substance P
TH	tyrosine hydroxylase
TrkA	tyrosine kinase A
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin type 1
TRPV1	transient receptor potential vanilloid subtype 1
VIP	vasoactive intestinal peptide
WGA-HRP	horse radish peroxidase conjugated with wheat germ agglutinin

1. INTRODUCTION

The skin is the largest sensory organ of the body richly endowed by sensory nerves which play a fundamental role in the sensation of and adaptation to changes of external and internal environment in order to maintain the integrity and homeostasis of the organism. Sensory nerve endings are sensitive and excited by a wide range of mechanical, chemical, and thermal stimuli. Primary sensory neurons comprise morphologically, functionally and neurochemically distinct populations innervating different tissues and organs. A major population of sensory nerves innervate the skin subserving specific sensory modalities including tactile, thermal and nociceptive sensations. Damage to cutaneous peripheral nerves results in complete or partial loss of skin sensation. The loss of sensory functions results from distinct degenerative alterations of sensory nerves depending on the type of the injury. Traumatic or surgical lesions of peripheral nerves may be followed by regenerative processes leading to partial or even complete recovery of function. Lesions induced by toxic agents or systemic, in particular metabolic diseases may cause specific and distinct impairments in skin sensation. Irrespective of the cause of damage inflicted upon sensory nerves, degeneration of afferent axons may be followed by regenerative processes of sensory nerves which may ultimately result in partial or full restitution of skin sensation.

Investigations into the mechanisms of nerve regeneration represent a research field of prime interest for both theoretical and practical reasons. The methods of study of nerve regeneration involve behavioral testing, electrophysiological techniques and histology of skin biopsies. Each of these techniques have advantages, but also significant shortcomings in the study of nerve regeneration, especially if longitudinal studies are to be performed. Indeed, most techniques used are subjective, imprecise and inappropriate for longitudinal studies. Therefore, we sought for a novel specific, objective and repeatable method suitable for the longitudinal study of cutaneous nerve regeneration.

Capsaicin-sensitive nociceptive afferents possess a dual function: on the one hand, they convey nociceptive impulses evoked by noxious thermal and chemical stimuli towards the central nervous system. On the other hand, these particular peptidergic sensory nerve endings, upon antidromic electrical nerve stimulation or direct application of irritant chemicals, such as mustard oil onto the skin, release vasoactive neuropeptides which induce vasodilatation and increase in vascular (venular) permeability. These vascular reactions, which are salient features

of nociceptor function, can be visualized and measured quantitatively by using scanning laser Doppler imaging and the vascular labeling method, respectively. By making use of these methods suitable for longitudinal studies, the present Thesis summarizes our findings on the regenerative propensity of cutaneous afferent nerves following peripheral nerve transection and perineural capsaicin treatment, which produce complete loss of sensation and specific loss of nociceptive functions, respectively.

1.1 Major characteristics and classification of primary sensory neurons

Primary sensory neurons detect signals of external and internal environment and convey this information toward the central nervous system. Morphologically, primary sensory neurons are pseudounipolar cells with perikarya residing in sensory ganglia of cranial and spinal nerves, and with central and peripheral axons terminating in the central nervous system and the peripheral tissues, respectively. The perikarya of spinal primary afferent neurons are located in the dorsal root ganglia (DRG). These neurons can be subdivided into different subpopulations according to their morphological, neurochemical and functional characteristics. In now classical studies, based on light- and electron microscopic observations, two basic subtypes of primary afferent neurons have been distinguished: large diameter light, type A cells with myelinated fibers and small diameter, dark type B cells with unmyelinated C-fibers (Lawson, 1979; Lawson and Waddell, 1991; Carr and Nagy, 1993; Basbaum *et al.*, 2009). Rodent large light A-type neurons, which are rich in neurofilaments, can be selectively labeled with the monoclonal antibody RT97 (Lawson *et al.*, 1984; Lawson and Waddell, 1991; Holger Sann *et al.*, 1995). Other markers of large A-cells include parvalbumin, carbonic anhydrase and GM1 ganglioside. Small dark neurons comprise peptidergic, non-peptidergic and tyrosine hydroxylase (TH)-expressing subpopulations (Basbaum *et al.*, 2009; Li *et al.*, 2016, 2018). Among specific markers of peptidergic small B-cells substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM) may be mentioned (Wiesenfeld-Hallin *et al.*, 1984; Basbaum *et al.*, 2009; Li *et al.*, 2018). Non-peptidergic small neurons are characterized by binding the plant lectin IB4 and may also contain fluoride-resistant acid phosphatase (FRAP), also known as thiamine monophosphatase (TMP), identified recently as prostatic acid phosphatase (Carr and Nagy, 1993; Zylka *et al.*, 2008; Basbaum *et al.*, 2009; Li *et al.*, 2018).

Another classification of DRG neurons is based on the expression pattern of neurotrophic factor receptors. Trophic factors play a fundamental role in the development and the regulation of the molecular traits of primary sensory neurons. However, during development, sensory neurons change their sensitivity to trophic factors. At early developmental stages almost all small diameter neurons express the nerve growth factor (NGF) receptor tyrosine kinase A (TrkA), and NGF is required to support their survival. In adult animals this receptor is still found in peptidergic small sized neurons, while in non-peptidergic subpopulation TrkA is downregulated and these cells express the c-Ret neurotrophin receptor which is the target of the glial-derived neurotrophic factor (GDNF) (Snider and McMahon, 1998; Basbaum *et al.*, 2009; Li *et al.*, 2018).

Recent studies based on single cell gene expression profiling of isolated mouse sensory neurons have revealed the need for a more complex categorization of primary sensory neurons. Assumption-free analyses of gene sequencing data obtained from DRG neurons by Usoskin and coworkers in 2015 (Usoskin *et al.*, 2015) and by Zeisel and coworkers in 2018 (Zeisel *et al.*, 2018) suggest up to 18 functionally distinct subtypes of primary sensory neurons. Specific marker maps were established at the major developmental stages of embryonic life and through early adulthood utilizing single-cell RNA sequencing allowing a cluster analysis-based unbiased identification of sensory neuron subpopulations at the molecular level (Usoskin *et al.*, 2015; Zeisel *et al.*, 2018; Sharma *et al.*, 2020). A detailed review of the functional properties of distinct subpopulations of primary sensory neurons is beyond the scope of this Thesis. We focus on nociceptive primary sensory neurons whose physiological and pathophysiological processes comprise the subject matter of this Thesis.

1.1.1 Functional properties of nociceptive primary sensory neurons

Nociception allows the nervous system to detect, encode and process intense thermal, mechanical and chemical stimuli by the activation of specific peripheral nerve fibers, called nociceptors (Basbaum *et al.*, 2009). The term nociceptor was introduced by Charles Scott Sherrington (Nobel Prize in Physiology or Medicine, 1932) in his monograph published in 1906. Nociceptors are afferent nerve endings located in a wide range of tissues including the skin, muscles, tendons, joints, visceral organs, etc., and are highly specialized to detect and convey specific stimuli which threaten to induce tissue damage. Nociceptors are activated only

when the stimulus intensity reaches the threshold to elicit pain sensation (nociception) (Burgess and Perl, 1967; Greenspan, 1997; Gu *et al.*, 2005; Oaklander and Siegel, 2005; Handwerker, 2006; Basbaum *et al.*, 2009). The neural activity induced by these stimuli may be interpreted as pain, discomfort or irritation.

Electrophysiological studies disclosed different classes of nociceptive afferents including Aδ mechanoreceptors, C-fiber mechano-heat nociceptors and C-fiber polymodal nociceptors. In the rat, the C-fiber polymodal nociceptor is the most abundant C-fiber nociceptor. Polymodal nociceptors respond to intense mechanical, noxious warm (hot) and irritant (painful) chemical stimuli (Bessou and Perl, 1969; Greenspan, 1997; Oaklander and Siegel, 2005; Lynn, 2009; Woller *et al.*, 2017). These potentially tissue damaging stimuli activate specific ion channels in the axonal membrane of the nociceptive free nerve endings which results in depolarization and eventually propagating action potentials. C-fiber polymodal nociceptors convey their signals through the sensory ganglia to the spinal or medullary dorsal horn (Gee, Lynn and Cotsell, 1997; Lynn, 2009; Woller *et al.*, 2017).

Besides their "sensory afferent" functions, polymodal nociceptors bear local regulatory, "sensory efferent" functions, too. Local regulatory functions are mainly attributed to (vasoactive) neuropeptides released by the stimulated nerve endings (Jancsó N., 1960; Jancsó, Jancsó-Gábor and Szolcsányi, 1968; Lembeck and Holzer, 1979; Brain *et al.*, 1986; Handwerker, 2006). Nociceptive primary afferents contain a wide range of neuropeptides, in particular, CGRP and various tachykinins among which SP is the most notable. In rats, the most frequent sensory neuropeptide is CGRP that is present in up to 50% of DRG neurons (O'Brien *et al.*, 1989; Gee, Lynn and Cotsell, 1997; Häbler *et al.*, 1999; Lynn, 2009; Sorkin *et al.*, 2018) and approximately 25% of the CGRP positive DRG neurons also express SP (Häbler *et al.*, 1999; Lynn, 2009; Sorkin *et al.*, 2018). Among primary sensory neurons, SP is expressed exclusively in CGRP-expressing afferents (Sorkin *et al.*, 2018). Both CGRP and SP possess proinflammatory potential (Kilo *et al.*, 1997; Lynn, 2009), however, they elicit different vascular reactions. CGRP induces vasodilation mainly through the relaxation of arterial smooth muscle, while SP increases the permeability of postcapillary venules and mediates plasma extravasation through activation of neurokinin 1 (NK1) receptors (Lembeck and Holzer, 1979; Brain *et al.*, 1986; Klede *et al.*, 2003; Handwerker, 2006). At higher concentrations, SP may lead to mast cell degranulation and local edema (Handwerker, 2006). Vasodilatation and plasma extravasation elicited by activation of peptidergic afferent nerve endings are collectively termed the neurogenic inflammatory response.

1.1.2 Chemosensitive primary sensory neurons

A subpopulation of primary sensory neurons is exquisitely sensitive to capsaicin (8-methyl-N-vanillyl-6-nonenamide), the pungent agent in red peppers (paprika). Capsaicin applied to the skin or mucous membranes elicits a burning sensation and/or pain. Neonatal systemic administration of capsaicin results in a selective degeneration of about 50 per cent of sensory neurons which belong to the population of small dark type B neurons (Jancsó, Kiraly and Jancsó-Gábor, 1977). The selective neurotoxic action of capsaicin and related vanilloid compounds has been exploited in different experimental paradigms, e.g. perineural capsaicin treatment, to defunctionalize nociceptive afferent neurons (Jancsó and Jancsóné Gábor, 1949; Jancsó and Sántha, 2015). This population of sensory neurons is known as chemosensitive or capsaicin-sensitive primary sensory neurons. The molecular mode of action of vanilloids was revealed only almost 50 years after the discovery of the unique pharmacological actions of capsaicin. It has been demonstrated that capsaicin activates a cation channel which has been termed the capsaicin receptor or the transient receptor potential vanilloid subtype 1 receptor (TRPV1) or simply the capsaicin receptor (Bevan and Szolcsányi, 1990; Caterina *et al.*, 1997; Kilo *et al.*, 1997; Jancsó, Oszlács and Sántha, 2011). These particular population of afferent neurons express a variety of neuropeptides including SP, neurokinin A (NKA), CGRP (Kilo *et al.*, 1997), vasoactive intestinal polypeptide (VIP) (Wallengren, Ekman and Sundler, 1987), SOM (Johansson and Vaalasti, 1987) or galanin (Ju *et al.*, 1987). This class of afferent neurons is involved in the mediation of local tissue reactions including cutaneous vascular changes such as antidromic vasodilation and neurogenic plasma protein extravasation (Jancsó *et al.*, 1987, 2009; Maggi *et al.*, 1987; Maggi and Meli, 1988; Koltzenburg, Häbler and Jänig, 1995; Holzer, 1998; Domoki *et al.*, 2003; Geppetti *et al.*, 2008).

1.2 TRP channels

Chemosensitive primary sensory neurons express various types of transient receptor potential (TRP) ion channels. These channels are fundamentally involved in the sensation of pain induced by chemical and thermal stimuli and are molecular integrators of harmful stimuli of different origin (Caterina *et al.*, 1997; Nagy *et al.*, 2009; Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013). TRP channels are non-selective cation channels that are highly permeable

to Ca^{2+} ions (Handwerker, 2006; Nagy *et al.*, 2009; Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013; Zhang *et al.*, 2023). Generally, in the TRP family the cation-selective ion channel consists of 6 transmembrane subunits (Nagy *et al.*, 2004, 2009; Handwerker, 2006; Liu *et al.*, 2006; Jancsó, Oszlács and Sántha, 2011), and 4 TRP molecule builds up a functional TRP ion channel (Nagy *et al.*, 2009). Acute activation of these channels depolarizes the cell membrane and causes an increase in intracellular Ca^{2+} concentration. This plays an important role in the activation of nociceptive nerve endings, the regulation of nociceptive threshold (sensitization/desensitization) by activating second messenger systems, the release of neuropeptides from the activated nerve terminals and, in certain cases, is also involved in degeneration of nerve endings. The significance of the discovery of the capsaicin-receptor TRPV1 channel and the related "sensory" TRP channels is also clearly demonstrated by the fact, that in 2021 the Noble prize in Physiology or Medicine was awarded to Professor David Julius, the leading person of TRP research.

For the present study, two types of TRP channels, the TRPV1 and TRPA1 have major importance and their characteristics will be dealt with in some detail relevant to the subject matter of this Thesis.

Some characteristics of the TRPV1 and TRPA1 receptors

Approximately 55% of the primary sensory neurons express the TRPV1 protein synthesized in the perikaryon (Caterina *et al.*, 1997; Nagy *et al.*, 2009), and transported by axoplasmic transport to the central and peripheral terminals (Carlton and Coggeshall, 2001; Nagy *et al.*, 2009). The TRPV1 channel has been detected in the plasma membrane, endoplasmic reticulum membrane (Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013) and cytoplasmic vesicles (Nagy *et al.*, 2004). Many organs and tissues are innervated by TRPV1-immunopositive fibers such as the skin, meninges(Dux *et al.*, 2020), airways, urinary tract, pancreas (Nagy *et al.*, 2009; Pecze, Blum and Schwaller, 2013), liver and smooth muscle (Pecze, Blum and Schwaller, 2013). Expression of TRPV1 was also reported in the brain (Nagy *et al.*, 2004, 2009; Cavanaugh *et al.*, 2011; Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013) and in various non-neuronal cells such as urothelial cells, T-lymphocytes and vascular smooth muscle cells (Nagy *et al.*, 2009; Cavanaugh *et al.*, 2011; Jancsó, Oszlács and

Sántha, 2011; Phan *et al.*, 2020). The TRPV1 receptor is polymodal, i.e., it can be activated by multiple chemical and physical factors (Caterina *et al.*, 1997, 1999; Woolf and Ma, 2007; Jancsó, Oszlács and Sántha, 2011). Activating stimuli are high temperature (>45°C) (Nagy *et al.*, 2004; Handwerker, 2006; Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013), acidic pH (Gunthorpe *et al.*, 2002; Nagy *et al.*, 2004; Handwerker, 2006; Pecze, Blum and Schwaller, 2013), and different exo- and endovanilloids such as capsaicin (Gunthorpe *et al.*, 2002; Nagy *et al.*, 2004; Handwerker, 2006; Jancsó, Oszlács and Sántha, 2011; Pecze, Blum and Schwaller, 2013), resiniferatoxin (Gunthorpe *et al.*, 2002; Nagy *et al.*, 2004; Pecze, Blum and Schwaller, 2013), and anandamide (Zygmunt *et al.*, 1999; Gunthorpe *et al.*, 2002; Nagy *et al.*, 2004; Woolf and Ma, 2007; Pecze, Blum and Schwaller, 2013). The function of the TRPV1 receptor is modulated by diverse intracellular regulatory mechanisms which change the sensitivity of the receptor to heat and chemical stimuli (Nagy *et al.*, 2004) causing sensitization or even desensitization of the receptor. A notable competitive antagonists of TRPV1 is capsazepine that blocks the functionality of the channel (Bevan *et al.*, 1992).

The transient receptor potential ankyrin type 1 (TRPA1) ion channel is characterized by ankyrin repeat domains located on the N-terminal. In mammals, TRPA1 is expressed in a subpopulation of TRPV1-positive peptidergic neurons (Guimaraes and Jordt, 2007). Approximately 35% of spinal ganglion neurons express the TRPA1 receptor(Jordt *et al.*, 2004). Although TRPA1 is considered to be a member of thermosensitive TRP channels, its thermal (cold) sensitivity has been disputed (Zhang *et al.*, 2022). A pivotal feature of this receptor is its sensitivity to various electrophilic chemical compounds, mostly of irritant nature. Therefore, it is regarded as a "promiscuous" sensory receptor sensitive to endogenous irritant and inflammatory agents and environmental pollutants and irritants. Under experimental conditions TRPA1 can be activated by mustard oil (allyl-isothiocyanate), allicin, acrolein and nitroxyl (HNO). These electrophilic activators form covalent bounds with the amino acid residues of the channel and activate the gating mechanism to allow the entry of cations, including Ca^{2+}. Mustard oil is the most commonly used activator of this channel. Application of mustard oil onto the skin induces acute pain and neurogenic inflammation (Bruce, 1913; Jancsó, Jancsó-Gábor and Szolcsányi, 1967; Bánvölgyi *et al.*, 2004). Mustard oil-induced neurogenic inflammation and capsaicin-induced neurogenic inflammation share similar characteristics; however, these two processes are mediated through different ion channels. An important difference is that while repeated capsaicin treatment inactivates the chemosensitive nerve endings (capsaicin desensitization), similar desensitization is not observed in the case of mustard oil (Jancsó, 1968).

1.3 Cutaneous vascular reactions mediated by sensory nerves

1.3.1 Innervation of skin and cutaneous blood vessels

The mammalian skin is the largest sensory organ that detects mechanical, thermal, chemical and noxious stimuli affecting the skin. The innervation of the skin can be demonstrated by immunohistochemistry using antibodies against panneural markers like protein gene product 9.5 (PGP9.5, ubiquitin carboxy-terminal hydrolase) or class III ß-tubulin (Oaklander and Siegel, 2005; Boros *et al.*, 2016). By making use of the selective neurotoxic effect of perineural capsaicin treatment, it was demonstrated that more than 90% of intraepidermal nerve endings are capsaicin-sensitive small-diameter unmyelinated C-fibers (Dux *et al.*, 1999; Nolano *et al.*, 1999; Oaklander and Siegel, 2005). Intraepidermal sensory axons may be classified into functionally distinct types of peptidergic and non-peptidergic afferents (Grelik, Allard and Ribeiro-da-Silva, 2005; Oaklander and Siegel, 2005; Bailey and Ribeiro-da-Silva, 2006; Woolf and Ma, 2007). Peptidergic (e.g., CGRP- and SP-containing) and non-peptidergic (e.g., IB4-positive and P2X3-expressing) fibers convey their information into distinct layers of the dorsal horn of the spinal cord (Oaklander and Siegel, 2005; Duraku *et al.*, 2013). Peptidergic fibers primarily terminate in Rexed's laminae I and outer part of laminae II, while non-peptidergic fibers terminate in the inner part of laminae II (Djouhri and Lawson, 2004; Bailey and Ribeiro-da-Silva, 2006). It is suggested that this segregation allows the central nervous system to specifically detect the stimulation of the epidermis in parallel with the identification of the depth of the origin of the stimulus (Oaklander and Siegel, 2005). Indeed, in the skin, a distinct laminar arrangement of afferent axons can also be observed. Non-peptidergic afferents terminate in the stratum granulosum, while peptidergic fibers innervate the deeper stratum spinosum (Dux *et al.*, 1999; Oaklander and Siegel, 2005; Duraku *et al.*, 2013).

In the dermis, arteries and veins are innervated by perivascular nerves of sensory and autonomic origin containing SP, CGRP, NKA, VIP, SOM as well as noradrenalin and neuropeptide-Y [NPY], respectively. The sensory neuropeptides SP and CGRP are often co-localized in both epidermal and perivascular nerve fibers. Sympathetic postganglionic axons arise from paravertebral ganglia and contain noradrenalin that causes vasoconstriction and confers sympathetic tone on cutaneous blood vessels acting via adrenergic receptors (Ruch *et al.*, 2003; Deng *et al.*, 2016). Sympathetic fibers are active even at rest, however, their firing frequency is

low (Rowell, 1977; Willette, Hieble and Sauermelch, 1991; Aalkjær, Nilsson and De Mey, 2021). Activation of thermoregulatory and cardiovascular regulatory mechanisms or general activation of the sympatho-adrenal system may cause maximal contraction of cutaneous blood vessels (Yu and Blessing, 1997; Ootsuka and Tanaka, 2015). Cutaneous blood flow is also affected by local humoral factors involving endothelium derived NO and endothelin and, under inflammatory conditions, inflammatory mediators such as histamine, bradykinin and prostaglandins, among others (Oaklander and Siegel, 2005; Gibbons and Freeman, 2012).

1.4 Neurogenic inflammation

Stricker observed in 1876 that stimulation of the distal stump of transected lumbar spinal dorsal roots resulted in prompt vasodilation in the innervated ipsilateral lower limb as measured by an increase in hind paw temperature. The phenomenon of sensory nerve mediated vasodilation has been confirmed later by Bayliss (Bayliss, 1901). Bruce's elegant classic experiments demonstrated the existence of irritant-induced sensory nerve mediated axon reflexes (1913). Based on these early observations Lewis proposed the axon reflex mechanism to explain the contribution of cutaneous nociceptive nerves to the development of the flare component of the famous "triple response" of the skin evoked by noxious stimulation (Lewis, Harris and Grant, 1927).

Further evidence for the role of sensory nerves in cutaneous vascular reactions was furnished by experiments on neurogenic inflammation. In the rodent skin and conjunctiva, direct chemical or antidromic electrical stimulation of peripheral sensory nerves elicits arteriolar vasodilation (Allnatt, Dickson and Lisney, 1990; Doubleday and Robinson, 1992; Jancsó, 1992; Domoki *et al.*, 2003; Lynn, 2009) and enhanced microvascular permeability resulting in plasma protein extravasation in postcapillary venules of 7–20 μm in diameter (Jänig and Lisney, 1989; Allnatt, Dickson and Lisney, 1990; Bharali and Lisney, 1992; Doubleday and Robinson, 1992; Jancsó, 1992; Domoki *et al.*, 2003). The pioneering observations of Miklós Jancsó furnished evidence for the sensory neurogenic origin of these reactions, since they were abolished after chronic denervation of the skin (Jancsó N., 1960; G. Jancsó, Király and Jancsó-Gábor, 1980; Jancsó and Kiraly, 1983; Pertovaara, 1988) and by capsaicin treatment (Jancsó N., 1960; Jancsó, 1968, 1992; Jancsó, Kiraly and Jancsó-Gábor, 1977; Gonzales *et al.*, 1991). Jancsó assumed that these

vascular reactions are mediated by an unidentified mediator substance, a "neurohumor", which is released from the stimulated sensory nerve terminals.

The identity of the substance(s) mediating these vascular responses was revealed only about a century later by Lembeck's group in Graz. Experiments in rats disclosed that antidromic vasodilatation and plasma extravasation could not be elicited in rats treated neonatally with capsaicin (Lembeck and Holzer, 1979; Gamse, Holzer and Lembeck, 1980), a treatment which eliminates peptidergic primary sensory neurons (Jancsó, Kiraly and Jancsó-Gábor, 1977). It was concluded that antidromic vasodilatation is mediated by capsaicin-sensitive (chemosensitive) C-fiber sensory nerves releasing SP upon stimulation (Lembeck and Holzer, 1979; Gamse, Holzer and Lembeck, 1980). Further studies disclosed that the major mediator of this response is another sensory neuropeptide, CGRP, which is also depleted by capsaicin treatment (Brain *et al.*, 1986; Gamse *et al.*, 1987). CGRP (Brain *et al.*, 1986) released from sensory nerves causes relaxation of smooth muscle of precapillary arterioles through a NO-independent mechanism (Ralevic *et al.*, 1992; Handwerker, 2006; Scott and Scott, 2009). It is worth noting that apart from vascular reactions, peptidergic capsaicin-sensitive afferents are also involved in other local regulatory functions including immune, trophic, proliferative and pro-inflammatory effects and contraction of smooth muscles (Roosterman *et al.*, 2006; Jancsó *et al.*, 2009).

1.5 The neurogenic inflammatory response as a tool for the study of cutaneous sensory innervation

The vascular labeling technique allows the direct visualization of the blood vessels showing increased vascular permeability induced by inflammatory mediators of non-neural or neural origin. This method was introduced by Miklós Jancsó to study histamine-induced inflammatory reactions (Jancsó, 1947). This approach made possible the demonstration of the innervated and the denervated regions of the skin after application of irritant agents which produce increased vascular permeability through the (sensory) neurogenic route, such as capsaicin and mustard oil (Dux *et al.*, 1999). Further studies disclosed a close association of colloidal silver labeled (permeable) venules with SP-containing sensory nerves (Jancsó, 1992). Besides the examination of the relationship between nerves and blood vessels, the vascular labeling technique is suitable for the morphofunctional characterization of the innervation territories of

capsaicin-sensitive nociceptive peptidergic afferents (Dux and Jancsó, 1994). This method offered a possibility for the direct, simultaneous and quantitative determination of the individual and the overlapping innervation territories of peripheral nerves in the rat hind paw. This was the first method allowing the direct examination of overlapping cutaneous innervation areas (Dux and Jancsó, 1994). Earlier, an indirect method was applied to determine the extent of overlapping innervation areas of peripheral nerves by comparing innervation territories of individual nerves on the ipsi- and contralateral sides (Wiesenfeld-Hallin, Kinnman and Aldskogius, 1988; Dux and Jancsó, 1994).

Another technique to visualize the innervation territories of chemosensitive C-fiber afferents is the Evans-blue extravasation technique (Jancsó, Kiraly and Jancsó-Gábor, 1977; G. Jancsó, Király and Jancsó-Gábor, 1980; Pertovaara, 1988; Wiesenfeld-Hallin, Kinnman and Aldskogius, 1988; Allnatt, Dickson and Lisney, 1990; Bharali and Lisney, 1992; Dux and Jancsó, 1994). This method may be also applied to examine the regenerative properties of these afferent nerves. The concept of this method is similar to the vascular labeling technique. The intravenously injected Evans-blue dye binds to serum albumin and leaves the blood vessels at sites of increased vascular permeability inducing a blue coloration of the (innervated) skin (Boros *et al.*, 2016). In rodents the vascular permeability increase is mediated by tachykinins, most prominently by SP, released from the activated peptidergic afferents (Lembeck and Holzer, 1979; Jancsó and Kiraly, 1983; Wiesenfeld-Hallin, Kinnman and Aldskogius, 1988; Allnatt, Dickson and Lisney, 1990; Bharali and Lisney, 1992; Dux and Jancsó, 1994).

1.5.1 Perineural capsaicin treatment

Perineural application of capsaicin is a special form of local capsaicin treatment by applying capsaicin directly onto peripheral nerves. (Jancsó and Király, 1980; Jancsó and Lawson, 1990; Jancsó, Oszlács and Sántha, 2011). At a concentration of 32 mM (1%), capsaicin leads to a selective and permanent (practically irreversible) functional blockade of C-fiber afferents running in that nerve (Jancsó and Király, 1980; Fitzgerald and Woolf, 1982; Baranowski, Lynn and Pini, 1986; Jancsó and Lawson, 1990; H Sann *et al.*, 1995; Domoki *et al.*, 2003; Jancsó, Oszlács and Sántha, 2011). It is worth to mention, that apart from capsaicin, perineural administration of other TRPV1 agonists, such as the ultrapotent capsaicin analogue

resiniferatoxin can also elicit similar regionally selective effects (Kissin, Bright and Bradley Jr., 2002).

Morphological consequences of perineural capsaicin treatment

Shortly after the capsaicin treatment the acute morphological changes are less marked: in the treated nerve swollen C-fibers are present and cell organelles are accumulated in the axons (Jancsó *et al.*, 1985; Jancsó, 1992; Jancsó, Oszlács and Sántha, 2011). However, profound alteration has been observed regarding the relationship between the C-fibers and glia (Schwann) cells 2–3 weeks following the treatment: C-fibers that normally are embedded into the cytoplasm of Schwann cells lose their tight connection with the glial cells, become unwrapped and are closely packed together with other bare C-fibers (Jancsó, 1992; Jancsó, Oszlács and Sántha, 2011). In contrast, there was no sign of damage in the structure of myelinated axons or the myelin structure confirming the selective effect of capsaicin on unmyelinated afferent axons. No immediate degeneration of peripheral chemosensitive axons is induced by perineural capsaicin treatment. However, 2–3 months after the treatment, a marked decrease in the number of C-fibers has been observed suggesting a delayed, dying back type of degeneration of capsaicin-sensitive axons (Jancsó and Lawson, 1990; Pini, Baranowski and Lynn, 1990; Jancsó, 1992; Jancsó, Oszlács and Sántha, 2011).

Following perineural capsaicin treatment of nerves supplying a defined area of the skin, the intraepidermal nerve fiber count is drastically (by 90%) reduced in the affected skin area as shown by quantitative immunohistochemical data (Jancsó, 1992; Dux *et al.*, 1999). Since the nerve fiber density was largely unaffected in the dermis which is innervated by mixed populations of sensory and autonomic efferent nerves, this observation also indicates that perineural capsaicin treatment induces a selective and robust destruction of cutaneous nociceptive C-fiber afferents. The number of intraepidermal axons remained reduced for at least 3 months after treatment suggesting a permanent and probably irreversible degeneration of nerve fibers (Jancsó, 1992; Dux *et al.*, 1999).

Functional consequences of perineural capsaicin treatment

Perineural capsaicin treatment causes profound but selective impairment of the exposed chemosensitive afferent nerves affecting both the sensory afferent and efferent functions (Jancsó and Király, 1980; Jancsó, 1992; Dux *et al.*, 1999). Three stages may be distinguished regarding the changes in C-fiber function. During the first stage capsaicin induces the activation

of C-fibers and causes antidromic neurogenic vasodilation and extravasation. In the second phase, conduction of nerve impulses by C-fibers is decreasing and eventually become blocked. The final phase is characterized by the loss of C-fiber functions distal to the site of capsaicin application. The defunctionalization and partial degeneration of the capsaicin-sensitive afferent C-fibers leads to an increase in chemical and thermal nociceptive thresholds (Jancsó and Király, 1980; Jancsó, 1992) and eventually chemical and thermal analgesia (Jancsó and Király, 1980; H Sann *et al.*, 1995; Jancsó, Oszlács and Sántha, 2011; Szigeti *et al.*, 2012) , and the complete loss of the neurogenic inflammatory response (Jancsó and Király, 1980; Jancsó and Lawson, 1990; Jancsó, 1992; H Sann *et al.*, 1995; Domoki *et al.*, 2003; Jancsó, Oszlács and Sántha, 2011; Szigeti *et al.*, 2012).

Perineural capsaicin treatment exerts a highly selective and marked effect on the neurochemical phenotype of chemosensitive primary sensory neurons (Jancsó, 1992; Jancsó, Oszlács and Sántha, 2011). A strong depletion of several neuropeptides (including SP and CGRP) has been demonstrated a few days after the treatment in parallel with the complete abolition of FRAP-activity within the dorsal root ganglia and spinal dorsal horn (Jancsó and Lawson, 1990), which may be attributed to a selective inhibition of axonal transport in the affected chemosensitive primary afferent neurons (Gamse *et al.*, 1982; Jancsó and Lawson, 1990; Jancsó, Oszlács and Sántha, 2011; Szigeti *et al.*, 2012). Intraneural transport blockage of numerous peptides and proteins, such as NGF, horseradish-peroxidase, SP, CGRP, SOM and FRAP has also been demonstrated (Jancsó, Oszlács and Sántha, 2011). An important aspect of perineural capsaicin treatment is its irreversible effects (Jancsó, 1992; Jancsó, Sántha and Gecse, 2002; Jancsó, Oszlács and Sántha, 2011). Following capsaicin-induced denervation, no signs of functional and morphological reinnervation have been observed. Interestingly, in contrast to regenerative phenomena observed after peripheral nerve transection, collateral sprouting of intact chemosensitive nerves innervating cutaneous regions adjacent to the chemodenervated skin has not been detected either (G. Jancsó, Király and Jancsó-Gábor, 1980; Pertovaara, 1988; Jancsó, Oszlács and Sántha, 2011). Importantly, the integrity of motor, autonomic and myelinated afferent nerves is fully preserved after perineural treatment with capsaicin (Jancsó *et al.*, 1987).

1.5.2 Consequences of peripheral nerve transection

Damage to peripheral nerves leads to denervation, i.e., abolition of motor, sensory and autonomic functions in tissues served by the affected nerve (Navarro, Vivó and Valero-Cabré,

2007; Allodi, Udina and Navarro, 2012). Interruption of the peripheral nerve induces characteristic changes at the distal and proximal ends of the damaged axon, as well as in its perikaryon (Aldskogius, Arvidsson and Grant, 1985; Navarro, Vivó and Valero-Cabré, 2007). Following complete nerve transection (neurotmesis according to Seddon's classification (Seddon, 1942)), nerve segments distal to the lesion undergo Wallerian degeneration (Aldskogius, Arvidsson and Grant, 1985; Bradbury, McMahon and Ramer, 2000; Navarro, Vivó and Valero-Cabré, 2007; Allodi, Udina and Navarro, 2012). As a result of this degenerative process, distal segments of the injured axons disintegrate. Schwann cells, as well as other cell types and the extracellular matrix and basement membrane constituting the scaffold of the peripheral nerve are retained and provide a cellular milieu which supports the potential regrowth of regenerating axonal processes (Jessen and Mirsky, 2016; Balakrishnan *et al.*, 2020; Rao *et al.*, 2022). Simultaneously, chromatolysis and retrograde reactions take place in the perikarya of the axotomized neurons associated with molecular and cellular changes (Navarro, Vivó and Valero-Cabré, 2007; Allodi, Udina and Navarro, 2012). First signs of degeneration appear within 24 hours of the nerve injury and continue for approximately 1–2 weeks progressing towards the distal axonal segments (Navarro, Vivó and Valero-Cabré, 2007).

Wallerian degeneration of the distal axonal segments and the subsequent reactive changes in the denervated nerve stump provide a microenvironment that facilitates axonal growth and regeneration. Regeneration of injured axons may result in partial or even complete reinnervation of the denervated skin and restoration of function (Navarro, Vivó and Valero-Cabré, 2007; Allodi, Udina and Navarro, 2012).

1.6 Possible mechanisms of reinnervation of the denervated skin area

Two basic neuronal mechanisms have been described contributing to the reinnervation of the denervated skin: reinnervation of the denervated skin by regeneration of the injured axons (Jancsó and Kiraly, 1983; Kinnman and Aldskogius, 1986; Doubleday and Robinson, 1992; Harper, Buchman and Owen, 1999; Navarro, Vivó and Valero-Cabré, 2007; Cobianchi, de Cruz and Navarro, 2014), and collateral reinnervation by intact axons of adjacent skin areas (Weddell, Guttmann and Gutmann, 1941; Livingston, 1947; Devor *et al.*, 1979; Jancsó and Kiraly, 1983; Kinnman and Aldskogius, 1986; Kinnman, 1987; Kuchel and Zigmond, 1991; Doubleday and Robinson, 1992; Harper, Buchman and Owen, 1999; Navarro, Vivó and Valero-

Cabré, 2007; Cobianchi, de Cruz and Navarro, 2014). If reinnervation cannot be accomplished by regeneration of the injured nerve, collateral sprouting of intact nerve fibers innervating adjacent skin areas may restore sensory functions in the denervated skin (Kinnman and Aldskogius, 1986; Harper, Buchman and Owen, 1999; Kovačič, Sketelj and Bajrović, 2003) as cutaneous sensory nerves are capable to invade skin regions outside their own innervation territories through sending collateral sprouts into the denervated skin (Kinnman, 1987).

Under physiological conditions, in intact nerves non-neuronal cells synthesize and secrete several trophic factors required for maintaining homeostatic environment for the neurons (Allodi, Udina and Navarro, 2012). Nerve injury is followed by the overexpression of such neurotrophic factors to promote axon regeneration and reinnervation of the target organ (Navarro, Vivó and Valero-Cabré, 2007; Allodi, Udina and Navarro, 2012). Such factors that enhance axonal regeneration may include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and insulin-like growth factors (IGF-I, IGF-II) (Navarro, Vivó and Valero-Cabré, 2007). It has been revealed that NGF is required for collateral sprouting of nociceptive and sympathetic fibers into the denervated skin (Kuchel and Zigmond, 1991; Doubleday and Robinson, 1992; Mearow and Kril, 1995; Harper, Buchman and Owen, 1999; Kovačič *et al.*, 2010; Allodi, Udina and Navarro, 2012) probably by acting as an initiating stimulus for axonal sprouting and elongation (Doubleday and Robinson, 1992; Harper, Buchman and Owen, 1999; Cobianchi, de Cruz and Navarro, 2014). Neutralizing endogenous NGF with anti-NGF antibody inhibits the collateral ingrowth of sensory nerves (Diamond, Holmes and Coughlin, 1992; Mearow and Kril, 1995; Harper, Buchman and Owen, 1999), while it does not have significant effect on the regeneration of the injured nerves (Mearow and Kril, 1995; Harper, Buchman and Owen, 1999). This suggests that different mechanisms are responsible for the regenerative processes and the collateral reinnervation.

Under favorable circumstances axonal regeneration and target reinnervation are attained following axotomy of peripheral nerves (Bradbury, McMahon and Ramer, 2000). Within 1–2 days, local factors, possibly others than NGF, initiate sprouting of the damaged axons proximal to the lesion where growth cones are formed and started to elongate. When the regenerating axons reach the distal stump, their growth is guided through the endoneurial tube together with Schwann-cells and the basal lamina (bands of Bügner) forming a regenerative unit (Navarro, Vivó and Valero-Cabré, 2007). The speed of axon regeneration is initially quite slow and it reaches a constant rate (2–3 mm/day) within 3–4 days after the injury (Bradbury, McMahon and Ramer, 2000; Navarro, Vivó and Valero-Cabré, 2007). Even under suboptimal

conditions (e.g. in case of complete transection of a nerve where the basal lamina is also damaged) several nerve fibers are able to reinnervate their respective peripheral target organ, however, this innervation is frequently topographically inadequate (Bradbury, McMahon and Ramer, 2000).

1.7 Functional and morphological examination of sensory reinnervation of previously denervated skin areas

In animal experiments various methods may be utilized to study the sensory deficit caused by denervation of skin areas and the subsequent recovery of sensory innervation of the denervated skin. These strategies include the detection of alterations in the thresholds to nociceptive mechanical and thermal stimuli (sensory testing based on the nociceptive behavioral response of animals) or taking skin biopsy for histological examinations (Nolano *et al.*, 1999; Kennedy *et al.*, 2010; Cobianchi, de Cruz and Navarro, 2014). However, these techniques are unsuitable to exactly outline the innervated and the denervated skin regions. Functional regeneration of chemosensitive nerves responsible for the neurogenic inflammatory response was investigated in the rat hind paw skin (Jancsó and Kiraly, 1983; Pertovaara, 1988). Initially, the ability of cutaneous sensory nerves to reinnervate target organs was studied by using histological techniques. In these studies, vital staining of nerve fibers with methylene blue dye (Weddell, Guttmann and Gutmann, 1941) and silver and gold impregnation techniques were used, although these methods were not specific enough for sensory axons. Later, anterograde labeling with horse radish peroxidase conjugated with wheat germ agglutinin (WGA-HRP) was utilized (Kinnman and Aldskogius, 1986). In these experiments it was shown that collaterals of intact sensory axons may sprout into the partially or completely denervated skin areas (Kinnman, 1987).

More recently, demonstration of the neurogenic inflammatory reaction was found to be more suitable to examine the extent of cutaneous sensory denervation. After nerve transection, crushing or perineural capsaicin treatment, neurogenic sensory vasodilation and plasma extravasation are completely abolished in the skin territory served by the affected nerve. In rats, spatial and temporal aspects of functional reinnervation following nerve crush and perineural capsaicin treatment were compared using the Evans blue extravasation technique that is suitable to demonstrate skin areas of increased vascular permeability induced by antidromic electrical

or direct chemical stimuli affecting nociceptive nerve endings (Jancsó and Kiraly, 1983; Bharali and Lisney, 1988; Brenan, Jones and Owain, 1988; Lisney, 1989). Although this method may exactly delineate the innervated skin areas, its use is limited in longitudinal studies.

1.7.1 Scanning laser Doppler perfusion imaging for study of neurogenic cutaneous vasodilation

A reliable method to demonstrate sensory neurogenic vasodilation in the skin is the measurement of cutaneous blood flow with laser Doppler flowmetry (Lynn, Schütterle and Pierau, 1996; Gee, Lynn and Cotsell, 1997; Lakatos *et al.*, 2020). Scanning laser Doppler imaging is a widely used technique to measure skin blood flow under various conditions involving both physiological and pathophysiological conditions. Laser Doppler flowmetry directly measures cutaneous blood perfusion in a non-invasive manner by detecting frequency (Doppler) shift induced by the scattering of the low-power monochromatic laser light by moving erythrocytes but not stationary tissue elements with a consequence of frequency-broadened laser light (Fredriksson, Larsson and Strömberg, 2009; Rajan *et al.*, 2009; Daly and Leahy, 2013; Allen and Howell, 2014). The intensity of shifted reflected light depends on the local concentration of moving erythrocytes, whereas the mean change in frequency is proportional to the average velocity of the moving red blood cells (Fredriksson, Larsson and Strömberg, 2009; Rajan *et al.*, 2009). Generally, an integrated value is calculated reflecting both the changes in red blood cell concentration and velocity, and the perfusion of the area of interest is expressed as an arbitrary perfusion unit (Rajan *et al.*, 2009; Daly and Leahy, 2013). The laser Doppler perfusion imager (LDI) is able to measure perfusion from a larger area by scanning the laser beam over the targeted area using scanning mirrors and computerized control and processing system. The tissue thickness that can be sampled by the laser light is approximately 1 mm (Fredriksson, Larsson and Strömberg, 2009). The perfusion map registered reflects skin areas with different blood perfusion intensities. Accordingly, a single scanned image reflects the momentary local differences in the microcirculation of the skin. Several factors influence the spatial resolution of the perfusion imaging with the smallest resolvable distance of approximately 0.1 mm (Rajan *et al.*, 2009; Allen and Howell, 2014). Sampling and analysis of time-series on the same preset skin area however is suitable to follow up even rapid (in the 10–100 s range) perfusion changes due to local or systemic neurovascular

reactions (Deng *et al.*, 2016). Since image series contain both temporal and spatial information on the cutaneous blood flow, LDI may also be utilized to demonstrate the topographical distribution of the changes in skin perfusion. In several earlier studies LDI has been utilized to characterize cutaneous vascular reactions elicited by direct electrical antidromic (Lynn, Faulstroh and Pierau, 1995) or orthodromic chemical stimulation (Lynn, Schütterle and Pierau, 1996; Sántha, Pierau and Jancsó, 1998; Van der Schueren *et al.*, 2007) of cutaneous sensory nerves (Illigens *et al.*, 2013).

In this study we have assessed the intensity and spatial distribution of mustard oil-induced changes in skin blood flow by using scanning laser Doppler imaging. As a TRPA1 receptor agonist, mustard oil activates intact nociceptive nerves leading to neurogenic vascular reactions through the release of the neuropeptides CGRP and SP from the sensory nerve endings. The cutaneous areas displaying sensory stimulation-induced neurogenic vasodilation exactly correlate with the innervation territory of the stimulated nerve. Based on these previous findings, the topographical localization of mustard oil-induced vasodilation by laser Doppler imaging technique can be a suitable method to assess the pattern of cutaneous innervation and also the functional integrity of the corresponding nerves. Our aim was to evaluate the regenerative propensity following transection and perineural capsaicin treatment of nerves serving the dorsal skin of the rat hind paw through longitudinal examination of changes in the topography and intensity of mustard oil-induced sensory neurogenic vasodilatation in the rat. In addition, the vascular labeling technique, utilizing colloidal silver, was also used to detect, by visual inspection and under the light microscope, the innervation pattern of the dorsal hind paw skin after denervation and in the course of nerve regeneration.

1.8 Aims

The overall aim of this study was to examine the mechanisms of functional reinnervation of the denervated skin by using novel techniques utilizing scanning laser Doppler imaging and the vascular labeling method which enable the longitudinal observation of the reinnervation process. The specific objectives of this study were as follows:

1. To evaluate and validate a non-invasive method, scanning laser Doppler imaging for longitudinal study of the function of cutaneous peptidergic nociceptive afferent nerves.
2. To evaluate scanning laser Doppler imaging for the longitudinal study of the functional reinnervation, by nociceptive peptidergic afferent nerves, of the denervated skin.
3. To examine the functional reinnervation, by nociceptive peptidergic afferent nerves, of the denervated skin following perineural capsaicin treatment of peripheral nerves using scanning laser Doppler imaging and the vascular labeling technique.

2 MATERIALS AND METHODS

2.1 Animals

Adult male Wistar rats (n = 34) weighing 250–280 g at the beginning of the experiments were used in this study. The animals were housed under a 12-h light/dark cycle with free access to food and water. All experiments were approved by the Ethics Committee for Animal Care at the University of Szeged as per the Council Regulation of 40/2013 (II. 14.) and were carried out in full accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering. The number of experimental animals was kept as low as possible.

2.2 Surgical interventions

For all surgical interventions a combination of ketamine (Calypsol, 70 mg/kg, Gedeon Richter, Budapest, Hungary) and xylazine (CP-Xylazin 2%, 10 mg/kg, Produlab Pharma, Raamsdonksveer, Netherlands) was administered intraperitoneally to anaesthesize the animals, and the hair of the right lateral thigh was shaved.
Two sets of experiments were performed to study the consequence of surgical transection or perineural capsaicin treatment of the peripheral nerve, respectively, on the cutaneous vascular reactions in separate groups of experimental animals.

Peripheral nerve transection

To study the cutaneous collateral reinnervation after complete denervation of the skin the animals were subjected to subsequent surgeries 5 weeks apart (Fig. 1.). After the measurement of the mustard oil-induced vascular reaction (baseline perfusion map, see later), the right saphenous nerve was exposed high in the thigh under sterile conditions and the nerve was transected distal to a ligature. A 0.5 cm long segment of the distal stump was removed to prevent regenerative regrowth of the nerve. After repeated measurements of the mustard oil-induced vascular reactions a second surgery was performed on the 40th postoperative day in order to eliminate the chemosensitive afferents of the ipsilateral sciatic nerve. The right sciatic nerve was exposed high in the thigh and isolated from the surrounding tissue with parafilm. The

isolated segment of the nerve was wrapped around with a small piece of gelfoam soaked with 0.1 ml of a 1% capsaicin solution (Sigma, Saint Louis, USA) dissolved in saline containing 6% ethanol and 8% Tween 80. After 20 min the gelfoam was removed, the wound was closed and the animals were returned to the animal house.

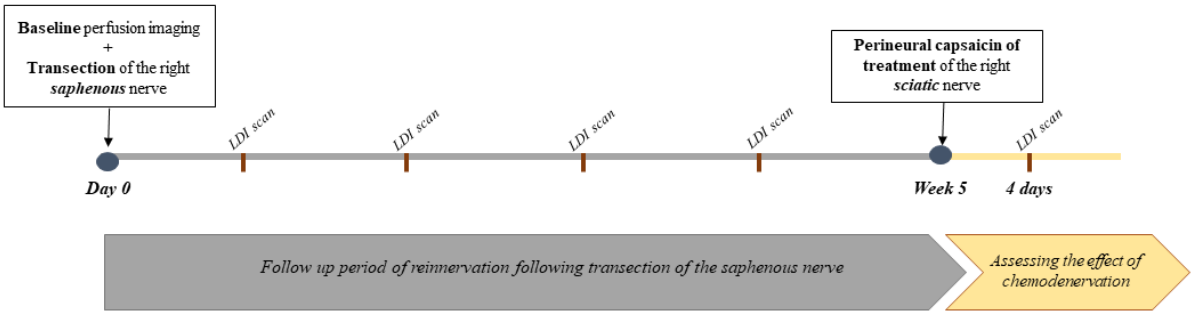

Figure 1 Timeline representing the study design for demonstration of the changes in the mustard oil induced cutaneous vascular reactions after transection of the saphenous nerve.

Perineural capsaicin treatment

To examine the functional reinnervation, by nociceptive peptidergic afferent nerves, of the denervated skin following perineural capsaicin treatment of the saphenous nerve the following experimental design was utilized (Fig. 2.). After the measurement of the mustard oil-induced vascular reactions in the intact skin (baseline perfusion map, see later), the right saphenous nerve was chemically denervated by performing perineural capsaicin treatment as described above. After repeated measurements of the mustard oil-induced vascular reactions (1^{st}, 4^{th}, 7^{th} p.o. weeks), the animals were subjected to a second surgery when their previously capsaicin treated saphenous nerve was transected distal to a ligation. After taking further LDI measurements (1^{st}, 2^{nd} and 4^{th} weeks after second surgery) the right sciatic nerve was treated perineurally with capsaicin as described above.

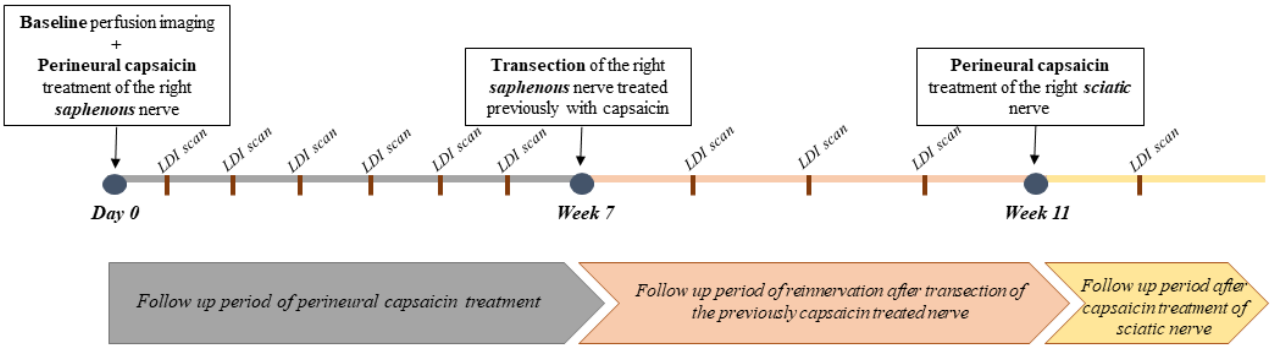

Figure 2 Timeline representing the consecutive steps within the 2nd experimental design for demonstration of the changes in the mustard oil induced cutaneous vascular reactions after perineural capsaicin treatment of the saphenous nerve.

2.3 Methods to examine the functions of cutaneous peptidergic nociceptive nerves and the progress of the reinnervation of denervated skin areas

2.3.1 Measurement of cutaneous blood flow with scanning laser Doppler flowmetry

Blood perfusion of the dorsal skin of the rat hindpaw was measured by scanning laser Doppler flowmetry. Consecutive perfusion images were captured with a PeriScan PIM3 scanning laser Doppler imager (Perimed, Järfälla, Sweden). Anaesthesized animals (ketamine, 70 mg/kg; xylazine 10 mg/kg; see before) were placed on a feed-back controlled heating pad (Biological Temperature Controler TMb5, Supertech, Hungary) to keep their body temperature relatively constant at 37±0.5 °C. Room temperature was kept at 22–23 °C. The rats were placed in a supine position, and their hindpaws were exposed to the laser beam. The dorsal surfaces of both hindpaws was scanned by using the repeated scan mode with 52 x 42 pixel frame size, the distance of the scanner aperture from the skin surface was set at 19 cm. The head of the scanner was positioned in the horizontal plane to ensure a laser beam line nearly perpendicular to the dorsal surface of the hindpaw.

Sequential perfusion image series were recorded by using repeated measurements mode, imaging frequency was set at 1 image/2 min. Imaging sessions to collect 12 perfusion images took 24 minutes: first three images were recorded to determine the baseline perfusion map and further 9 images were captured to register the perfusion changes evoked by epicutaneous mustard oil stimulation. Mustard oil (5% in liquid paraffin) was applied on the unshaved hairy skin of the paws using a cotton swab.

Basal cutaneous tissue perfusion and changes in skin blood flow induced by mustard oil were recorded in arbitrary perfusion units (PU) and expressed as per cent change relative to baseline. The value of the PU integrates the linear velocity values and the concentration of moving erythrocytes in the skin volume fraction detected by the scanner at any instances (Rajan *et al.*, 2009). Baseline values were obtained by calculating the average of three subsequent measurements before the application of mustard oil. For quantitative evaluation to determine the proportion of skin areas showing neurogenic vasodilation, images displaying the maximum vasodilatatory responses were used in each experiment. Scanning laser Doppler images were taken before surgery and 1–40 days after saphenous nerve transection or perineural capsaicin treatment. After finishing the sequential measurement of the recovery of sensory vasodilatation (4th week post surgery) an additional measurement was made 4–7 days after perineural treatment of the sciatic nerve with capsaicin or the transection of the previously capsaicin treated saphenous nerve.

2.3.2 Quantitative evaluation of the cutaneous denervation and reinnervation by using LDI perfusion images captured from stimulated skin areas

The innervation territory of the saphenous nerve was defined on the basis of perfusion images taken 4 days after transection or capsaicin treatment of the saphenous nerve. In each experiment the color-coded perfusion images showing the maximal vasodilatatory response after mustard oil application were selected for further processing with the ImagePro 6.2 image analysis software (MediaCybernetics, Rockville, Maryland, USA). To characterize the spatial extension of the reinnervation process the proportions of innervated and denervated skin areas were determined by using the color-coded perfusion images recorded at the time of maximal vasodilation. Original perfusion image files obtained at different time points of the study were converted to relative perfusion change maps by normalizing all pixel data with the 90 percentile of the recorded maximal response. Using the images captured on the 4^{th} day after saphenous nerve transection or perineural capsaicin treatment colour segmentation was applied on the relative perfusion images to demarcate and separate areas showing no or minimal vasodilation from those exhibiting large (or maximal) perfusion increases. To perform this segmentation, the 20 percentile of the pixel values collected from the lateral (intact) side was calculated to set a threshold value for each individual images. Pixels showing relative increases lower than the 20 percentile of the intact side were considered as denervated. This step was followed by the

generation of a binary mask representing the size and topography of denervated cutaneous areas corresponding to the innervation area of the saphenous nerve (Fig. 3). Functional reinnervation was characterized by measuring the intensity of the vasodilatatory response (expressed as maximal relative increase in the skin blood flow) in the saphenous skin area as defined above.

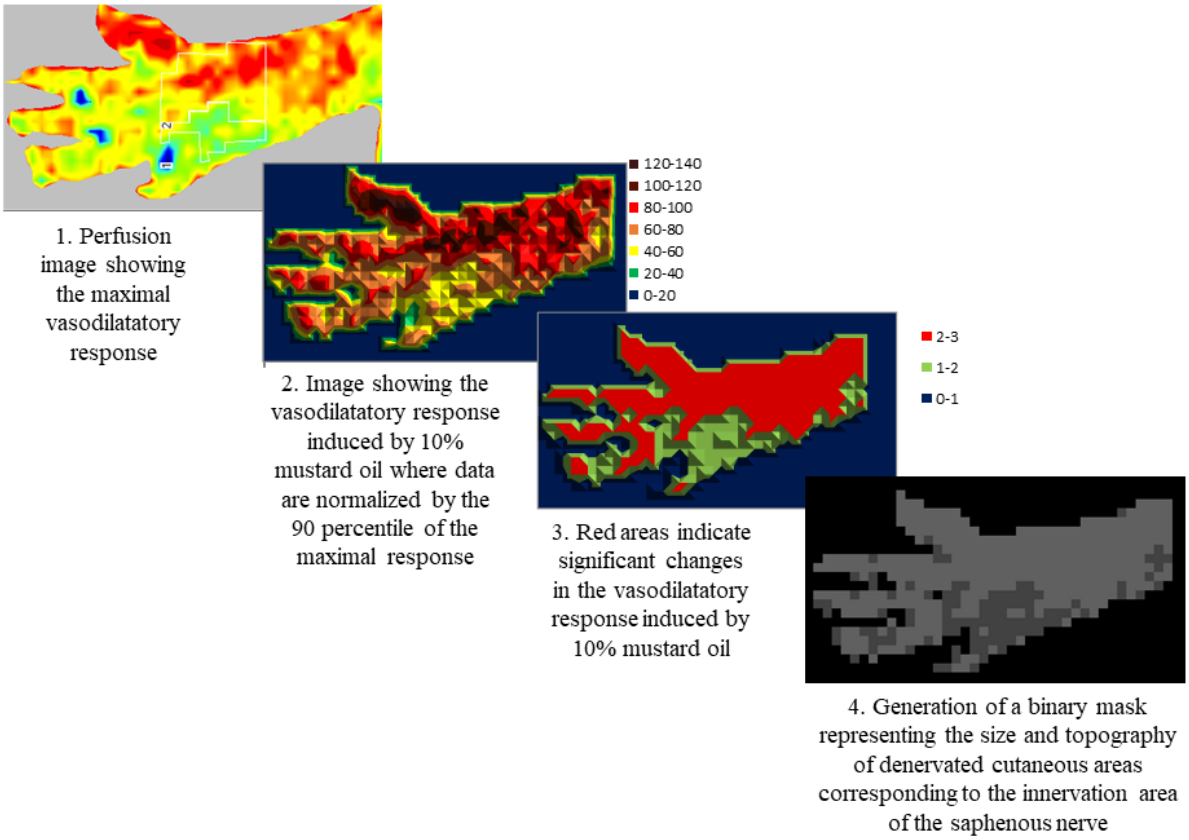

Figure 3 Images extracted from each step of the data evaluation process.

2.3.3 Immunohistochemical demonstration of the sensory innervation of the skin

The animals were deeply anaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The skin of the hind feet was excised and postfixed in the same fixative for 2 hours at 4 °C and then placed into a phosphate-buffered 30% sucrose solution for cryoprotection. Serial sections of the dorsal hindpaw skin in 20 µm thickness were prepared with a cryostat (Leica CM 1950, Leica Biosystems, Wetzlar, Germany). Sections were mounted on gelatin-coated glass slides then rinsed twice in phosphate-buffered saline (PBS) and incubated overnight with the primary antibodies with 1% Triton X100 added. Antibodies

against β-tubulin III (mouse monoclonal, 1:500, Sigma-Aldrich GmbH, St. Louis, Missouri, United States) and CGRP (rabbit polyclonal, 1:4500, Sigma-Aldrich GmbH, St. Louis, Missouri, United States) were used to label all the nerve fibers and the peptidergic fibers, respectively. After rinsing in PBS the sections were incubated for 2 hours with the secondary antibodies (donkey anti-rabbit and anti-mouse IgGs labeled with CY3 and DL488, respectively, 1:500) diluted in PBS containing 1% TritonX100. The sections were rinsed in PBS then the slides were covered with ProLong Gold Antifade Mountant with DAPI (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts, United States).
The sections were examined under a laser-scanning confocal fluorescence microscope (Zeiss LSM 700, Germany). Z-stack image series with tile scan mode were obtained.

2.3.4 Visualization of neurogenic plasma extravasation and vascular permeability changes by vascular labeling technique

Vascular labeling technique utilizing colloidal silver solution was used to obtain an accurate and detailed picture of the morphological distribution of increased vascular permeability evoked by mustard oil-induced chemical stimulation of cutaneous sensory nerves the. In anaesthetized animals 1% solution of colloidal silver (Sigma-Aldrich, Saint Louis, Missouri, USA, 50 mg/kg b.w.) was administered intravenously to the tail vein at the time of epicutaneous application of 5% mustard oil onto the dorsal surface of the hind feet. Skin areas with increased vascular permeability due to intact innervation showed brownish color while the reaction was absent in denervated skin regions. Photographs were taken for further analysis of the ratio of denervated and innervated skin areas using the Image Pro software. For long survival 2 weeks later and for short survival 30 minutes later the animals were sacrificed and were perfused transcardially with 4% paraformaldehyde. Skin of the hind paws were removed. Small sections were excised, fixed in methanol, cleared with xylene and mounted in Canada balsam for light microscopic examination. We have also performed a series of 1% colloidal silver administration on an experimental group (n = 3) for the purpose of following up the territorial changes of the two cutaneous nerves.

2.4 Statistics

Data represent means ± S.E.M. of 5–9 independent measurements. For statistical comparisons of the mustard oil-induced vasodilatatory responses one-way ANOVA test was run followed by multiple comparisons using the Dunett's post-hoc analysis or Fisher’s Least Significant Difference test. In all groups, normality was proved by the Shapiro-Wilk test and homogeneity of variances was confirmed by Levene’s test in advance of performing ANOVA. Statistical analysis was performed by using Statistica 6.4 software (Dell Inc., Tulsa, Oklahoma, U.S.A.).

3 RESULTS

3.1 Characteristics of the mustard oil-induced vascular reactions

Perfusion of the dorsal skin of the rat hind paw was essentially uniform as assessed with the scanning laser Doppler imager. The coefficients of variance of the basal perfusion values recorded on subsequent perfusion images representing the basal blood perfusion of the hind paw were 0.043 ± 0.28 (medial aspect) and 0.054 ± 0.027 (lateral aspect) with no significant difference between the two sides ($p = 0.19$). Basal tissue perfusion and changes in blood flow induced by mustard oil (5% in liquid paraffin) were recorded in arbitrary perfusion units (PU) and expressed as per cent change relative to baseline (Lakatos *et al.*, 2020) (Fig. 4). Epicutaneous application of 5% mustard oil onto the dorsal skin of the hind paws of intact rats resulted in marked immediate vasodilation in the dorsal skin of the hind paws. The vasodilatatory response peaked within 2–4 minutes (Fig. 4 B, K). Peak perfusion values varied among animals depending on the basic perfusion. Maximal increase in perfusion was 80–85% with no significant difference between the medial and lateral aspects of the dorsal hind paw skin. Skin perfusion values recovered to baseline within 15–20 minutes after the onset of vasodilatation. The results of the LDI measurements are displayed in panel A–E and K of Fig. 4 and panel A of Fig. 5.

3.2 Effect of nerve transection and capsaicin-induced selective chemodenervation on mustard oil-induced vasodilatation

To obtain reference perfusion images, in all animals subjected to denervation of the saphenous nerve, mustard oil-induced vasodilatatory responses were measured before surgery. Following nerve transection or selective chemodenervation, significant differences were not observed in basal blood flow values between the denervated (saphenous) and the intact (lateral sciatic) skin areas of the dorsal skin of the hind paw. However, 3 days after saphenous nerve transection, the magnitude of the mustard oil-induced vasodilatatory response significantly decreased in the medial (denervated) skin area, as compared with the lateral dorsal paw skin innervated by the intact sciatic nerve (Fig. 4F–J, L). A similar decrease in the perfusion of the medial but not the

lateral dorsal skin area was observed after perineural capsaicin treatment (selective chemodenervation) of the saphenous nerve (Fig. 5B, C). The time course of the vasodilatatory response in the intact lateral areas of the dorsal hind paw skin was similar to that measured in control animals. The results showed that both transection and perineural capsaicin treatment of the saphenous nerve resulted in a significant decrease in sensory neurogenic vasodilation in the medial (denervated) part of the dorsal skin of the hind paw. This finding may be explained by an impairment of the efferent vasodilatatory function of peptidergic afferent nerves serving the medial part of the dorsal skin of the rat hind paw.

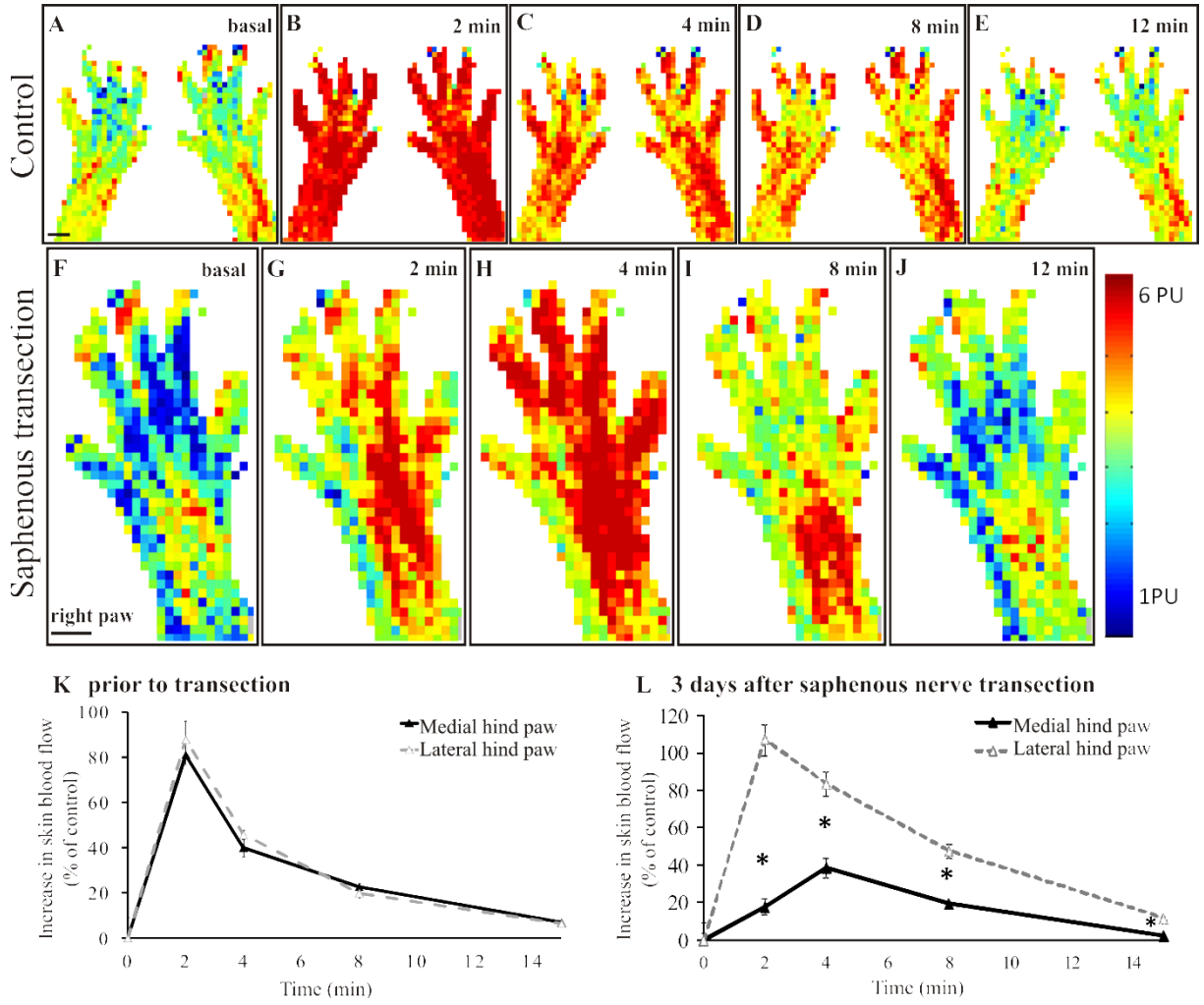

*Figure 4 Time course and spatial distribution of the vasodilatatory response elicited by the epicutaneous application of mustard oil onto the dorsal skin of the hind paws of a control rat as shown by the perfusion images (**A–E**) recorded with the scanning laser Doppler flowmeter. Increase in blood flow of similar magnitude in the medial and lateral regions of the hind paw skin has been observed. The line graph (**K**) depicts the time course of the increase in cutaneous blood flow relative to baseline value. Filled and empty triangles represent values measured in*

*the medial (saphenous innervated) and lateral (sciatic innervated) skin areas, respectively (n = 6). **F–J** represent the reduced mustard oil-induced vasodilatation in the medial aspect of the dorsal hind paw skin 3 days after transection of the saphenous nerve as shown by perfusion images recorded with the scanning laser Doppler flowmeter. The line graph (**L**) shows the time course of the vasodilatatory response in the medial (filled triangles) and lateral (empty triangles) aspects of the dorsal hind paw skin (*p < 0.05; n = 6). Scale bar on A and F represents 5 mm.*

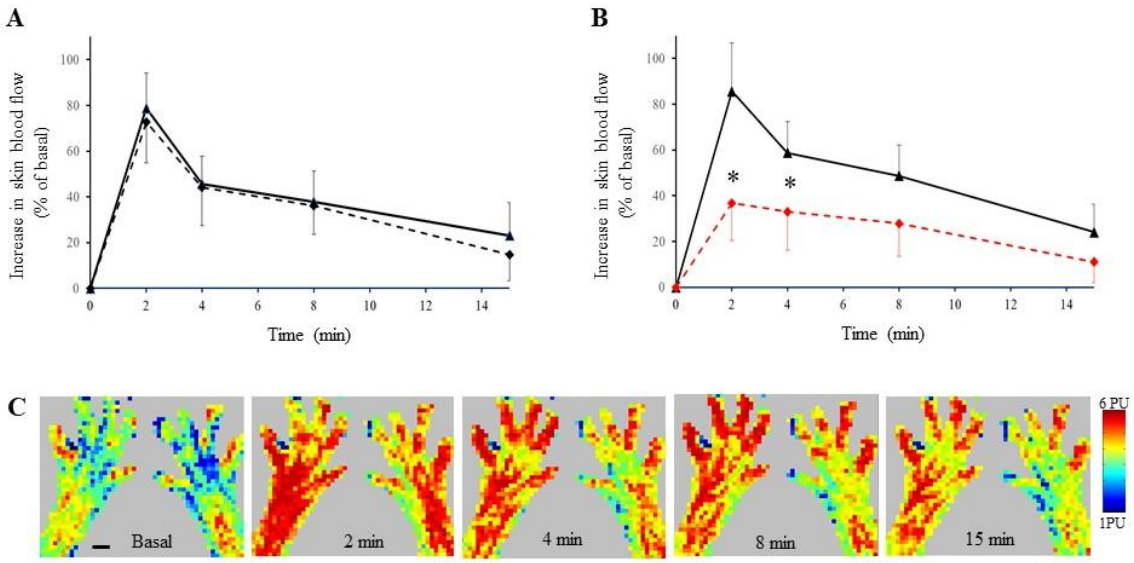

*Figure 5 (**A**) Time course of mustard oil–induced increases in perfusion of the medial (dashed line) and lateral (solid line) parts of the dorsal skin of the right hind paws of rats (n = 5). (**B**) Time course of mustard oil–induced increases in perfusion of the medial (dashed red line) and lateral (solid line) parts of the dorsal skin of the right hind paws of rats 1 week after perineural treatment of the right saphenous nerve (n = 5). *: significantly different from the perfusion of the lateral part of the dorsum of the hind paw skin. (**C**) A series of perfusion images illustrating the time course of mustard oil–induced increases in blood flow in the dorsum of a rat hind paw. The right saphenous nerve was treated with capsaicin 5 days before the experiment. Scale bar represents 5 mm.*

3.3 Reinnervation of the denervated skin following nerve transection

Following the transection of the saphenous nerve the vasodilatatory response showed a progressive and marked recovery towards control values initially in the saphenous (medial) skin area bordering the innervation territory of the (intact) sciatic nerve. The medial spreading of the

area displaying mustard oil-induced vasodilation gradually continued and about 4 weeks after saphenous nerve transection the spatial distribution of the vasodilatatory response resembled that obtained prior to denervation (Fig. 6A–E). In addition, similar time course in the increase of mustard oil-induced maximal perfusion values were observed in the medial and lateral aspects of the dorsal hind paw skin following sciatic nerve transection (Fig. 6G). These findings may be explained by an ingrowth, into the saphenous territory, of collateral nerve sprouts from intact nerve fibers of the adjacent innervation territory of the sciatic nerve. To support the contention that reinnervation of the denervated (saphenous) nerve area occurs through collateral sprouting of sciatic afferents, perineural capsaicin treatment of the sciatic nerve was performed to defunctionalize chemosensitive afferents running in that nerve. Defunctionalization of sciatic afferents resulted in the elimination of the sensory neurogenic vasodilatatory response not only in the innervation territory of the sciatic nerve but also in the medial aspect of the hind paw which is innervated by the saphenous nerve in the intact animal (Fig. 6F, H). These results strongly indicate that recovery of the vasodilatatory response, i.e. functional reinnervation of the saphenous nerve innervation territory may be attributed to collateral sprouting of sciatic afferents.

Reinnervation of the chemodenervated skin by collateral sprouting occurs only after transection of the capsaicin-treated nerve

Similar to transection of the saphenous nerve, perineural treatment with capsaicin resulted in a complete abolition of the mustard oil-induced vasodilatatory response after 3 days. However, in contrast to nerve transection, no tendency for recovery of the vasodilatatory response was observed after perineural capsaicin. Indeed, the proportion of the denervated skin area showed no significant change even after a long postoperative period of 7 weeks after perineural capsaicin (Fig. 7).

An intriguing finding of the experiments presented in this Thesis was the observation of the reinnervation of the chemodenervated skin following the transection of the capsaicin treated nerve. Evaluation of perfusion images revealed that 2–4 weeks following the transection of the previously capsaicin-treated saphenous nerve, the proportion of the denervated skin area decreased gradually. In turn, in the chemodenervated area, the proportion of the reinnervated area increased gradually and peaked 4 weeks later at around 63.04 ±7.13% (Fig. 8).

To reveal the possible mechanism of reinnervation of the (saphenous) skin, the sciatic nerve was treated perineurally with capsaicin to defunctionalize sciatic sensory afferents. This resulted in an abolition of the vasodilatatory response not only in the skin area innervated by the sciatic nerve, but also in the (reinnervated) saphenous skin (Fig. 8C). Hence, these experiments clearly indicated that reinnervation of the chemodenervated saphenous skin area was accomplished through collateral sprouting of intact sciatic afferent axons which normally do not innervate that area of the hind paw skin.

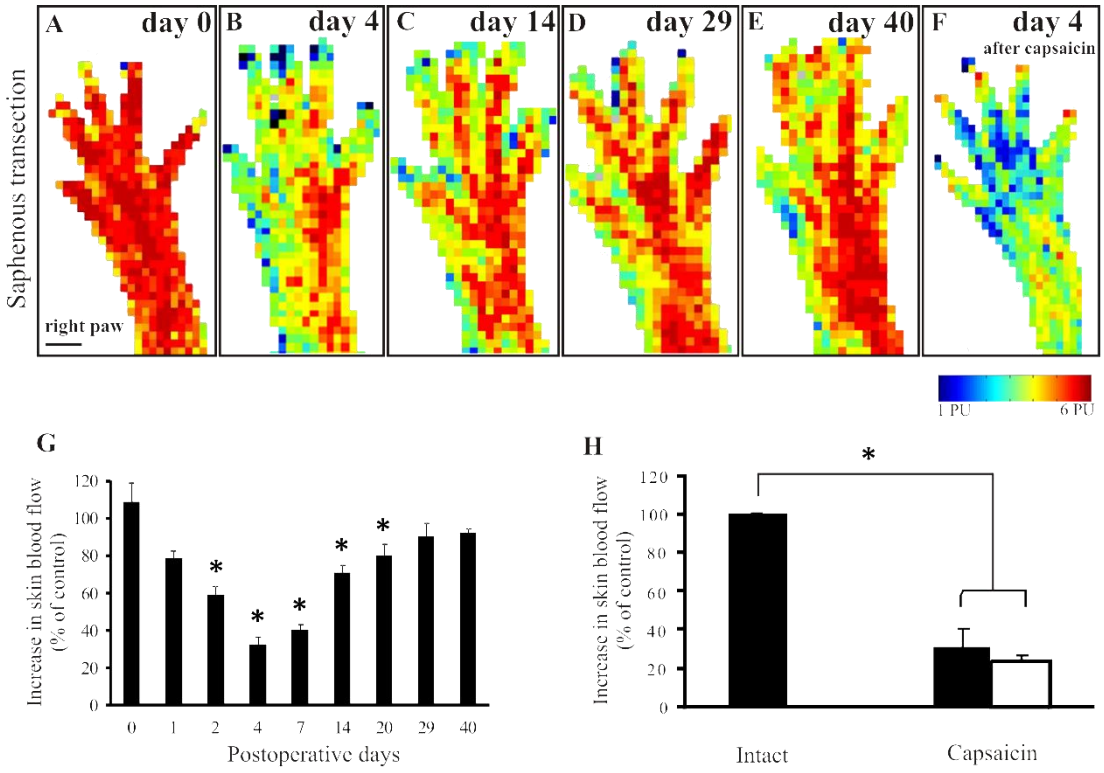

*Figure 6 Mustard oil-induced changes in blood flow of the dorsal hind paw skin after transection and ligation of the saphenous nerve. Original perfusion images demonstrate mustard oil-induced vasodilatatory responses before (**A**), and 4, 14, 29, and 40 (**B–E**) days after surgery. A gradual recovery of the vasodilatatory response in the medial aspect of the hind paw can be observed. The effect of the selective defunctionalization by perineural capsaicin treatment of sciatic afferents on the mustard oil-induced vasodilation is shown by image **F**. Note the marked reduction of the vasodilatatory response in both the lateral and the medial aspects of the dorsal hind paw skin served by the sciatic and saphenous nerves, respectively, after capsaicin treatment. **(G)** The histogram shows the quantitative data on changes in the vasodilatatory responses following saphenous nerve transection. Note the marked decrease shortly after transection and the gradual recovery over a period of 4–5 weeks,*

*indicating denervation and reinnervation of the denervated saphenous skin area. (**H**) 40 days after saphenous nerve transection and 4 days after perineural capsaicin treatment of the sciatic nerve significant reductions of mustard oil-induced vasodilatation in both the medial (filled column) and lateral (empty column) aspect of the dorsal hind paw skin were detected as compared to the vasodilatatory response measured in the contralateral dorsal hind paw skin ($p < 0.05$; $n = 8$). Scale bar represents 5 mm.*

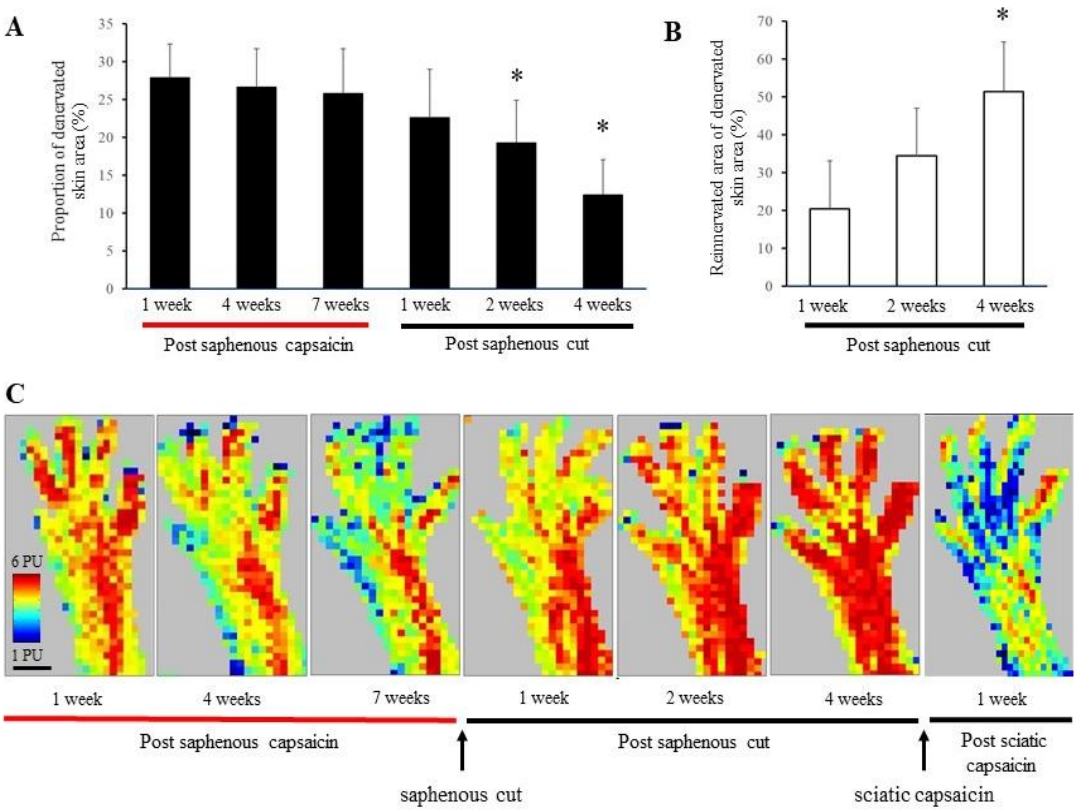

*Figure 7 (**A**) Bar charts show the proportion of denervated skin areas following perineural capsaicin treatment of the saphenous nerve and subsequent transection of that nerve 7 weeks later ($n = 9$). *: significantly different from the skin area determined 1 week after perineural capsaicin treatment. (**B**) Calculation of reinnervated skin areas following transection of the saphenous nerve treated perineurally with capsaicin 7 weeks previously. *: significantly different from the denervated area determined 1 week after perineural capsaicin treatment. (**C**) A series of laser Doppler scanning perfusion images of the rat hind paw following perineural capsaicin treatment of the saphenous nerve and subsequent transection of the saphenous nerve and perineural capsaicin treatment of the sciatic nerve 7 and 11 weeks later, respectively. Scale bar represents 5 mm.*

To confirm the results obtained by laser Doppler imaging, the mustard oil-induced vascular labeling technique was applied and the topographical distribution of the labeling pattern was repeatedly assessed (Fig. 8). Intravenous injection of a colloidal silver solution followed by the application of mustard oil onto the skin produced clear-cut vascular labeling of permeable subepidermal small venules in skin areas of intact sensory innervation but vascular labeling could not be elicited with mustard oil in the denervated skin. Vascular labeling with colloidal silver can be observed not only under the light microscope (image taken from a whole mount specimen, Fig. 8A) but also macroscopically due to the brownish coloration of the skin containing permeable small blood vessels (venules) (Fig. 8B). By photographing the skin and measuring the brownish and the uncolored skin areas with a computer software, the innervated and denervated skin territories can be quantitatively determined. The results obtained with the vascular labeling technique and scanning laser Doppler imaging were essentially similar (Fig. 8C). Following perineural capsaicin treatment and subsequent transection of the saphenous nerve a series of photographs was captured over a period of 11 weeks to visually follow-up changes in the size and topography of the denervated skin regions and the gradual recovery of the vascular labeling in the previously denervated cutaneous areas. Following perineural capsaicin treatment of the saphenous nerve, a marked decrease of "silver stained" skin area was detected indicating the loss of vascular labeling in the chemodenervated skin. Recovery of vascular labeling was not observed even after a survival period of 4 weeks. However, after transection of the capsaicin treated nerve a gradual and significant recovery of vascular labeling was observed, indicating reinnervation of the skin. Quantitative data showed that the proportion of the chemodenervated skin area gradually decreased parallel with a gradual increase in the proportion of the reinnervated area amounting to about 70 per cent of the skin area served by the saphenous nerve (Fig. 8D). These findings supported LDI data showing that reinnervation of the chemodenervated (saphenous) skin area resulted from collateral sprouting of intact sciatic afferents into the denervated skin.

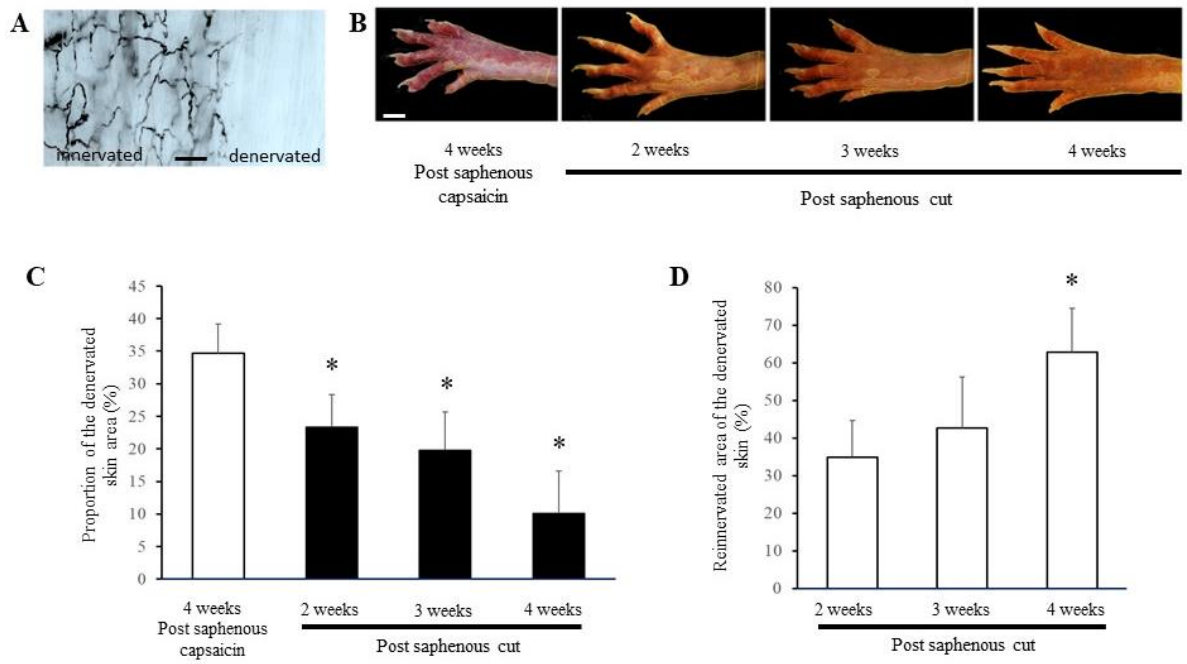

*Figure 8 Figure **A** represents a microphotograph illustrating vascular labeling of the hind paw skin of a rat following an intravenous injection of a colloidal silver solution. Colloidal silver–labeled small blood vessels (venules) are present in the innervated but not the denervated skin. (**B**) A series of photographs taken from the right hind paw of a rat to illustrate the appearance of mustard oil–induced vascular labeling in the innervated skin areas 4 weeks after perineural capsaicin treatment of the saphenous nerve and after a subsequent transection of that nerve. Scale bar represents 5 mm. The quantitative data showing the proportions of the denervated and reinnervated skin areas are shown in (**C**) (n = 4, *: significantly different from the skin area determined 4 weeks after perineural capsaicin treatment) and (**D**) (n = 4, *: significantly different from the skin area determined 4 weeks after perineural capsaicin treatment), respectively.*

3.4 Immunohistochemical study of degeneration and regeneration of cutaneous nerves after peripheral nerve lesions

Tubulin III- and CGRP-immunohistochemistry can be applied for the demonstration of nerve fibers innervating the epidermis, hair follicles and small blood vessels of the skin. Tubulin III is a reliable general marker of all types of nerve fibers in a variety of tissues, whereas CGRP is a specific marker of the major population of the peptidergic afferent nerves.

Vascular labeling technique utilizing colloidal silver was used to identify skin regions exhibiting mustard oil-induced vascular reactions in innervated skin area. On the partially denervated hind paw skin the distribution of the mustard oil-induced hyperaemic response captured by LDI (Fig. 9A) corresponded well with the pattern of vascular labeling 4 days after transection of the saphenous nerve (Fig. 9B). At the microscopic level many subepidermal microvessels were labeled with dense deposits of colloidal silver in the intact, but in the denervated skin region (Fig. 9C, D). Four days after nerve transection in the lateral hindpaw skin identified by the presence of vascular labeling immunohistochemical staining revealed many tubulin- and/or CGRP immunoreactive nerve fibers in close apposition to silver labeled blood vessels. Soliter fibers in the epidermis and fibers or fiber bundles were observed around hair follicles and small blood vessels of the innervated skin (Fig. 9E–H). Nerve transection resulted in a depletion of epidermal nerve fibers from the denervated saphenous skin area 4 days after the surgery (data not shown). The first signs of reinnervation were detected 14 days following saphenous nerve transection; few tubulin III- and CGRP-immunopositive epidermal and subepidermal nerve fibers could be observed in the previously denervated skin area (Fig. 9I-K). Restitution of innervation progressed in the next 2–4 weeks. Regeneration of sensory nerves was functional as evidenced by the re-appearance of vascular labeling after intravenous injection of colloidal silver prior to the application of mustard oil onto the skin. These nerves proved to be of sciatic origin since they were completely lost after perineural capsaicin treatment of the sciatic nerve (data not shown). These observations are in line with the suggestion of the laser Doppler imaging experiments that reinnervation of the denervated saphenous skin area was accomplished through collateral sprouting of intact sciatic sensory axons.

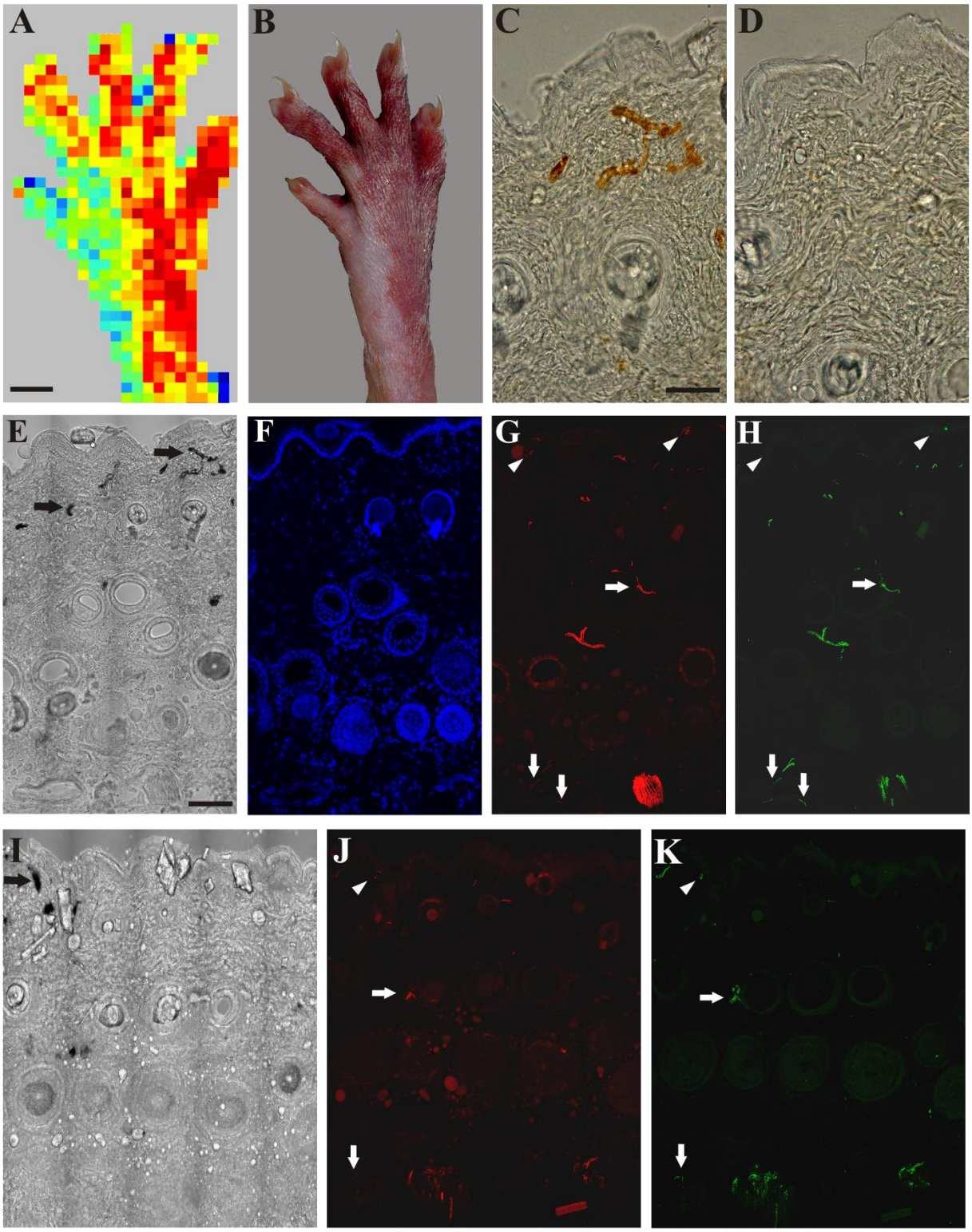

*Figure 9 (**A**) Application of mustard oil induced hyperaemic response in the innervated (sciatic) but not in the denervated (saphenous) skin area as captured by scanning laser Doppler imaging. (**B**) Mustard oil-induced vascular labeling appeared as brownish coloration of the skin in the innervated (sciatic) but not in denervated (saphenous) skin. (**C, D**) Bright field photomicrographs showing sections from the lateral intact (**C**) and medial denervated regions*

*of the hind paw skin after transection of the saphenous nerve (**D**). Deposition of colloidal silver in subepidermal small venules indicate increased vascular permeability of small postcapillary venules elicited by mustard oil application in the intact dorsal hind paw skin (**C**). Deposits of colloidal silver cannot be observed in the denervated area 4 days after saphenous nerve transection (**D**). (**E–H**) Bright field (**E**) and immunofluorescence (**F–H**) photomicrographs showing the lateral (sciatic) skin area (**E–H**) after transection of the saphenous nerve. (**I–K**) Bright field (**I**) and immunofluorescence (**J, K**) photomicrographs showing the medial (saphenous) skin area 14 days after transection and ligation of the saphenous nerve. Note the localization of ß-tubulin III (red) and CGRP-immunoreactive (green) nerve fibers in the epidermis (arrowheads), and around hair follicles and small arteries (arrows) in the innervated (lateral, sciatic) area of the dorsal hind paw skin identified by the presence of silver-labeled venules (arrows in **E**). Fourteen days after transection and ligation of the saphenous nerve, some silver-labeled venules (arrow in **I**) and some ß-tubulin III and CGRP-immunoreactive epidermal (arrowheads in **J, K**) and dermal (arrows in **J, K**) nerve fibers can be observed in the medial (saphenous) skin area indicative of (collateral) regeneration. Scale bars indicate 5 mm in A and B and 50 μm in C,E. Scale bars in C,E apply for C,D and E–K, respectively.*

Unlike nerve transection, selective chemodenervation with perineural capsaicin treatment produced a robust, but not complete depletion of epidermal and subepidermal nerve fibers, as compared with the adjacent, sciatic innervation area (lateral side of the paw, Fig. 10A–C). In the denervated saphenous area (note absence of the vascular labeling, Fig. 10D) residual fibers positive for tubulin but not for CGRP could be observed 4 days after perineural capsaicin treatment (Fig. 10E, F). In contrast with nerve transection, the CGRP-immunoreactivity did not recover, but remained absent for the whole 7-week follow-up period when transection of the capsaicin-treated nerve was made (data not shown). Two weeks following the transection of the previously capsaicin-treated saphenous nerve a few CGRP-positive fibers and vascular labeling with colloidal silver reappeared on the medial side originally innervated by the transected saphenous nerve (Fig. 10G–I). Restitution of CGRP-immunoreactivity and vascular labeling may be explained by collateral sprouting of intact sciatic afferents into the denervated saphenous skin area, since the saphenous nerve was cut and ligated. Reappearance of vascular labeling indicates that collateral nerve sprouts of sciatic afferents were functional. Treating the intact sciatic nerve with capsaicin almost completely abolished the previously restituted vascular labeling and CGRP-immunoreactivity both in the medial and lateral sides (Fig. 10J–L).

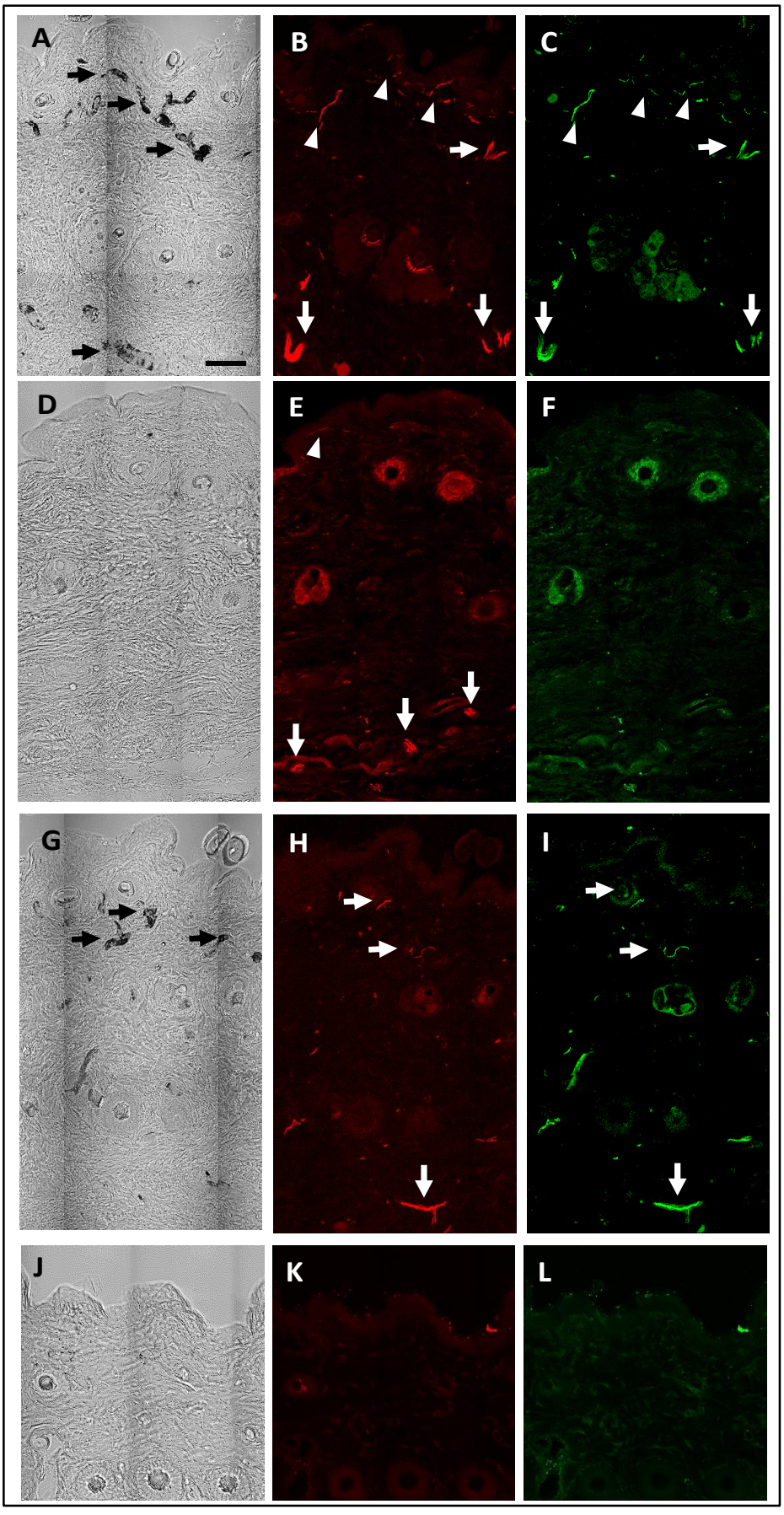
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*Figure 10 Bright field (**A**) and immunofluorescence (**B, C**) photomicrographs showing the intact sciatic innervation area. ß-tubulin III (red, **B**) and CGRP-immunoreactive (green, **C**) nerve fibers are localized in the epidermis (arrowheads), and around hair follicles and small blood vessels, probably arteries (arrows) of the dorsal hind paw skin. Deposition of colloidal silver in subepidermal small venules indicate increased vascular permeability of small postcapillary venules elicited by mustard oil application (arrows in **A**). (**D-F**) Brigh field (**D**) and immunofluorescence (**E, F**) photomicrographs showing the medial (saphenous) innervation area 4 days after perineural capsaicin treatment of the saphenous nerve. Note the absence of silver-labeled venules (**D**) and CGRP-immunoreactivity (**F**), while some tubulin-immunoreactivity can be observed in the deeper layers, but not in the epidermis of the denervated skin (E). (**G-I**) Two weeks after the transection of the previously capsaicin-treated saphenous nerve, reappearance of CGRP-immunoreactivity and vascular labeling (**G**) can be observed (**I**) on the medial side originally innervated by the saphenous nerve indicative of (collateral) regeneration by the adjacent intact sciatic afferents. (**J–L**) Four days after the perineural capsaicin treatment of the sciatic nerve, complete disappearance of vascular labeling (**J**) and CGRP-immunostaining (**L**) was observed, suggesting that the previously seen restitution was due to collateral reinnervation by the sciatic nerve. Scale bar represents 50 µm and applies for each photomicrographs.*

4 DISCUSSION

Information on the functional status of cutaneous nerves is of fundamental clinical and experimental importance in assessing degenerative and regenerative processes of sensory nerves and the progression of diseases affecting the peripheral nervous system. Different techniques may be applied to study sensory functions of skin nerves involved in the sensation of specific modalities, such as touch, vibration cold, warm, itch and pain. Many of these modalities can be studied by relatively simple means using e.g., the tuning fork for vibration, thermodes for cold and warm sensations and von Frey hairs for touch and pain. Although sensory functions of the skin and, after selective denervation procedures, cutaneous innervation territories of peripheral nerves can be assessed with these methods, these techniques are neither accurate nor objective. For example, assessment of thermal sensitivity using contact thermodes has a relatively low spatial resolution and is subjective. In both clinical and experimental studies determination of the borders of the innervation territories of peripheral nerves by applying punctate touch or painful stimuli is inconvenient and inaccurate. These technical difficulties come even more to the front if longitudinal studies are to be performed.

The visualization of the neurogenic inflammatory response for objective study of the function and spatial distribution of a particular population of peptidergic cutaneous nociceptors has been introduced by Jancsó and Király (1981). In the skin with intact sensory innervation, cutaneous application of mustard oil, following an intravenous injection of Evans blue dye solution, results in a dark blue discoloration of the skin due to the extravasation of the Evans blue dye through small blood vessels (venules) of increased vascular permeability (Jancsó and Kiraly, 1983; Bharali and Lisney, 1988; Brenan, Jones and Owain, 1988; Lisney, 1989). Electrophysiological observations indicated that the area showing extravasation exactly corresponds to the innervation territory of the stimulated nerve (Pertovaara, 1988) or nerve ending (Kenins, 1982). In the denervated skin, neurogenic plasma extravasation cannot be evoked with mustard oil and, therefore innervated and denervated skin areas can be clearly defined by the presence and absence, respectively, of Evans blue coloration of the skin. The spatial distribution of small blood vessels (venules) of enhanced vascular permeability following the cutaneous administration of mustard oil can also be visualized by using the vascular labeling technique (Dux and Jancsó, 1994). Vascular labeling combines the stimulation of nerve endings with the simultaneous intravenous administration of a suitable colloidal substance, such as colloidal

silver. Upon stimulation of nociceptive nerve endings with mustard oil, colloidal silver accumulates in the wall of the permeable blood vessels which can be easily visualized under the light microscope in transparent preparations (Jancsó, 1955; Jancsó N., 1960; Dux and Jancsó, 1994). The innervated territories of the skin can also be seen with the naked eye and photographed owing to the brownish discoloration of the skin.

In the present Thesis we have applied a novel approach to demonstrate the cutaneous innervation territories of hindlimb nerves utilizing the visualization of mustard oil-induced sensory neurogenic vasodilatation. We have presented evidence for scanning LDI being a reliable method for the study of the functional status of peptidergic cutaneous nociceptive nerves enabling the longitudinal observation of denervation and reinnervation processes in the skin following peripheral nerve injuries.

Measurement of cutaneous blood flow with scanning LDI

The development of the technique of scanning LDI has permitted the direct visualization of cutaneous blood flow in both man and experimental animals. Based on the principle of laser Doppler velocimetry, LDI technique allows the direct observation of the perfusion pattern of a given skin area with a relatively high temporal resolution. LDI measures the frequency broadening of the incident laser light due to its scattering by moving red blood cells. By recording of red blood cell velocities and concentration, the Doppler frequency shifts can be measured, which are proportional with a blood flow-related variable expressed in arbitrary perfusion units.

Scanning LDI has already been used earlier to examine mechanisms of neurogenic sensory vasodilatation in the skin(Lynn, Schütterle and Pierau, 1996; Sántha, Pierau and Jancsó, 1998, 1999). The major advantages of the LDI technique are that it is non-invasive, measures changes in microcirculation without requiring direct contact with the tissue, and detects perfusion in real time. LDI generates a perfusion map by scanning, by the laser beam, of the selected area of skin into a depth of about 1 mm (Rajan *et al.*, 2009). A single image with a pixel frame size of 52×42, as used in our studies, takes approximately 2 minutes to capture and the spatial resolution of the image is typically 1 mm (at high-resolution it is 0.1 mm) and a relatively large area (up to 52 mm x 52 mm) can be scanned. This resolution is appropriate to delineate the borders of a denervated skin area in animal studies and in clinical setup for human

measurements. To calculate changes in perfusion, control baseline flow data are compared with the maximal vasodilatatory response induced by stimulation of sensory nerves. Since perfusion measured with the scanning LDI technique cannot be given in absolute values, measurements of blood flow are expressed in perfusion units as per cent changes in perfusion (Rajan *et al.*, 2009).

Sensory neurogenic vasodilatation elicited by antidromic electrical stimulation of sensory nerves or by application of irritants, such as mustard oil onto the skin, is initiated by activation of C-fiber polymodal nociceptors in the rat (Kenins, 1982). The contribution of some Adelta afferents has also been demonstrated (Lynn, 2009). The afferents involved in these vascular reactions are peptidergic. It is generally accepted that sensory neurogenic vasodilatation and plasma extravasation are mediated by afferent nerve fibers containing CGRP and SP, respectively (Brain *et al.*, 1986; Brain, 1997; Boros *et al.*, 2009; Jancsó *et al.*, 2009). In the skin, SP- and CGRP-containing peptidergic epidermal axons have a relatively simple morphology with few if any arborizations, whereas non-peptidergic axons have a more complex tortuous course (Dux *et al.*, 1999; Zylka, Rice and Anderson, 2005). Primary afferent neurons involved in the mediation of the neurogenic inflammatory responses are capsaicin-sensitive and express the TRPV1 and/or the TRPA1 receptors (Gamse, Holzer and Lembeck, 1980; G. Jancsó, Király and Jancsó-Gábor, 1980; Dux and Jancsó, 1994; Brain, 1997; Dux *et al.*, 1999; Jancsó, Oszlács and Sántha, 2011; Boros *et al.*, 2016), the activation of which results in the release of CGRP and/or SP from their peripheral (and central) nerve endings. These vascular responses represent the sensory-efferent, local regulatory functions of nociceptive afferent nerves which are regarded as reliable measures of the functional status of nociceptive primary sensory neurons (Jancsó N., 1960; Jancsó, Kiraly and Jancsó-Gábor, 1977; G Jancsó, Király and Jancsó-Gábor, 1980; Maggi and Meli, 1988; Bevan and Szolcsányi, 1990; Holzer, 1998; Nagy *et al.*, 2004; Jancsó *et al.*, 2009). In man, sensory neurogenic vasodilatation manifests as the flare component of Lewis's triple response of the skin, which is frequently examined in the clinical practice to assess sensory functions of the skin (Jancsó, Jancsó-Gábor and Szolcsányi, 1968; Jancsó and Janka, 1981; Jancsó, Husz and Simon, 1983a, 1983b; Namer *et al.*, 2013). Hence, the findings of the present experiments may bear of relevance to the pathophysiology of cutaneous sensation in man.

Following nerve injury, two modes of reinnervation of the denervated skin has been revealed. On the one hand, reinnervation of the denervated skin may be effected through regeneration of the injured nerve, i.e., by regenerative sprouting. On the other hand, reinnervation may result from collateral sprouting, i.e., the invasion of the denervated skin by sprouting intact nerve

fibers serving skin areas adjacent to the denervated skin region. The restitution of the function of the denervated skin through collateral sprouting has been demonstrated in both man and experimental animals (Brenan, 1986; Inbal *et al.*, 1987; Diamond *et al.*, 1992; Diamond, Holmes and Coughlin, 1992).

The phenomenon of collateral sprouting has been demonstrated following crush and transection of a nerve. Nerve crush is followed by reinnervation of the denervated skin through regenerative sprouting of the injured nerve if regeneration is permitted. In contrast, if regeneration is prevented by e.g. ligating the lesioned nerve, the restitution of sensory functions is achieved by collateral sprouting of intact neighboring axons serving skin areas adjacent to the denervated skin. However, not all types of sensory nerves undergo remarkable collateral spread as thick mechanoreceptive Aβ fibers seem to lack sprouting ability; only thin myelinated Aδ fibers and unmyelinated C fibers are capable of this reaction (Devor *et al.*, 1979; Jancsó and Kiraly, 1983; Brenan, 1986; Diamond *et al.*, 1987; Pertovaara, 1988; Kinnman *et al.*, 1992).

The observations presented in this Thesis furnished evidence for the suitability of the scanning LDI technique in longitudinal investigations of the local regulatory, vasodilatatory functions of peptidergic nociceptive nerves in the rat skin. Further, the technique, as applied in our experiments, proved to be a reliable method for the study of degenerative and regenerative processes of cutaneous nerves in the rat.

4.1 Cutaneous blood flow at rest and after stimulation of sensory nerve endings with mustard oil

In the naïve rat, scanning LDI revealed an essentially uniform perfusion of the hindlimb dorsal skin under resting conditions. Stimulation of cutaneous nociceptive nerve endings by applying mustard oil onto the skin markedly increased local blood flow resulting in an immediate albeit transient hyperaemic reaction. In a previous study from our research group, using classical single channel (point) laser Doppler flowmetry, epicutaneous application of mustard oil evoked a vasodilatatory reaction of similar time course, magnitude and duration (Boros *et al.*, 2016). This suggests that both methods measure skin perfusion reliably, but scanning LDI has the added advantage of showing the spatial distribution of cutaneous perfusion changes.

Mustard oil exerts its vasodilatatory effect through activation of the TRPA1 nociceptive ion channel expressed in peptidergic nociceptive sensory nerves which also express the TRPV1 channel (Bautista *et al.*, 2006). Recent reports indicated that in addition to the TRPA1 receptor, mustard oil activates also the TRPV1 nociceptive ion channel (Everaerts *et al.*, 2011; Gonzalez-Cano *et al.*, 2020). Further studies disclosed the predominant roles of TRPA1 receptor in the vasodilatatory, and the TRPV1 receptor in the nociceptive effects of mustard oil (Everaerts *et al.*, 2011; Gees *et al.*, 2013; Boros *et al.*, 2016). Mustard oil induced vasodilatation elicited through activation of the TRPA1 receptor is mediated by CGRP, a vasodilatatory peptide released from the nociceptive nerve endings (Brain *et al.*, 1985; Sauerstein *et al.*, 2000; Pozsgai *et al.*, 2010). Besides pharmacological evidence (Pozsgai *et al.*, 2012; Aubdool *et al.*, 2016), this is also supported by the close association of CGRP with blood vessels (arteries) in the rat skin (H Sann *et al.*, 1995). Interestingly, in the avian skin mustard oil produced similar increases in blood flow only after administration of galanin antagonists, indicating an inhibitory tone exerted by galanin contained in and released from sensory nerves (Sántha, Pierau and Jancsó, 1998). A similar inhibition by somatostatin of cutaneous vascular reactions at distant, but not at the stimulation sites has also been demonstrated in the rat (Lembeck, Donnerer and Barthó, 1982; Szolcsányi *et al.*, 1998).

4.2 Functional reinnervation of the denervated skin after peripheral nerve transection

Transection and ligation of the saphenous nerve resulted in a dramatic, although not complete reduction of mustard oil-induced vasodilatation in the denervated skin. By day 1 after saphenous nerve section, a vasodilatatory response was still observed, which was comparable to that observed in intact skin areas. This finding suggests that cutaneous nerve endings are still functional, i.e. release vasoactive neuropeptides upon stimulation at this early phase of axonal degeneration. A significant decrease in mustard oil-induced vasodilation was observed in the denervated skin 3–4 days after nerve transection and was essentially abolished by the 4th post-operative day. The denervation-induced decrease in the vasodilatatory response may be accounted for by the depletion of vasodilatatory sensory neuropeptides, mainly CGRP from the denervated skin due to Wallerian degeneration of sensory nerves (Jancsó and Kiraly, 1983; Sta *et al.*, 2014; Lakatos *et al.*, 2020). The modest vasodilatatory reaction remaining after nerve

transection may probably be attributed to non-neuronal mechanisms or to activation of TRPV1 in non-neural elements of the vascular bed (Bánvölgyi *et al.*, 2004; Grant *et al.*, 2005; Everaerts *et al.*, 2011; Gees *et al.*, 2013; Tóth *et al.*, 2014). The vasodilatatory response remained unaffected in the ipsilateral sciatic innervation territory as well as in the contralateral hind paw skin. These observations are in line with earlier findings using the Evans blue method or the vascular labeling technique or antidromic electrical stimulation of the saphenous nerve (Jancsó and Kiraly, 1983; Brenan, 1986; Pertovaara, 1988; Dux *et al.*, 1999).

Signs of reinnervation of the denervated skin were first observed at the 2nd postoperative week with a gradual and time-dependent reappearance of the vasodilatatory response as assessed by repeated measurements of the mustard oil-induced vasodilatation using scanning LDI. The size of the skin area displaying negligible vasodilatatory responses gradually decreased and after approximately 3–4 weeks the topography and intensity of the vasodilatatory response were similar to the pre-operative condition. This time-course of functional recovery is consistent with observations of earlier studies utilizing behavioral testing or the Evans blue technique to examine functional cutaneous nerve regeneration (Devor *et al.*, 1979; Bharali and Lisney, 1988). Since the transected nerve was ligated, reinnervation of the denervated skin by regeneration of the nerve was impeded. Hence, the most likely mechanism of reinnervation of the denervated skin is the collateral sprouting of intact sensory axons of adjacent skin areas into the denervated skin. This is strongly supported by the finding that defunctionalization, by perineural capsaicin treatment, of sciatic nerve afferents innervating the adjacent lateral skin area resulted in the loss of mustard oil-induced vasodilatatory response in the entire dorsal skin of the hind paw (Devor *et al.*, 1979; Diamond *et al.*, 1987; Pertovaara, 1988).

Reinnervation of the denervated skin by collateral sprouting was also demonstrated in our immunohistochemical experiments. Examination of tubulin- and CGRP-immunoreactive cutaneous axons were performed in intact, denervated and reinnervated skin following induction of neurogenic inflammation with mustard oil. In support of the functional observations with scanning LDI, tubulin- and CGRP-immunoreactive nerve fibers could be demonstrated in the previously denervated saphenous skin territory. Regenerating CGRP-immunoreactive nerve fibers could not be detected in the saphenous skin area after treatment of the sciatic nerve with capsaicin. These findings support the notion that, after transection of the saphenous nerve, the denervated skin was reinnervated by collateral sprouting of sciatic nerve afferents serving the neighboring skin areas.

4.3 Reinnervation of the chemodenervated skin

Perineural application of vanilloid agonists such as capsaicin or resiniferatoxin to peripheral nerves induces nociceptor analgesia, i.e., a selective regional analgesia without affecting motor and sensory functions unrelated to pain. The selective sensory deficit is confined to the innervation territory of the treated nerve and includes chemo- and thermo-analgesia and the inhibition of neurogenic sensory vasodilatation and plasma protein extravasation (G. Jancsó, Király and Jancsó-Gábor, 1980; Fitzgerald and Woolf, 1982; Gamse *et al.*, 1982; Chung *et al.*, 1985; Dux *et al.*, 1999; Kissin, Bright and Bradley Jr., 2002; Domoki *et al.*, 2003; Kissin, 2008). The effects of perineural capsaicin are mediated through TRPV1 receptors located in the axolemma of peripheral nerves (Bernardini *et al.*, 2004). Perineural treatment of the rat saphenous nerve with 1% capsaicin resulted in a loss of 32–36 per cent of unmyelinated C-fibers as revealed by counting of unmyelinated axons in electron micrographs of nerve cross-sections (Scadding, 1980; Jancsó and Lawson, 1990; Lynn, 1990; Pini, Baranowski and Lynn, 1990). This suggests that the perineural capsaicin induced loss of function of capsaicin-sensitive afferents may be attributed to both a loss of unmyelinated axons and depletion of vasoactive neuropeptides (SP, CGRP) from these afferents (G. Jancsó, Király and Jancsó-Gábor, 1980; Gamse *et al.*, 1982; Jancsó and Lawson, 1990; Dux *et al.*, 1999). Local application of capsaicin on peripheral nerves results in a prompt blockade of axoplasmic transport of SP and SOM, but not noradrenaline or acetylcholinesterase indicating the selective effect of capsaicin on nociceptive primary sensory neurons (Gamse *et al.*, 1982).

The present experiments showed an abolition of mustard oil-induced vasodilatation in skin areas served by the capsaicin treated saphenous nerve 3 days postoperatively. This finding support the notion that sensory neurogenic vasodilatation is mediated by capsaicin-sensitive (chemosensitive), TRPV1-expressing neurons (Gamse, Holzer and Lembeck, 1980; Domoki *et al.*, 2003; Sousa-Valente and Brain, 2018). In contrast to peripheral nerve transection, recovery of vascular responses was not observed by using the Evans blue-based technique (Pertovaara, 1988). This finding was confirmed in the present study using scanning laser Doppler imaging of mustard oil-induced neurogenic sensory vasodilation in the rat hind paw skin following perineural capsaicin treatment of the saphenous nerve. Thus, the mustard oil-induced neurogenic vasodilatatory response was completely and permanently lost in the skin area served by the affected (saphenous) nerve. During the 7-week follow-up period, the vasodilatatory response did not recover. This suggests that neither regenerative regrowth of saphenous nerve

afferents, nor collateral sprouting of intact neighboring sciatic afferents ensued to reinnervate the chemodenervated skin. However, following the transection and ligation of the capsaicin-treated saphenous nerve, the mustard oil-induced vasodilatatory response showed a gradual and significant recovery in both intensity and spatial extent similarly to that observed in experiments on saphenous nerve transection. Since the ligation of the transected saphenous nerve prevented its regeneration, the recovery of the vasodilatatory response may be a consequence of reinnervation of the denervated skin through collateral sprouting, i.e. invasion of adjacent intact sciatic nerve afferents into the saphenous skin territory (Lakatos *et al.*, 2020; Sántha *et al.*, 2022). This is strongly supported by the observation that subsequent perineural capsaicin treatment of the sciatic nerve resulted in the elimination of the vasodilatatory response not only in the sciatic, but also in the saphenous skin territories (Sántha *et al.*, 2022).

Similar results were obtained by using the vascular labeling technique to delineate innervated/denervated areas of the dorsal hind paw skin. As vascular labeling induced by mustard oil requires intact peptidergic sensory innervation (G Jancsó, Király and Jancsó-Gábor, 1980; Dux and Jancsó, 1994; Dux *et al.*, 1999), the measurement of silver-stained skin areas provides information on the functional status of cutaneous peptidergic nerves. In the saphenous nerve innervation territory vascular labeling was completely abolished 3-4 days after capsaicin treatment of the saphenous nerve as assessed by both visual inspection and light microscopy of transparent skin preparations. The size of the (denervated) area devoid of vascular labeling did not change for at least 4 weeks after capsaicin treatment, indicating a lack of regeneration within this time period. Similarly to the findings obtained with repeated scanning LDI measurements, the denervated area markedly and progressively decreased 2–4 weeks after transection of the (capsaicin-treated) saphenous nerve, indicating reinnervation of the denervated skin by collateral sprouting of sciatic nerve afferents.

These observations suggest novel pathophysiological/neuroplastic changes which commence after chemodenervation of the skin by perineural treatment with capsaicin. The phenomenon of inhibition of reinnervation of the denervated skin by collateral sprouting of adjacent intact nerves may be referred to as collateral inhibition of nerve regeneration. The findings also suggest a permissive effect of transection of the capsaicin-treated (saphenous) nerve promoting collateral sprouting. The exact mechanism of this permissive effect is not known at present. We assume that non-degenerating unmyelinated and myelinated nerve fibers which persist in the chemodenervated skin may inhibit the sprouting of adjacent intact sciatic nerve fibers into the

denervated skin area. Similar processes resulting in failure of regeneration may be involved in certain human neuropathies which affect only subpopulations of afferent nerves.

Collateral sprouting has been found to be promoted through sprouting-associated transcriptomic changes that differ from that of regenerating neurons. The process of collateral sprouting has been associated with specific growth mechanisms triggered by Wallerian degeneration. In the peripheral nervous system, Wallerian degeneration plays a critical role in axonal regeneration. In spinal cord injury models it has been demonstrated that delayed Wallerian degeneration reduces functional recovery (Collyer *et al.*, 2014). Mechanical and toxic stimuli induced Wallerian degeneration involves axonal degeneration, differentiation of Schwann cells to a phenotype allowing for repairing, myelin disintegration, macrophage invasion, and secretion of neurotrophic factors. The production of trophic factors and/or cytokines triggered by degenerating axons are essential for the collateral sprouting of sensory axons in degenerating nerves (Lemaitre *et al.*, 2020) or denervated target tissue (Diamond *et al.*, 1987). A major factor in the mechanism of successful reinnervation through collateral spouting is that resident Schwann cells must become permissive to allow for the elongation of regenerating axons as normally mature Schwann cells repress axonal growth. Schwann cell dedifferentiation induced by axonal destruction significantly contributes to initiation of sprouting at the site of injury (Court and Alvarez, 2000). The lack of Wallerian degeneration of myelinated and capsaicin-insensitive unmyelinated afferents and sympathetic axons upon chemodenervation with capsaicin and consequent failure of the production of specific signaling molecules, e. g. cytokines and chemokines, and the sprouting suppressing effects of mature Schwann cells may provide an explanation for the failure of intact sciatic afferents to invade the partially denervated adjacent skin areas. Transecting the nerve previously treated with capsaicin leads to Wallerian degeneration of those fibers that at first were spared by the capsaicin-induced chemodenervation. This may arrest the inhibitory influences discussed above and create a tissue environment favorable for the collateral invasion of afferent axons from the adjacent intact skin territory. The demonstration that different cytokine profiles were detected in nerves following transection and perineural capsaicin, respectively, support this assumption (Cheepudomwit *et al.*, 2008).

5 CONCLUSION

Examination of the functional status of nociceptive sensory nerves is essential in experimental and clinical studies on pathologies affecting the somatosensory system. In this Thesis we furnished evidence for visualization of cutaneous neurogenic inflammatory responses being a reliable approach for the study of a particular population of afferent nerves which are activated by painful chemical and thermal stimuli and mediate neurogenic sensory vasodilatation and neurogenic plasma extravasation. We have established a novel experimental paradigm for the study of the function and topographical distribution of this particular nociceptive afferents. Measurement of mustard oil-induced sensory neurogenic vasodilatation by repeated scanning Laser Doppler perfusion imaging and visualization of neurogenic plasma extravasation by the vascular labeling technique proved to be reliable and objective methods to assess the functional status, integrity, spatial distribution and regenerative propensity of cutaneous nociceptive nerves. These methods are suitable for the longitudinal follow-up of changes in both the function and topographical distribution of this particular nociceptive nerves following peripheral nerve injuries, such as nerve transection or capsaicin-induced selective chemodenervation.

By using these novel experimental paradigms, we confirmed previous findings on differences in regenerative propensities of sensory nerves following surgical and chemical lesioning of peripheral sensory nerves. After transection of the saphenous nerve, reinnervation of the denervated skin area occurred by means of collateral sprouting of intact chemosensitive afferents innervating adjacent skin areas. By contrast, collateral sprouting could not be observed in skin areas chemodenervated by perineural capsaicin treatment. An important observation of the present study is that transection and ligation of the previously capsaicin treated nerve induced intact sciatic afferents to invade the denervated skin area through collateral sprouting.

These findings suggest a novel neuroplastic phenomenon of collateral inhibition of nerve regeneration that may be attributed to an inhibitory effect of capsaicin-*in*sensitive sensory nerves persisting in the chemodenervated skin. Further studies are warranted to provide information on the exact mechanism(s) of collateral inhibition and the permissive conditioning lesion effect promoting collateral sprouting. Experiments in this line may expand our knowledge on axonal regenerative processes under neuropathic conditions brought about by nerve injuries of various origins including traumatic, toxic or metabolic nerve damage in man, too.

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I.

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ORIGINAL RESEARCH
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Longitudinal Study of Functional Reinnervation of the Denervated Skin by Collateral Sprouting of Peptidergic Nociceptive Nerves Utilizing Laser Doppler Imaging

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Restitution of cutaneous sensory function is accomplished by neural regenerative processes of distinct mechanisms following peripheral nerve lesions. Although methods available for the study of functional cutaneous nerve regeneration are specific and accurate, they are unsuitable for the longitudinal follow-up of the temporal and spatial aspects of the reinnervation process. Therefore, the aim of this study was to develop a new, non-invasive approach for the longitudinal examination of cutaneous nerve regeneration utilizing the determination of changes in the sensory neurogenic vasodilatatory response, a salient feature of calcitonin gene-related peptide-containing nociceptive afferent nerves, with scanning laser Doppler flowmetry. Scanning laser Doppler imaging was applied to measure the intensity and spatial extent of sensory neurogenic vasodilatation elicited by the application of mustard oil onto the dorsal skin of the rat hindpaw. Mustard oil induced reproducible and uniform increases in skin perfusion reaching maximum values at 2–4 min after application whereafter the blood flow gradually returned to control level after about 8–10 min. Transection and ligation of the saphenous nerve largely eliminated the vasodilatatory response in the medial aspect of the dorsal skin of the hindpaw. In the 2^{nd} to 4^{th} weeks after injury, the mustard oil-induced vasodilatatory reaction gradually recovered. Since regeneration of the saphenous nerve was prevented, the recovery of the vasodilatatory response may be accounted for by the collateral sprouting of neighboring intact sciatic afferent nerve fibers. This was supported by the elimination of the vasodilatatory response in both the saphenous and sciatic innervation territories following local treatment of the sciatic nerve with capsaicin to defunctionalize nociceptive afferent fibers. The present findings demonstrate that this novel technique utilizing scanning laser Doppler flowmetry to quantitatively measure cutaneous sensory neurogenic vasodilatation, a vascular response mediated by peptidergic nociceptive nerves, is a reliable non-invasive approach for the longitudinal study of nerve regeneration in the skin.

Keywords: sensory innervation, nociception, cutaneous vasodilatation, nerve injury, collateral sprouting, scanning laser Doppler flowmetry, TRPV1, TRPA1

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Abbreviations: CGRP, calcitonin gene-related peptide; PU, perfusion unit; TRPA1, transient receptor potential ankyrin type 1 receptor; TRPV1, transient receptor potential vanilloid type 1 receptor.

INTRODUCTION

Lesions of peripheral nerves are often inflicted by common traumatic injuries or toxic agents of environmental and medicinal origins resulting in axonal degeneration and consequent loss of neural function. Restitution of cutaneous sensory functions may ensue rapidly after nerve injury. The return of sensation may be studied with various methods including measurements of changes in the thresholds to nociceptive mechanical and thermal stimuli or histological examination of skin biopsy specimens (Nolano et al., 1999; Kennedy et al., 2010; Cobianchi et al., 2014). Alternatively, reinnervation of denervated skin territories may be evaluated with the Evans blue technique by demonstrating skin areas of increased vascular permeability induced by antidromic electrical or direct chemical stimulation of nociceptive nerve endings (Jancsó and Király, 1983; Bharali and Lisney, 1988; Brenan et al., 1988; Lisney, 1989). Although these techniques are specific and accurate in determining skin areas innervated by nociceptive afferents, they have significant limitations. Techniques utilizing measurements of nociceptive thresholds and evaluation of biopsy specimens are unsuitable for the exact delineation of innervated and denervated skin regions. In contrast, the Evans blue method exactly outlines the innervated skin areas, but similarly to the biopsy technique, its use is limited in longitudinal studies.

Peptidergic chemosensitive primary sensory neurons which express the nociceptive ion channels transient receptor potential vanilloid type 1 (TRPV1) and transient receptor potential ankyrin type 1 (TRPA1) comprise a unique population of sensory neurons with dual nociceptive and secretory functions. Besides the transmission of nociceptive stimuli toward the central nervous system, chemosensitive sensory nerve endings through the release of neuropeptides such as substance P and calcitonin gene-related peptide are involved in mediation of local tissue reactions, including vascular changes (Jancsó et al., 1987; Maggi et al., 1987; Maggi and Meli, 1988; Holzer, 1998; Geppetti et al., 2008; Jancsó et al., 2009). Electrophysiological studies also disclosed that vasodilator afferent nerves are nociceptive C-fibers mostly comprised of nociceptor polymodal units in the rat (Gee et al., 1997). Ample experimental evidence indicates that sensory neurogenic plasma extravasation and sensory neurogenic vasodilatation are elicited by substance P and calcitonin gene-related peptide, respectively, released from activated chemosensitive afferent nerves (Lembeck and Holzer, 1979; Gamse et al., 1980; Brain et al., 1985; Holzer, 1998; Boros et al., 2016). Cutaneous sensory neurogenic vasodilatation can be reliably demonstrated and measured utilizing measurement of skin blood flow with laser Doppler flowmetry (Lynn et al., 1996; Gee et al., 1997).

Scanning laser Doppler flowmetry is a reliable and useful technique for the measurement of cutaneous blood flow under physiological and pathophysiological conditions including thermoregulatory vascular responses in the intact and denervated skin in both man and animals (Hu et al., 2012; Deng et al., 2016). Laser Doppler flowmetry directly measures the cutaneous blood perfusion based on the detection of frequency ("Doppler") shift of low-power monochromatic laser light reflected from the moving erythrocytes, but not from the stationary tissue elements in the skin (Rajan et al., 2009; Daly and Leahy, 2013; Allen and Howell, 2014). The magnitude of the intensity of shifted reflected light is proportional to the local concentration of moving erythrocytes, whereas the mean of frequency change is proportional to the average velocity of moving red blood cells (Fredriksson et al., 2009; Rajan et al., 2009). Usually an integrated value reflecting simultaneously the changes in red blood cell concentration and velocity is calculated and the tissue perfusion is expressed as an arbitrary perfusion unit (Rajan et al., 2009; Daly and Leahy, 2013). Typical laser Doppler flowmetry assesses blood flow in the microcirculation of the superficial approximately 1 mm thick layer of the skin (Fredriksson et al., 2009). Laser Doppler perfusion imaging is also based on this principle, however a point-to-point sampling on a pre-defined skin area is performed by using scanning mirrors and computerized control and processing system. The recorded perfusion map shows the local distribution of skin areas exhibiting different blood perfusion intensities. The spatial resolution of the perfusion imaging is influenced by numerous factors, but the shortest distance which could be resolved is close to the 0.1 mm range (Rajan et al., 2009; Allen and Howell, 2014). This technique is suitable not only for the measurement of changes in skin blood flow but also to determine the topographical distribution of the changes in skin perfusion. Examination of cutaneous vasodilatatory responses elicited through orthodromic chemical stimulation of afferent nerve endings is a reliable technique for the demonstration of the functional innervation of the skin and the identification of innervated and denervated skin areas (Domoki et al., 2003; Illigens et al., 2013). The present experiments were initiated in an attempt to evaluate and validate scanning laser Doppler imaging as a novel non-invasive approach for the longitudinal study of the functional regeneration of cutaneous nociceptive nerves.

To support the experimental findings on degeneration and regeneration of cutaneous sensory nerves as assessed with the technique of scanning laser Doppler flowmetry, further experiments were performed using immunohistochemistry. The method of vascular labeling was utilized to identify innervated and denervated skin areas following transection of the saphenous nerve. This technique is based on the visualization of colloidal silver deposited in the basal membrane of permeable small blood vessels, mostly postcapillary venules following the epicutaneous application of mustard oil to induce neurogenic inflammation (Jancsó, 1960; Jancsó et al., 1980a). Neurogenic inflammation is a collective term for neurogenic sensory vasodilatation, mediated primarily by CGRP, and neurogenic plasma extravasation, mediated by substance P upon orthodromic or antidromic stimulation of sensory nerves (Jancsó, 1960; Jancsó et al., 1967, 2009; Lembeck and Holzer, 1979; Brain et al., 1985; Maggi and Meli, 1988; Holzer, 1998). Vascular labeling is a salient feature of increased vascular permeability (Jancsó, 1955; Majno et al., 1961). Detection of vascular labeling is a reliable measure to gather information on the functional state of sensory nerves mediating these vascular reactions, since CGRP is co-localized in almost all substance P-containing nerve fibers (Ju et al., 1987). The permeable blood vessels can be easily identified under the light microscope by the presence of colloidal silver in their walls

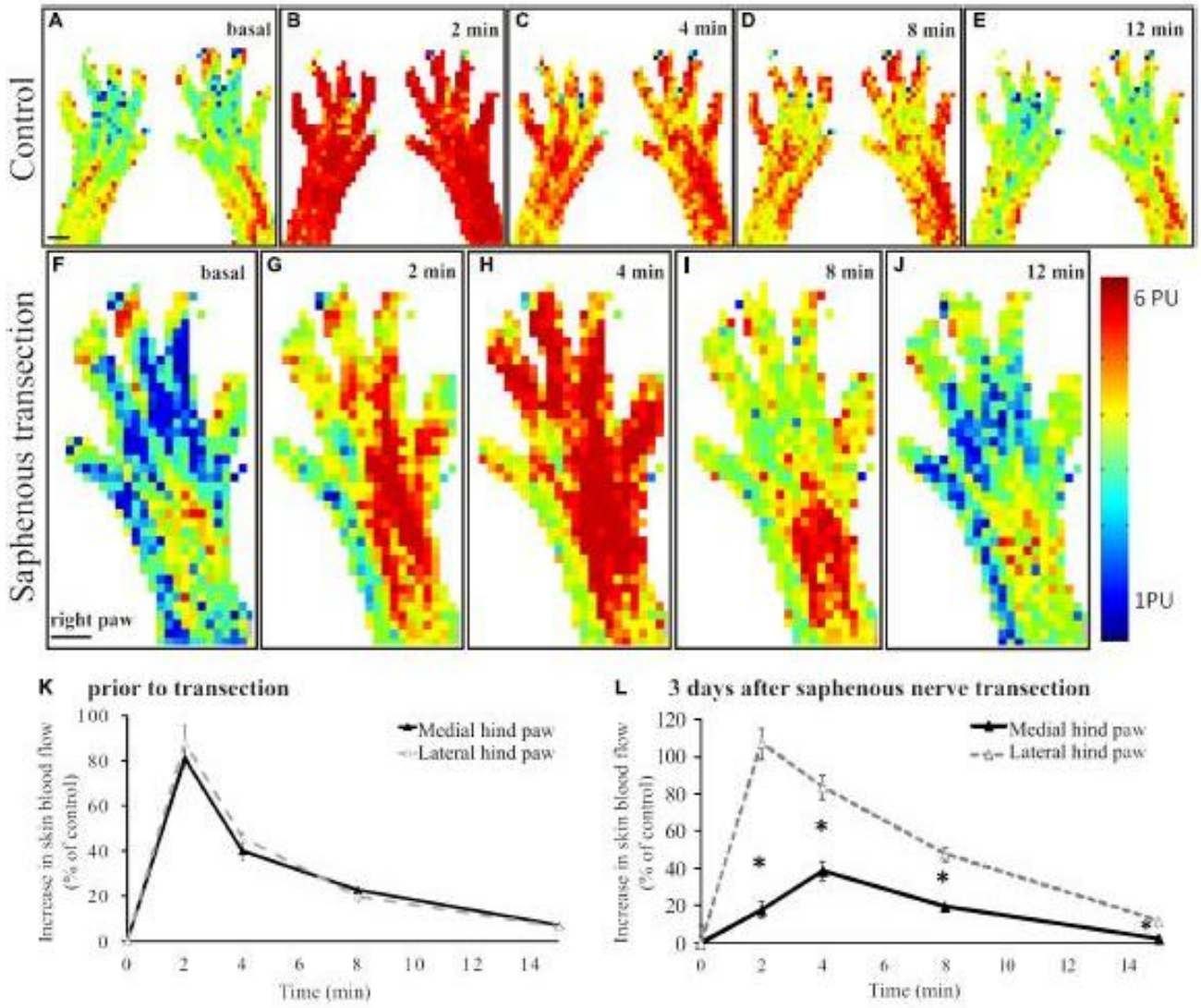

FIGURE 1 | A–E: Original perfusion images recorded with the scanning laser Doppler flowmeter illustrating the time course and spatial distribution of the vasodilatatory response elicited by the epicutaneous application of mustard oil onto the dorsal skin of the hindpaws of a control rat. Note the increase in blood flow of similar magnitude in the medial and lateral regions of the hindpaw skin. The line graph **(K)** depicts the time course of the increase in cutaneous blood flow relative to baseline value. Filled and empty triangles represent values measured in the medial (saphenous innervated) and lateral (sciatic innervated) skin areas, respectively ($n = 6$). **F–J:** Original perfusion images recorded with the scanning laser Doppler flowmeter illustrating the reduced mustard oil-induced vasodilatation in the medial aspect of the dorsal hindpaw skin three days after transection of the saphenous nerve. The line graph **(L)** shows the time course of the vasodilatatory response in the medial (filled triangles) and lateral (empty triangles) aspects of the dorsal hindpaw skin (*$p < 0.05$; $n = 6$). Scale bar on A and F represents 5 mm.

(Dux and Jancsó, 1994). Neurogenic inflammation and vascular labeling cannot be induced in the denervated skin (Jancsó, 1955, 1960; Jancsó et al., 1967, 1980a, 1987; Pertovaara, 1988; Sann et al., 1995; Dux et al., 1998).

MATERIALS AND METHODS

Animals

In total of eight adult male Wistar rats weighing 250–280 g at the beginning of the experiments were used in this study. The animals were maintained under a 12-h light/dark cycle with free access to food and water. The experiments were approved by the Ethics Committee for Animal Care at the University of Szeged as per the Council Regulation of 40/2013 (II. 14.) and were carried out in full accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering. The number of experimental animals was kept as low as possible.

Surgery

For surgical interventions rats were anesthetized with a combination of ketamine (Calypsol, 70 mg/kg, i.p., Gedeon Richter, Budapest, Hungary) and xylazine (CP-Xylazin 2%, 10 mg/kg, i.p., Produlab Pharma, Raamsdonksveer, Netherlands).

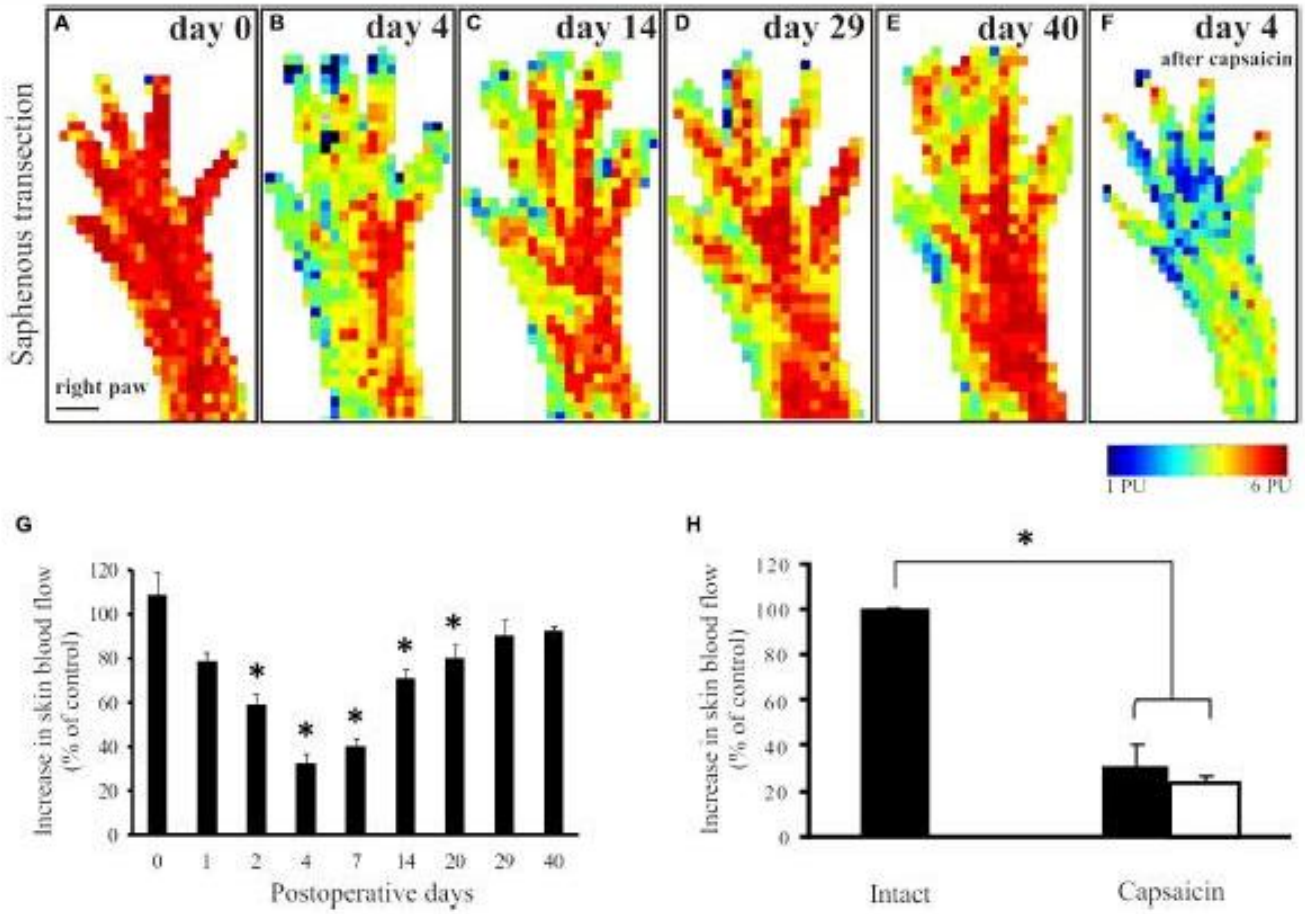

FIGURE 2 | Mustard oil-induced changes in blood flow of the dorsal hindpaw skin after transection and ligation of the saphenous nerve. Original perfusion images demonstrate mustard oil-induced vasodilatatory responses before **(A)**, and 4 **(B)**, 14 **(C)**, 29 **(D)**, and 40 **(E)** days after surgery. Note the gradual recovery of the vasodilatatory response in the medial aspect of the hindpaw. Image **F** illustrates the effect of the selective defunctionalization by perineural capsaicin treatment of sciatic afferents on the mustard oil-induced vasodilation. Note the marked reduction of the vasodilatatory response in both the lateral and the medial aspects of the dorsal hindpaw skin served by the sciatic and saphenous nerves, respectively, after capsaicin treatment. **G:** The histogram shows the quantitative data on changes in the vasodilatatory responses following saphenous nerve transection. Note the marked decrease shortly after transection and the gradual recovery over a period of 4–5 weeks, indicating denervation and reinnervation of the denervated saphenous skin area. **H:** Forty days after saphenous nerve transection and four days after perineural capsaicin treatment of the sciatic nerve significant reductions of mustard oil-induced vasodilatation in both the medial (filled column) and lateral (empty column) aspect of the dorsal hindpaw skin were detected as compared to the vasodilatatory response measured in the contralateral dorsal hindpaw skin (*$p < 0.05$; $n = 8$). Scale bar on A represents 5 mm.

Peripheral Nerve Transection

The right saphenous nerve was exposed high in the thigh and transected distal to a ligature. To prevent regeneration of the nerve, a 0.5 cm long segment of the distal stump was removed. The wound was then closed and the rat was returned to the animal house.

Perineural Capsaicin Treatment

Perineural application of capsaicin was performed as described by Jancsó et al. (1980b). Briefly, the right saphenous nerve was exposed high in the thigh, isolated from the surrounding tissues with Parafilm® (Sigma-Aldrich) and wrapped with a small piece of gelfoam soaked with 0.1 ml of a 1% solution of capsaicin (Sigma, Saint Louis, United States) dissolved in saline containing 6% ethanol and 8% Tween 80. After 20 min, the gelfoam was removed, the wound was closed, and the rat was returned to the animal house.

Measurement of Cutaneous Blood Flow With Scanning Laser Doppler Flowmetry

Scanning laser Doppler flowmetry was used to measure cutaneous blood flow in the dorsal skin of the rat hindpaw by capturing consecutive perfusion images with a PeriScan PIM3 scanning laser Doppler imager (Perimed, Järfälla, Sweden). The rats were anesthetized with a combination of ketamine and xylazine and then were placed on a heating pad to keep their body temperature at 37 ± 0.5°C. Room temperature was kept at 22–23°C. The dorsal surface of both hindpaws was scanned by using the repeated scan mode with 52 × 42 pixel frame size. Distance of the scanner aperture from the skin surface was set to 19 cm and the scanner was positioned to ensure that

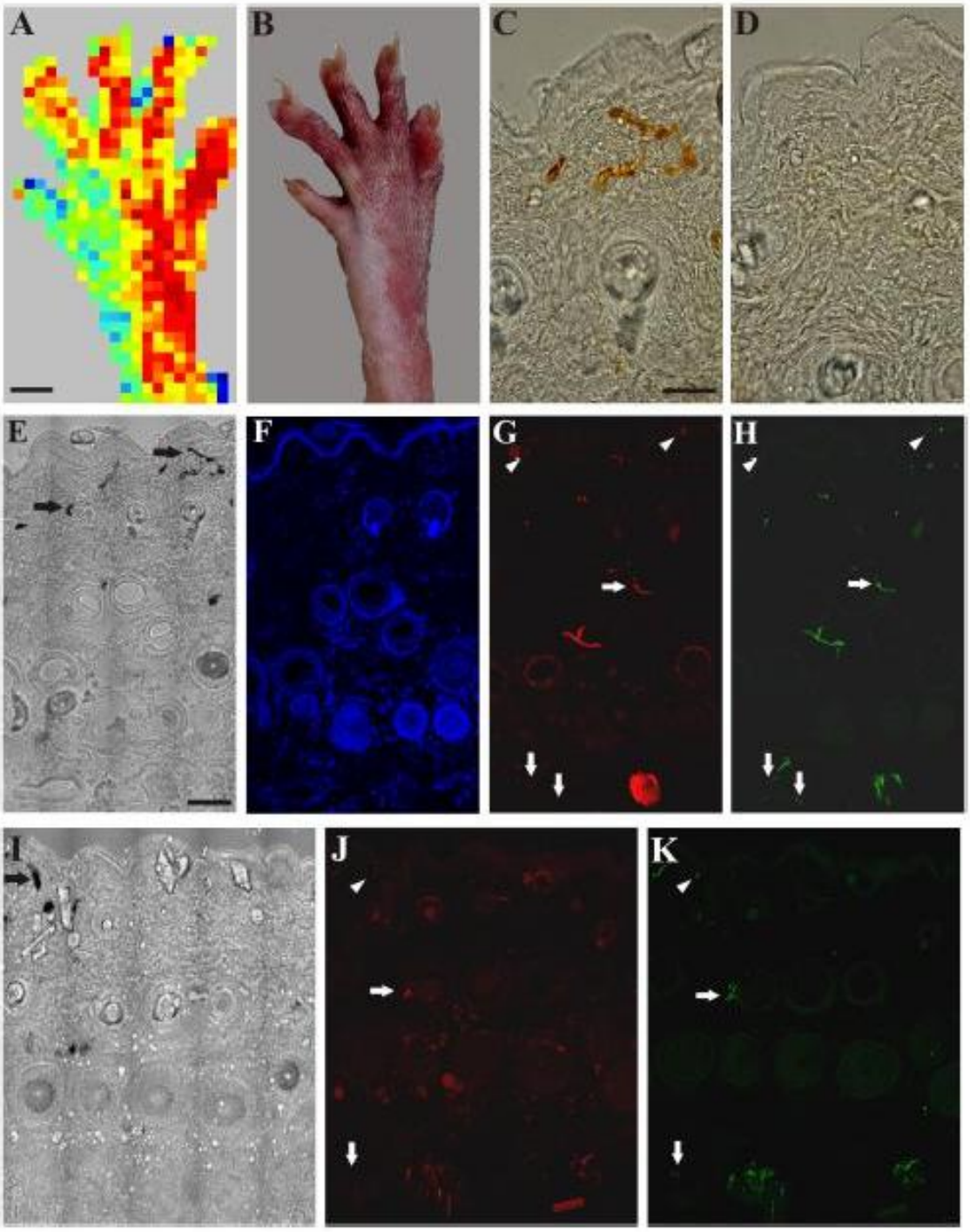

FIGURE 3 | A: Application of mustard oil increased skin blood flow in the innervated (sciatic) but not in the denervated (saphenous) skin area of the right hind paw as assessed with scanning laser Doppler imaging. **B:** Brownish coloration of the skin due to silver accumulation in the innervated (sciatic) but not in denervated (saphenous) skin area of the dorsal hind paw skin elicited by mustard oil. **C,D**: Bright field photomicrographs showing sections from the lateral intact **(C)** and medial denervated regions of the hind paw skin after transection of the saphenous nerve **(D)**. Silver-labeled small venules indicate increased vascular permeability of small postcapillary venules elicited by mustard oil application in the intact dorsal hind paw skin **(C)**. Silver-labeled blood vessels cannot be observed in the denervated skin 4 days after saphenous nerve transection **(D)**. **E–H**: Bright field **(E)** and immunofluorescence **(F–H)** photomicrographs showing the lateral (sciatic) skin area of the dorsal hind paw skin **(E–H)** after transection of the saphenous nerve. **I–K**: Bright field **(I)** and immunofluorescence **(J,K)** photomicrographs showing the medial (saphenous) skin area of the dorsal hind paw skin of a rat 15 days after transection and ligation of the saphenous nerve. Note the localization of ß-tubulin III (red) and CGRP-immunoreactive (green) nerve fibers in the epidermis (arrowheads), and around hair follicles and small arteries (arrows) in the innervated (lateral, sciatic) area of the dorsal hind paw skin identified by the presence of silver-labeled (permeable) venules (arrows in **E**). Fifteen days after transection and ligation of the saphenous nerve, some silver-labeled venules (arrow in **I**) and some ß-tubulin III and CGRP-immunoreactive epidermal (arrowheads in **J,K**) and dermal (arrows in **J,K**) nerve fibers can be observed in the medial (saphenous) skin area of the dorsal hind paw skin indicative of (collateral) regeneration. Scale bars indicate 5 mm in **A** and **B** and 50 μm in **C,E**. Scale bars in **C,E** apply for **C,D** and **E–K**, respectively.

the laser beam was perpendicular to the skin surface. Perfusion images were captured in every 2 min and measurements took 15–20 min in each animal. All flow values were expressed as means ± S.E.M. Basal tissue perfusion and changes in blood flow induced by mustard oil (5% in liquid paraffin) were recorded in arbitrary perfusion units (PU) and expressed as per cent change relative to baseline. The value of the PU integrates the linear velocity values and the concentration of moving erythrocytes in the skin volume fraction detected by the scanner at any instances (Rajan et al., 2009). Mustard oil was applied onto the intact unshaved hairy skin of the paw. Baseline values were obtained by calculating the average of three subsequent measurements before the application of mustard oil. For quantitative evaluation, images displaying the maximum vasodilatatory responses were used in each experiment.

Scanning laser Doppler images were taken before surgery and 1–40 days after saphenous nerve transection. After finishing the sequential measurement of the recovery of sensory vasodilatation (4th week post surgery) an additional measurement was made 4 days after perineural treatment of the sciatic nerve with capsaicin.

The innervation territory of the saphenous nerve was defined on the basis of perfusion images taken 4 days after transection the saphenous nerve. In each experiment the color-coded perfusion images showing the maximal vasodilatatory response were selected for further processing with the ImagePro 6.2 image analysis software (MediaCybernetics, Rockville, MD, United States). After subtracting background pixel values, color segmentation was applied on the perfusion image to demarcate and separate areas showing no or minimal vasodilation from those exhibiting large (or maximal) perfusion increases. This step was followed by the generation of a binary mask representing the size and topography of denervated cutaneous areas corresponding to the innervation area of the saphenous nerve. Functional reinnervation was characterized by measuring the intensity of the vasodilatatory response in the saphenous skin area as defined above.

Demonstration of Cutaneous Nerve Fibers and Permeable Blood Vessels After Induction of Neurogenic Inflammation With Mustard Oil

Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and the dorsal skin of the hind paw was painted with mustard oil (allyl isothiocyanate, 5% in liquid paraffin) after an intravenous injection of a colloidal silver solution (1% in 5% glucose, 100 mg/kg). Twenty min later the animals were terminally anesthetized and perfused transcardially with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. The dorsal hind paw skin was removed and postfixed for another 2 h. After washing in phosphate buffer containing 30% sucrose overnight, frozen sections were cut and free-floating sections were processed for immunohistochemical staining with antibodies against tubulin and CGRP. Monoclonal mouse anti-ß-tubulin III and polyclonal rabbit anti-CGRP antibodies were obtained from Sigma-Aldrich (St. Louis, Missouri, United States) and used at a dilution of 1:4500. Sections were mounted on slides and covered with ProLong® Gold antifade medium (Invitrogen, Carlsbad, Calif., United States). Specimens were examined under a Zeiss LSM 700 confocal laser scanning microscope. Tile scan and z-stack maximum intensity projection images were obtained to illustrate the findings.

Statistics

Data represent means ± S.E.M. of 5–9 independent measurements. For statistical comparisons of the mustard oil-induced vasodilatatory responses one-way ANOVA was performed followed by multiple comparisons using the Dunett's *post hoc* analysis. In all groups, normality was proved by the Shapiro-Wilk test and homogeneity of variances was confirmed by Levene's test in advance of performing ANOVA. Statistical analysis was performed by using Statistica 6.4 software (Dell Inc., Tulsa, OK, United States).

RESULTS

Scanning Laser Doppler Imaging of Cutaneous Blood Flow of the Dorsal Skin of the Rat Hindpaw: The Effect of Sensory Denervation

Scanning laser Doppler imaging revealed a largely uniform perfusion in the intact rat hindpaw skin (**Figure 1A**). The coefficients of variance of the basal perfusion values recorded on subsequent perfusion images representing the basal blood perfusion of the hind paw were 0.043 ± 0.28 (medial aspect) and 0.054 ± 0.027 (lateral aspect) with no significant difference between the two sides (p = 0.19). Application of mustard oil onto the skin elicited a marked increase in cutaneous blood flow with a maximum at 2–4 min (**Figures 1B,K**). The maximum increase in blood flow amounted to 87 ± 18% of the initial basal value and returned to the pre-application level after about 15–20 min (**Figures 1C–E,K**). This vasodilatatory response could be repeatedly elicited by mustard oil resulting in similar perfusion patterns (data not shown). Basal blood flow measured in the denervated (saphenous) skin area was similar to that of the intact lateral (sciatic) area of the dorsal skin of the hindpaw (**Figure 1F**). However the mustard oil-induced vasodilatatory response was strongly reduced in the medial aspect of the dorsal hindpaw skin 3 days after saphenous nerve denervation (**Figures 1G,H** decline of the response is shown on **Figures 1I,J**). As illustrated in **Figure 1L**, the magnitude of the vasodilatatory response was reduced significantly in the medial aspect of the dorsal hindpaw skin served by the saphenous nerve, as compared with the lateral side innervated by the intact sciatic nerve.

Effect of Saphenous Nerve Transection on Mustard Oil-Induced Vasodilation in the Dorsal Hindpaw Skin

In animals to be subjected to transection and ligation of the saphenous nerve, mustard oil-induced vasodilatatory responses

were measured shortly before surgery to obtain reference perfusion images (**Figure 2A**). Basal blood flow values did not differ significantly in the denervated and innervated areas of the dorsal hindpaw skin (**Figure 1F**). Four days but not one day after saphenous nerve transection mustard oil-induced increase in cutaneous blood flow was markedly reduced in the medial part of the dorsal hindpaw skin served by the saphenous nerve. Perfusion values in the skin area normally served by the saphenous nerve amounted to about 32 ± 4% of the values measured in the lateral hindpaw skin innervated by the intact sciatic nerve (**Figures 2B,G**). The vasodilatatory response gradually recovered within the medial skin area of the dorsal hindpaw. The re-appearance of mustard oil-induced vasodilation first commenced in the lateral aspect of the saphenous innervation territory immediately adjacent to the skin area innervated by the intact sciatic nerve (**Figures 2C,D**). The area displaying mustard oil-induced vasodilation gradually spread toward the medial aspect of the dorsal hindpaw skin and finally, after about 4 weeks the topography of the vasodilatatory response was similar to that seen before surgery (**Figure 2E**). To identify the origin of nerve fibers which reinnervated the medial part of the dorsal hindpaw skin served normally by the saphenous nerve, a second surgery was performed. The sciatic nerve was treated locally with capsaicin to defunctionalize chemosensitive afferent nerves (Jancsó et al., 1980b; Gamse et al., 1980) which mediate the sensory neurogenic vasodilatatory response (Lembeck and Holzer, 1979; Lynn et al., 1996; Domoki et al., 2003). Examination of the mustard oil-induced vasodilatatory response 3–4 days after perineural capsaicin revealed a marked reduction of the vasodilatatory response not only in the lateral skin area, innervated by the sciatic nerve, but also in the medial skin area normally served by the saphenous nerve (**Figures 2F,H**). This finding strongly indicates that recovery of the vasodilatatory response in the medial part of the dorsal hindpaw skin, normally served by the saphenous nerve, may be attributed to sciatic afferents which innervate the denervated saphenous skin area through collateral sprouting.

Immunohistochemical Demonstration of Degeneration and Regeneration of Cutaneous Nerves After Peripheral Nerve Lesions

Application of mustard oil resulted in clear-cut vascular labeling in subepidermal small blood vessels of skin areas of intact sensory innervation (perfusion image: **Figure 3A**). Silver deposits were observed in the wall of permeable small blood vessels (**Figures 3B,C**). In contrast, in the denervated skin vascular labeling could not be observed (**Figures 3B,D**). Immunohistochemistry revealed many tubulin- and/or CGRP-immunoreactive nerve fibers in the epidermis, around hair follicles and small blood vessels of the innervated skin (**Figures 3E–H**). Four days after nerve transection, nerve fibers were absent in the denervated saphenous skin areas, but the innervation of the skin area served by the intact sciatic nerve was similar to control (data are not shown). Fourteen days after saphenous nerve transection, a few silver-labeled blood vessels could be detected in the previously denervated skin area parallel with the re-appearance of a few epidermal and subepidermal tubulin- and CGRP-immunoreactive nerve fibers indicating regeneration of the denervated skin area (**Figures 3I–K**). These nerves were completely depleted after perineural treatment of the sciatic nerve with capsaicin. Hence, these immunohistochemical findings extend and support our observations on the regeneration of the denervated skin as assessed with the aid of scanning laser Doppler flowmetry.

DISCUSSION

Longitudinal evaluation of restitution of cutaneous sensory function is essential to unravel the progress and mechanisms of nerve regeneration following peripheral nerve lesions of various origins. The findings of the present study demonstrate that repeated scanning laser Doppler imaging of cutaneous blood flow is a reliable method for the longitudinal examination of the progress of regeneration of a particular population of peptidergic nociceptive cutaneous nerves. Stimulation of cutaneous nociceptive nerve endings with mustard oil results in a marked increase of local blood flow in intact but not denervated skin areas. Previous studies have shown that mustard oil is a potent activator of the TRPA1 nociceptive ion channel (Jordt et al., 2004; Bautista et al., 2006). Although mustard oil was regarded as a selective agonist of the TRPA1 receptor, recent findings challenged this view by showing activation also of the TRPV1 receptor by this compound suggesting that besides the TRPA1 receptor, the TRPV1 receptor is also involved in the transmission of nociceptive impulses elicited by mustard oil (Everaerts et al., 2011; Gees et al., 2013). However, these studies also disclosed that mustard oil-induced inflammation is mainly mediated by the TRPA1 receptor (Everaerts et al., 2011; Gees et al., 2013). This is in accord with our previous observations showing that the vasodilatatory effect of this agent is largely mediated by the activation of the TRPA1 receptor in the rat hindpaw skin (Boros et al., 2016). The vasodilation elicited by activation of the TRPA1 receptor by mustard oil is mediated by the potent vasodilatatory peptide CGRP contained and released from the stimulated nociceptive nerve endings (Brain et al., 1985; Sauerstein et al., 2000; Pozsgai et al., 2010). This notion is also supported by the close spatial correlation of the distribution of CGRP-containing chemosensitive afferent nerves and vascular changes associated with neurogenic inflammation in the rat skin (Sann et al., 1995).

Denervated skin areas displayed markedly attenuated vasodilatatory responses upon exposure to mustard oil. The slight mustard oil-induced increase in blood flow measured in the denervated skin areas may be attributed to possible non-neural mechanism(s) or, alternatively, to activation of TRPV1 receptors (Grant et al., 2005; Everaerts et al., 2011; Gees et al., 2013).

Mustard oil-induced vasodilation was markedly reduced in the denervated skin 3–4 days after nerve transection and was abolished completely by the 4th post-operative

day. The vasodilatatory response was similar to controls 1 day after nerve transection, since at this post-lesion time cutaneous nerve endings are still functional until the onset of the rapid phase of Wallerian degeneration 1–3 days after injury (Jancsó and Király, 1983; Sta et al., 2014). Repeated measurements of mustard oil-induced vasodilatatory responses revealed a gradual, time-dependent re-appearance of the vasodilatatory response in the denervated skin. Following saphenous nerve transection, the size of the skin area displaying negligable vasodilatatory responses after denervation gradually decreased. By the 3rd-4th post-operative week, the vasodilatatory response was similar to the control in both size and intensity. To furnish independent data on the reliability of scanning laser Doppler flowmetry to examine cutaneous nerve function, immunohistochemical demonstration of tubulin- and CGRP-positive nerves were performed in intact, denervated and reinnervated skin after induction of neurogenic inflammation with mustard oil. In accord with previous findings, small blood vessels, mainly postcapillary venules were labeled with colloidal silver. No such labeled venules were seen in the denervated skin. Re-appearance of tubulin- and CGRP-immunoreactive nerve fibers in the previously denervated saphenous skin area supported our findings obtained with scanning laser Doppler flowmetry. The nerve fibers in the saphenous skin area were completely depleted after perineural treatment of the sciatic nerve with capsaicin, a treatment which induces a complete elimination of nociceptive afferent nerves (Jancsó et al., 1980b; Dux et al., 1998; Domoki et al., 2003; Kang et al., 2010). Hence, immunohistochemical findings supported our observations obtained with scanning laser Doppler flowmetry. This time-course of functional recovery observed in the previously denervated saphenous innervation area is similar to that observed in studies using behavioral testing or the Evans blue technique to examine the temporal characteristics of functional cutaneous nerve regeneration (Devor et al., 1979; Bharali and Lisney, 1988).

Previous studies have demonstrated that reinnervation of denervated skin areas may be effected by two different mechanisms: regeneration of the injured nerve and reinnervation by collateral sprouting (Devor et al., 1979; Brenan, 1986; Diamond et al., 1987; Kinnman et al., 1992). The re-innervation of the denervated skin following nerve crush occurs through regenerative sprouting of the injured nerve. The restitution of function is almost complete after nerve crush (Devor et al., 1979; Wiesenfeld-Hallin et al., 1988). If regeneration of the injured nerve is prevented by ligation of the transected nerve, as in the present study, restitution of sensory function in the denervated skin is accomplished through collateral sprouting of axons of an intact peripheral nerve serving skin areas adjacent to the denervated skin (Devor et al., 1979; Diamond et al., 1987; Pertovaara, 1988). Under the experimental conditions of the present study, intact sciatic nerve axons were expected to invade, by collateral sprouting, the denervated saphenous skin area. Our findings indicate that, indeed, this is the case. In the denervated saphenous nerve territory, the mustard oil-induced vasodilatatory response gradually recovered within a period of 1-4 weeks after transection and ligation of the saphenous nerve and, importantly, this could be largely abolished by perineural treatment of the sciatic nerve with capsaicin. Perineural capsaicin treatment was used to selectively defunctionalize sciatic nociceptive afferents (Jancsó et al., 1980a; Jancsó et al., 2008; Jancsó and Király, 1983; Petsche et al., 1983; Pini and Lynn, 1991; Domoki et al., 2003) sparing efferent autonomic and motor nerve fibers (Jancsó et al., 1987). Thus, inhibition by perineural capsaicin treatment of the mustard oil induced vasodilation not only in the lateral, but also in the medial aspect of the dorsal skin of the hindpaw, strongly indicates that reinnervation of the denervated saphenous skin area was accomplished by collateral sprouting of adjacent intact sciatic afferent fibers. This observation is in accord with previous findings which applied the Evans blue technique and behavioral testing to demonstrate the reinnervation of the denervated skin by collateral sprouting of nociceptive afferent nerves of neighboring skin areas (Devor et al., 1979; Diamond et al., 1987; Pertovaara, 1988).

In conclusion, the present observations indicate that repeated scanning laser Doppler imaging of the mustard oil-induced vasodilatatory response is a reliable technique for the longitudinal study of cutaneous nerve regeneration being suitable for the follow-up of changes of both the topographical distribution and the intensity of the vasodilatatory response, most probably proportional with cutaneous innervation density. An obvious limitation of this technique is that it does not provide information on the regeneration of other types of cutaneous sensory nerves, for example myelinated mechanoreceptors. Noteworthy, it has been shown that low threshold myelinated afferents lack the ability to collaterally grow into a denervated skin area (Jackson and Diamond, 1984). In addition, however, previous findings suggested that regeneration of different types of cutaneous nerves does not occur simultaneously; unmyelinated fibers regenerate more rapidly as compared to myelinated axons (Allt, 1976; Duraku et al., 2013). Human and rodent nociceptive cutaneous nerves share many functional and neurochemical traits, including the sensitivity to chemical irritants such as mustard oil and capsaicin (Jancsó, 1960; Jancsó et al., 1985; Hou et al., 2002). In the human skin mustard oil produces local and axon reflex vasodilation in the intact but not in the denervated skin (Jancsó and Janka, 1981; Jancsó et al., 1983, 1985). Demonstration of mustard oil-induced sensory neurogenic vasodilation has been used to detect the functional condition of cutaneous sensory nerves in man under physiological and pathological conditions (Jancsó and Janka, 1981; Jancsó et al., 1983; Westerman et al., 1987). Hence, with some modifications, the approach presented in this report may be applied also in human studies aimed at the examination of cutaneous nerve function affected by toxic environmental and medicinal agents, such as anticancer chemoterapeutics or by pathologies such as diabetes mellitus.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author, PS.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Care at the University of Szeged as per the Council Regulation of 40/2013 (II. 14.) and were carried out in full accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

AUTHOR CONTRIBUTIONS

GJ, PS, and SL were responsible for the study concept and design. SL, ÁH, ID, GJ and PS were responsible for the data collection and analysis. SL, ÁH, GJ, and PS interpreted the data, and revised the manuscript for intellectual content. All authors were involved in manuscript editing and have approved the final version for submission.

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II.

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Perineural Capsaicin Treatment Inhibits Collateral Sprouting of Intact Cutaneous Nociceptive Afferents

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Abstract: Perineural treatment of peripheral nerves with capsaicin produces a long-lasting selective regional thermo- and chemo-analgesia and elimination of the neurogenic inflammatory response involving degeneration of nociceptive afferent fibers. In this study, we examined longitudinal changes in mustard oil–induced sensory neurogenic vasodilatation and plasma extravasation following perineural capsaicin treatment of the rat saphenous nerve utilizing scanning laser Doppler imaging and vascular labeling with colloidal silver. Capsaicin treatment resulted in a marked decrease in mustard oil–induced vasodilatation in the skin area served by the saphenous nerve. Repeated imaging of the vasodilatatory response showed no recovery for at least 7 weeks. However, following transection and ligation of the capsaicin-treated saphenous nerve, a substantial recovery of the vasodilatatory response was observed, suggesting a reinnervation of the chemodenervated skin area by collateral sprouting of neighboring intact sciatic nerve afferents. Elimination of the recovered vascular reaction by capsaicin treatment of the sciatic nerve supported this conclusion. Similar results have been obtained by using the vascular labeling technique. These findings indicate an inhibitory effect of persisting cutaneous nerve fibers on the collateral sprouting of intact nerve fibers into the chemodenervated skin area. These observations may bear implications for the development of sensory disturbances following peripheral nerve injuries.

Keywords: collateral sprouting; neurogenic inflammation; capsaicin TRPV1; TRPA1; scanning laser Doppler perfusion imaging

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1. Introduction

Injuries inflicted upon peripheral nerves result in the immediate functional deterioration of sensory functions often associated with short-term degenerative and delayed regenerative processes. Damage to peripheral nerves may cause either an indiscriminate lesion in all types of nerve fibers or may be directed towards a specific class or classes of axons. Nerve transection and crush produce loss of function in all nerve fibers running in the affected nerve, whereas in metabolic (diabetes) or toxic (alcohol, antitumor drugs, antibiotics, toxic metals) neuropathies specific classes of axons may be affected [1–3]. Capsaicin, the pungent agent in red peppers, is a selective neurotoxin acting through the transient receptor potential vanilloid type 1 receptor (TRPV1), selectively targeting C-fiber nociceptive afferent axons [4]. Local application of capsaicin and related vanilloids onto peripheral nerve trunks produces a selective defunctionalization and/or chemodenervation of nociceptive afferent axons, resulting in an apparently permanent regional nociceptor analgesia characterized by a loss of sensitivity to pain-producing chemical irritants, profound decrease in heat-pain sensitivity and the abolition of the neurogenic inflammatory response strictly confined to the innervation territory of the affected nerve [5–10]. Immunohistochemical and quantitative electron microscopic findings revealed that this may

be, at least in part, accounted for by a loss of unmyelinated, C-fiber afferent axons in the capsaicin-treated nerve and the skin area innervated by that nerve [7,11,12]. It was also shown that, unlike after nerve transection or crush lesions [13,14], the denervated skin area showed no signs of functional reinnervation by regenerative or collateral sprouting of the affected or the adjacent intact nerves, respectively [15]. However, due to the lack of techniques capable of a quantitative, longitudinal evaluation of the process of functional reinnervation of the denervated skin by peptidergic nociceptive C-fibers, the temporal and spatial patterns of degenerative and regenerative processes could not be investigated. Recently, we have developed a novel experimental approach which permits the longitudinal assessment of the functional regeneration of peptidergic nociceptive afferents following peripheral nerve transection. The technique is based on the assessment of the intensity and spatial distribution of mustard oil–induced changes in skin blood flow by utilizing scanning laser Doppler imaging [16]. Mustard oil is an agonist of transient receptor potential ankyrin type 1 (TRPA1) receptors [17], which are expressed in all TRPV1 receptor–expressing nociceptive primary sensory neurons [18,19]. Importantly, mustard oil has also been shown to be a potent activator of the TRPV1 receptor [20]. Activation of these receptors by mustard oil results in the release of the neuropeptides calcitonin gene–related peptide (CGRP) and substance P from sensory nerve endings, which in turn elicit neurogenic sensory vasodilatation and plasma extravasation, collectively termed the neurogenic inflammatory response, a salient feature of peptidergic nociceptive afferent function [21–24]. Previous studies have shown that skin areas delineated by mustard oil–induced increased vascular permeability as assessed with the Evans blue method [14,15,25–27] or the vascular labeling technique [7] correspond to the innervation territories of nerve endings stimulated by antidromic electrical nerve stimulation or the direct application of chemical irritants onto the skin. Importantly, cutaneous areas displaying sensory nerve stimulation–induced vasodilatation and plasma extravasation are strictly coincident with the innervation territory of the stimulated nerve [7,16,28]. Therefore, topographical localization of mustard oil–induced vasodilatation and plasma extravasation can be used to assess the pattern of cutaneous innervation in naïve and nerve-injured animals [14–16,26].

The present study was initiated to elucidate the regenerative propensity of intact and capsaicin-treated nerves serving the dorsum of the rat hind paw through longitudinal examination of changes in the topography and intensity of mustard oil–induced sensory neurogenic vasodilatation and plasma extravasation in the rat. Application of the novel experimental paradigm utilizing scanning laser Doppler imaging was expected to reveal the time course and mechanisms of collateral sprouting of cutaneous afferents following a specific chemical lesion of nociceptive sensory neurons. The findings may also be of interest with respect to the possible therapeutic application of TRPV1 agonists for pain relief.

2. Materials and Methods

Experiments were performed on adult male Wistar rats (n = 18) weighing 250–280 g at the beginning of the experiments. The animals were kept in a 12 h light/dark cycle in standard cages with free access to food and water. All experiments were carried out according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Council Regulation 40/2013 (II. 14.). The experimental protocol was reviewed by the Ethics Committee for Animal Care at the University of Szeged. The number of experimental animals was kept as low as possible.

2.1. Surgery

Rats were anaesthetized with an intraperitoneal injection of a combination of ketamine (Calypsol, 70 mg/kg, Gedeon Richter, Budapest, Hungary) and xylazine (CP-Xylazin 2%, 10 mg/kg, Produlab Pharma, Raamsdonksveer, The Netherlands). Animals were placed on a heating pad to keep their body temperature at a constant level of 37 ± 0.5 °C. Room temperature was kept at 22–23 °C.

2.2. Perineural Capsaicin Treatment

The right saphenous nerve was exposed in the thigh, isolated from the surrounding tissues with Parafilm® (Sigma-Aldrich, St. Louis, MO, USA) and a small piece of gel foam soaked with 0.1 mL of a 1% solution of capsaicin (Sigma-Aldrich, St. Louis, MO, USA; dissolved in saline containing 6% ethanol and 8% Tween 80) was applied around the nerve. After 20 min, the gel foam was removed, the wound was closed, and the rat was returned to the animal cage (Figure 1A).

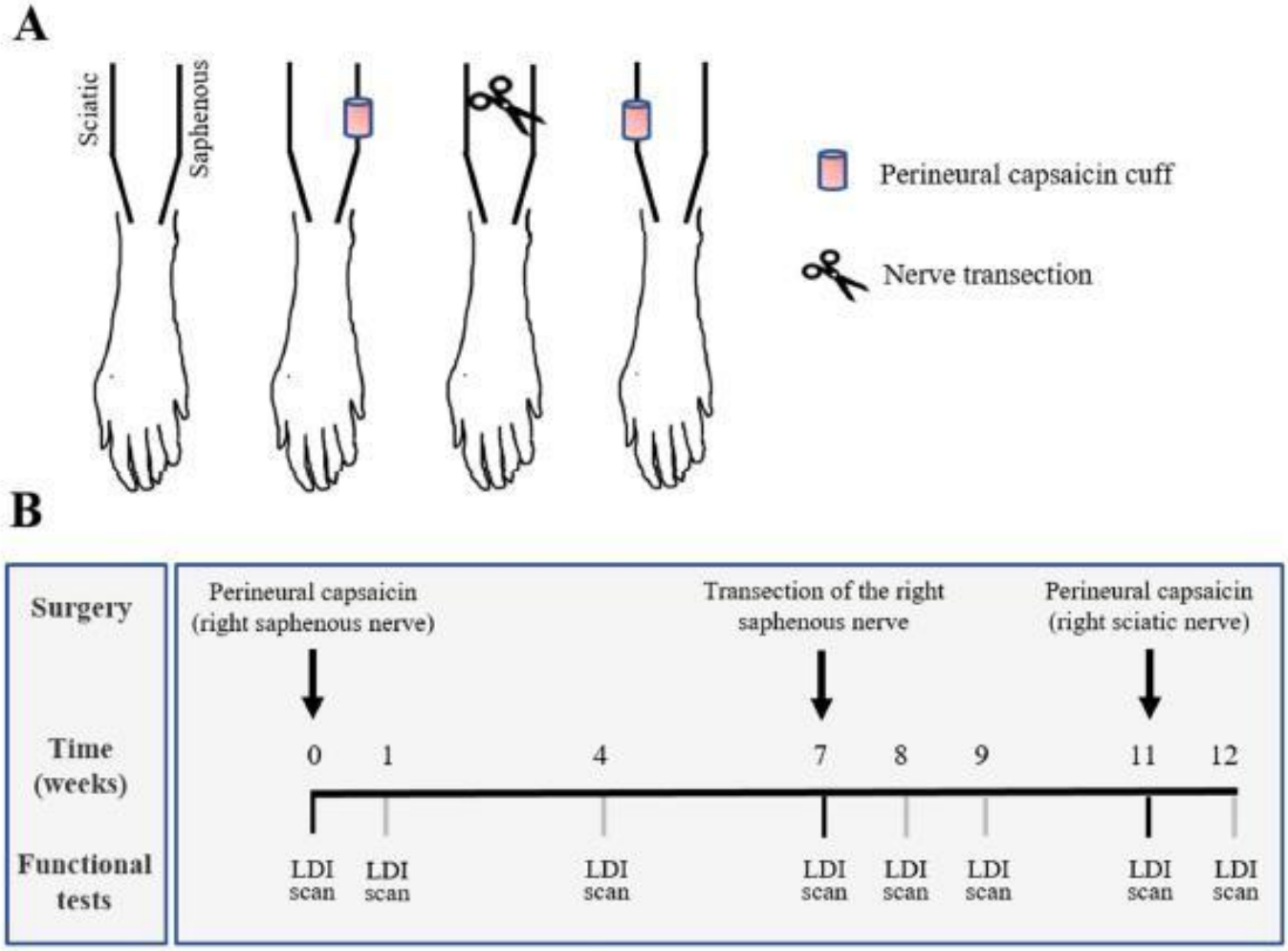

Figure 1. (**A**) Schematic illustration of the types and sequence of surgical interventions applied in this study. (**B**) Timeline of the experiments illustrating the sequence of surgical interventions and functional testing with laser Doppler imaging (LDI) following perineural capsaicin treatment of the saphenous nerve.

2.3. Peripheral Nerve Transection

After a follow-up period of 7 weeks, the previously capsaicin-treated right saphenous nerve was exposed high in the thigh and transected distal to a ligature (Figure 1A). To prevent regeneration of the nerve, a 0.5 cm long segment of the distal stump was removed. The wound was then closed and the rat was returned to the animal cage.

2.4. Measurement of Cutaneous Blood Flow with Scanning Laser Doppler Flowmetry

Rats were anaesthetized with a combination of ketamine (Calypsol, 70 mg/kg) and xylazine (CP-Xylazin 2%, 10 mg/kg). Scanning laser Doppler flowmetry using a PeriScan PIM3 scanning laser Doppler imager (Perimed, Järfälla, Sweden) was utilized to determine

changes in the intensity and topographical distribution of chemically-induced increases in cutaneous blood flow following peripheral nerve lesions. The dorsal surface of both hind paws was scanned by using the repeated scan mode with 52 × 42 pixel frame size. Distance of the scanner aperture from the skin surface was set to 19 cm and the laser beam was perpendicular to the skin surface. Perfusion images were captured every 2 min and measurements took 15–20 min for each animal. All flow values were expressed as means ± S.D. Basal tissue perfusion and changes in blood flow induced by painting the skin of the dorsum of the hind paw with mustard oil (5% in liquid paraffin) were recorded in arbitrary perfusion units (PU) and expressed as per cent change relative to baseline. The linear velocity values and the concentration of moving erythrocytes in the skin volume fraction detected by the scanner at any instances are combined in the PU value [29]. The average of three subsequent measurements before the application of mustard oil was calculated to determine baseline value. Images displaying the maximum vasodilatatory responses were used in each experiment to quantitatively evaluate the reactions. Scanning laser Doppler images were taken before surgery and 1–7 weeks after perineural capsaicin treatment of the saphenous nerve. Additional measurements were made 1–4 weeks after transection of the saphenous nerve previously treated with capsaicin (Figure 1B).

2.5. Evaluation of Perfusion Data

The innervation territory of the saphenous nerve was defined on the basis of perfusion images taken 1 week after perineural capsaicin treatment of the saphenous nerve. Color-coded perfusion images representing the maximal vasodilatation were further processed with the ImagePro 6.2 software (MediaCybernetics, Rockville, MD, USA). Changes in the intensity of the vasodilatatory responses measured in the saphenous and sciatic skin areas were considered as evidence of functional reinnervation.

2.6. Separation of Innervated and Denervated Skin Areas

The color-coded perfusion images showing the maximal vasodilatatory response were used to determine the proportion of innervated and denervated skin areas, respectively. Data of all pixels were normalized to the 90th percentiles of the maximal response, then the 20th percentile of the lateral (intact) side was calculated to define a threshold value for each individual image. Pixels with data lower than the 20th percentiles of the intact side were considered as denervated to separate areas showing no or minimal increase in vasodilation from those exhibiting large (or maximal) perfusion increases. This step was followed by the generation of a binary mask representing the size and topography of denervated cutaneous areas corresponding to the innervation area of the saphenous nerve.

2.7. Vascular Labeling Technique

To study the size and topographical distribution of the innervated and denervated skin areas, another functional-morphological method, the vascular labeling technique was also used. This technique enables the visualization of small blood vessels of increased permeability to colloid particles. Hence, vascular labeling is a salient feature of the (neurogenic) inflammatory response [25,30]. Vascular labeling experiments were performed 4 weeks after perineural capsaicin treatment of the saphenous nerve, and 2, 3 and 4 weeks after transection of that same nerve. Briefly, the anesthetized animals were injected intravenously with 1% solution of colloidal silver (Sigma-Aldrich, St. Louis, MO, USA; 50 mg/kg b.w.) and the right hind paw skin was treated with 5% mustard oil solution immediately after completion of the injection. The vascular labeling of small blood vessels (venules) exhibiting increased permeability was observed as intense brownish color of the innervated skin in sharp contrast with the denervated skin regions, where the reaction is absent. Close-up photographs were made to document the extension of cutaneous vascular labeling. The size of the total skin area of the hind paw and the proportions of the innervated and denervated skin areas were determined with planimetry using ImagePro software. To visualize the topographical distribution of vascular labeling under the light microscope, the dorsal

skin of the hind paw was removed, flattened, dehydrated in graded alcohols and made transparent with xylene. The specimens were mounted on glass slides and cover-slipped with Canada balsam.

2.8. Statistics

Data represent means ± S.D. of 4–9 independent measurements. For statistical comparisons of the mustard oil–induced vasodilatatory responses and the change in the proportions of innervated and denervated skin areas, respectively, the one-way ANOVA test was performed followed by multiple comparisons using Fisher's least significant difference test (time course of mustard oil–induced vasodilation) or Dunnett's post hoc analysis. In all groups, normality was proved by the Shapiro–Wilk test and homogeneity of variances was confirmed by Levene's test in advance of performing ANOVA. Statistical analysis was performed by using Statistica 13.0 software (Dell Inc., Tulsa, OK, USA).

3. Results

3.1. Effect of Perineural Capsaicin Treatment of the Saphenous Nerve on Mustard Oil–Induced Neurogenic Vasodilatation in the Rat Hindpaw

In accord with previous findings, changes in cutaneous blood flow could be reliably demonstrated with scanning laser Doppler imaging. Application of mustard oil onto the dorsum of the intact rat hind paw resulted in an increase in skin perfusion which reached its maximum after 2 min and amounted to 78.76 ± 15.42 and 72.64 ± 17.85 percent of the basal value in the lateral and medial parts, respectively, of the dorsal skin of the right hind paw. The vasodilatatory response gradually subsided and returned to the initial basal value after about 15 min (Figure 2A). One week after perineural capsaicin treatment of the right saphenous nerve, the dorsal skin of the intact (left) hind paw and the lateral part of the dorsal skin of the right hind paw displayed marked increases in blood flow. In contrast, in the medial part of the dorsal skin of the righthind paw, mustard oil–induced increase in perfusion was markedly and significantly reduced amounting only to 39.03 ± 16.38 percent of the basal value (Figure 2B). A series of perfusion images taken from the dorsal skin of the hind paws 1 week after perineural capsaicin treatment of the right saphenous nerve illustrates these changes (Figure 2C). These findings demonstrate that perineural capsaicin treatment of the saphenous nerve resulted in an impairment of the "efferent" vasodilatatory function of peptidergic afferent nerves which innervate the medial aspect of the dorsum of the rat hind paw.

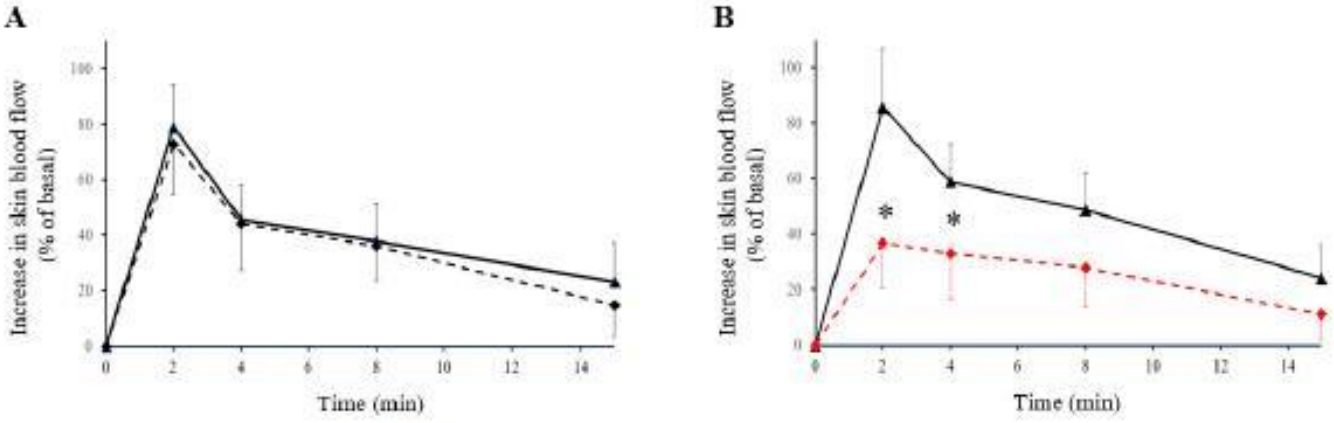

Figure 2. *Cont.*

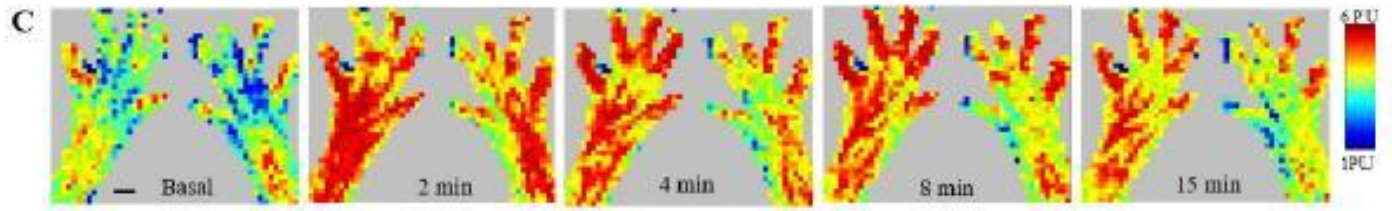

Figure 2. (**A**) Time course of mustard oil–induced increases in perfusion of the medial (dashed line) and lateral (solid line) parts of the dorsal skin of the right hind paws of rats ($n = 5$). (**B**) Time course of mustard oil–induced increases in perfusion of the medial (dashed red line) and lateral (solid line) parts of the dorsal skin of the right hind paws of rats 1 week after perineural treatment of the right saphenous nerve ($n = 5$). *: significantly different from the perfusion of the lateral part of the dorsum of the hind paw skin. (**C**) A series of perfusion images illustrating the time course of mustard oil–induced increases in blood flow in the dorsum of a rat hind paw. The right saphenous nerve was treated with capsaicin 5 days before the experiment. Scale bar represents 5 mm.

3.2. Effect of Transection of the Capsaicin-Treated Saphenous Nerve on Mustard Oil–Induced Neurogenic Vasodilatation in the Rat Hindpaw

Figure 3A shows that there is no significant change in the proportion of denervated skin areas up to 7 weeks after perineural capsaicin treatment. Since no signs of functional restitution was observed even 7 weeks after perineural capsaicin, it can be assumed that the marked and permanent reduction of the vasodilatatory response may result from degenerative changes in the affected saphenous nerve afferents serving the medial part of the dorsum of the hind paw. Hence, innervated and denervated skin areas of the dorsal hind paw skin were delineated as described in the Methods section. Further, we speculated that nerve fibers other than peptidergic capsaicin-sensitive afferents, which persist in capsaicin-treated nerve and skin, may impede collateral invasion of intact afferent nerve fibers from the neighboring sciatic innervation territory of the skin. Therefore, we transected the (right) saphenous nerve 7 weeks after capsaicin treatment and measured the vasodilatatory responses thereafter. Evaluation of perfusion images disclosed a gradual decrease in the proportion of denervated skin areas 2–4 weeks after transection of the saphenous nerve (Figure 3A). Accordingly, in the chemodenervated skin area, the proportion of reinnervated areas gradually increased and after 4 weeks peaked at around 40 percent (Figure 3B). The temporal and spatial characteristics of the effect of perineural capsaicin treatment of the saphenous nerve and the reinnervation process of the chemodenervated area are illustrated in the series of perfusion images in Figure 3C.

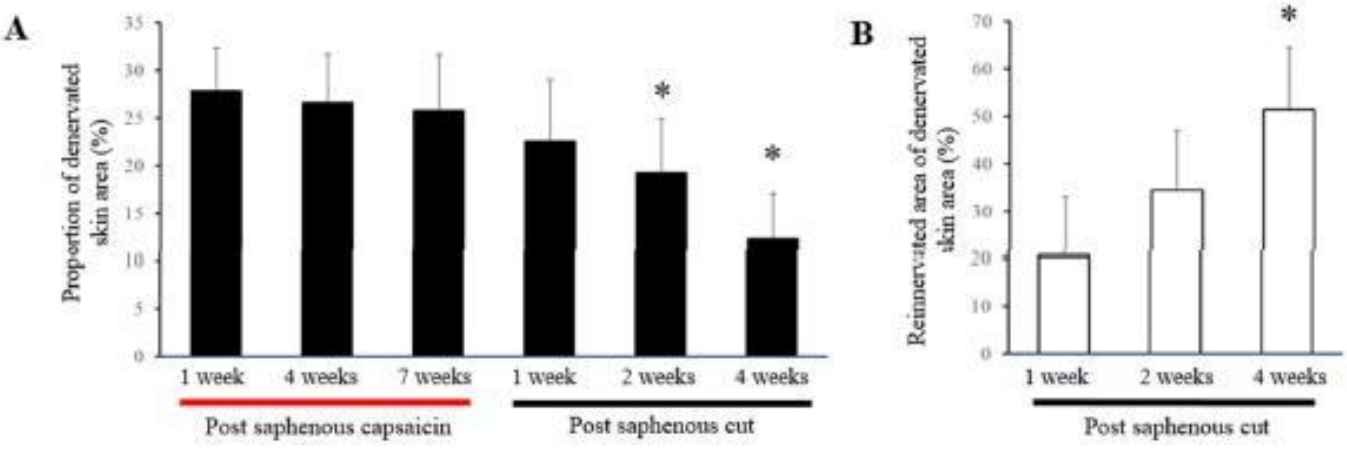

Figure 3. *Cont.*

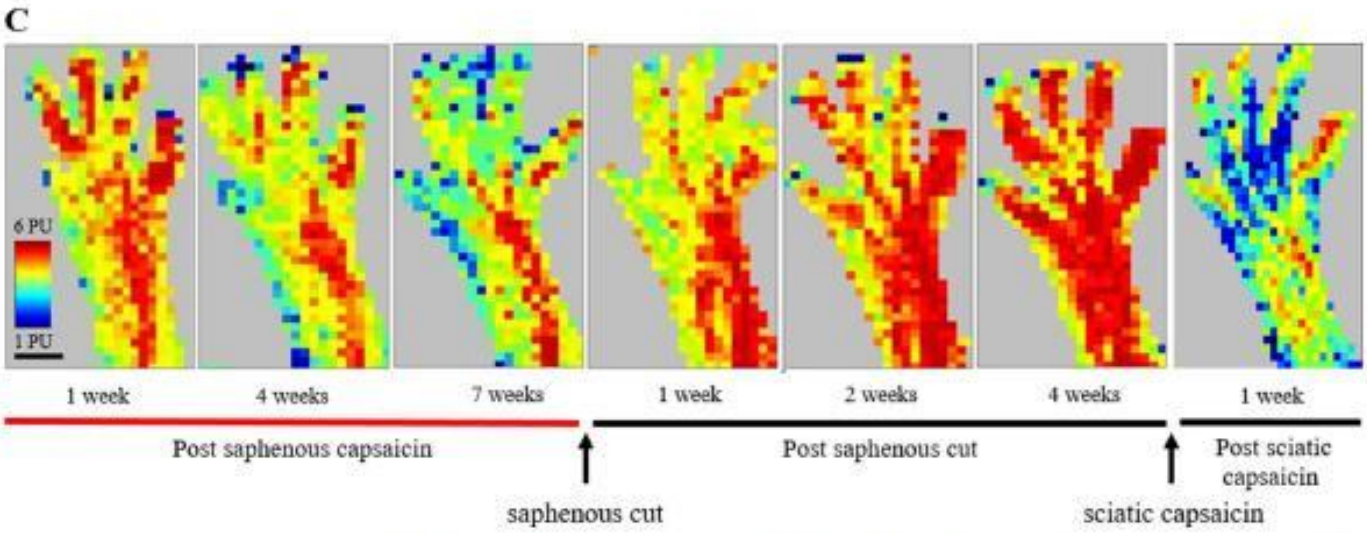

Figure 3. (**A**) Calculation of denervated skin areas following perineural capsaicin treatment of the saphenous nerve and subsequent transection of that nerve 7 weeks later ($n = 9$). *: significantly different from the skin area determined 1 week after perineural capsaicin treatment. (**B**) Calculation of reinnervated skin areas following transection of the saphenous nerve treated perineurally with capsaicin 7 weeks previously ($n = 7$). *: significantly different from the denervated area determined 1 week after perineural capsaicin treatment. (**C**) A series of laser Doppler scanning perfusion images of the rat hind paw following perineural capsaicin treatment of the saphenous nerve and subsequent transection of the saphenous nerve and perineural capsaicin treatment of the sciatic nerve 7 and 11 weeks later, respectively. Scale bar represents 5 mm.

These findings suggest a reinnervation of the chemodenervated skin by collateral sprouting of intact sciatic afferents, provided that the capsaicin-treated saphenous nerve is transected. This is supported by the finding that perineural capsaicin treatment of the sciatic nerve abolished the recovered vasodilatatory response within the confines of the capsaicin-treated saphenous nerve (Figure 3C).

3.3. Effect of Perineural Capsaicin Treatment of the Saphenous Nerve on Mustard Oil–Induced Vascular Labeling in the Rat Hindpaw

In accord with earlier reports, cutaneous application of mustard oil produces a clear-cut vascular labeling of small blood vessels (venules) in innervated but not chemodenervated skin areas following an intravenous injection of colloidal silver [7,16]. This is illustrated in Figure 3A. Importantly, labeling by colloidal silver can also be observed macroscopically, photographed and the innervated and denervated areas measured with a computer program. The results obtained with the vascular labeling technique and scanning laser Doppler imaging were essentially similar. The size and topography of the denervated skin area, as well as the gradual recovery of the vascular labeling, is illustrated in a series of photographs taken at different time intervals after perineural capsaicin treatment and subsequent transection of the saphenous nerve (Figure 4B). The quantitative data show a substantial reinnervation of the chemodenervated skin following transection of the capsaicin-treated (saphenous) nerve. Hence, the proportion of the chemodenervated skin area gradually decreased, whereas in the chemodenervated skin area the proportion of the reinnervated territory gradually increased after transection of the capsaicin-treated saphenous nerve (Figure 4C,D).

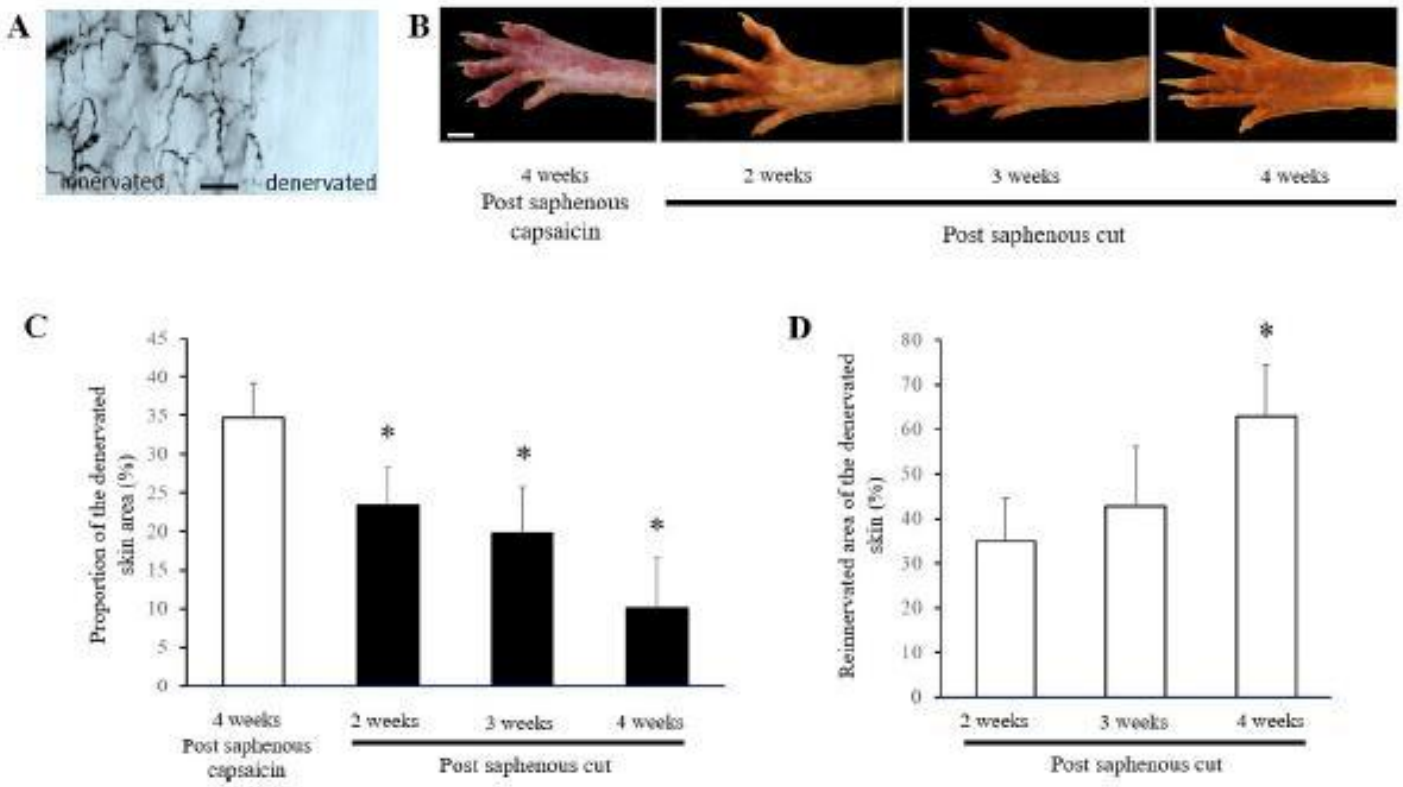

Figure 4. (**A**) A microphotograph illustrating vascular labeling of the hind paw skin of a rat following an intravenous injection of a colloidal silver solution. Colloidal silver–labeled small blood vessels (venules) are present in the innervated but not the denervated skin. (**B**) A series of photographs taken from the right hind paw of a rat to illustrate the appearance of mustard oil–induced vascular labeling in the innervated skin areas 4 weeks after perineural capsaicin treatment of the saphenous nerve and after a subsequent transection of that nerve. Scale bar represents 5 mm. The quantitative data showing the proportions of the denervated and reinnervated skin areas are shown in (**C**) (n = 4, *: significantly different from the skin area determined 4 weeks after perineural capsaicin treatment) and (**D**) (n = 4, *: significantly different from the skin area determined 4 weeks after perineural capsaicin treatment), respectively.

4. Discussion

Perineural treatment with capsaicin or other vanilloids has been shown to produce marked nociceptor analgesia, i.e., chemo- and thermo-analgesia and inhibition of neurogenic plasma extravasation confined to the innervation territory of the treated nerve [5–10,31]. The effect of capsaicin on peripheral nociceptive C-fibers is mediated by TRPV1 [32] and is associated with a decrease in afferent nerve fibers. Quantitative electron microscopic studies on rat saphenous nerves treated with capsaicin revealed a 32–36 percent loss of unmyelinated axons [11,12]. This indicates that not all capsaicin-sensitive afferent axons are lost after perineural treatment with capsaicin, since the proportion of capsaicin-sensitive afferents amount to 64–70 percent in the saphenous nerve [33,34]. Collectively, these observations suggested that, following perineural treatment with capsaicin, the loss of function of capsaicin-sensitive afferents may be accounted for by both a loss of unmyelinated axons [7,11,12] and depletion of vasoactive neuropeptides substance P and CGRP from capsaicin-sensitive cutaneous afferents [7,31].

The possible restitution of the function of peptidergic afferents in a skin area chemodenervated by perineural application of capsaicin has been examined in previous studies by making use of neurogenic plasma extravasation to study cutaneous nerve regeneration. These experiments demonstrated a complete disappearance of neurogenic plasma extravasation in the cutaneous innervation territories served by the affected nerve. In contrast to

peripheral nerve transection or crush, no tendency for recovery of the vascular response was observed [15]. A significant drawback to the application of the Evans blue–based technique for the demonstration of cutaneous plasma extravasation is that it is unsuitable for longitudinal studies in the same animal.

In the present study, the experimental paradigm based on scanning laser Doppler imaging of mustard oil–induced neurogenic sensory vasodilatation in the rat hind paw permitted the longitudinal examination of changes in the vasodilatatory response, i.e., the putative functional regeneration of peptidergic, in particular the CGRP-containing sensory nerves which mediate this vascular reaction [22–24]. This approach has recently been successfully used to reveal the time course and mechanisms of the functional regeneration of peptidergic cutaneous nerves following neurotmesis of the saphenous nerve in the rat. It was demonstrated that transection and ligation of the saphenous nerve resulted in a substantial reduction of the vasodilatatory response in the innervation territory of the nerve, which, however, displayed a gradual, albeit not complete, recovery towards control after 4–6 weeks. Evidence was presented that this recovery resulted from collateral sprouting of intact sciatic afferents into the denervated saphenous skin area [16].

Perineural treatment of the saphenous nerve with capsaicin resulted in a complete and permanent loss of mustard oil–induced neurogenic sensory vasodilatation in the skin area served by that nerve. Further, no tendency for recovery of the vasodilatatory response was observed up to a survival period of 7 weeks, suggesting that neither the afferents running in the treated saphenous nerve nor the intact sciatic afferents innervating the adjacent skin area invaded the denervated skin via regenerative or collateral sprouting, respectively.

The most intriguing finding of the present study is, however, the restoration of the regenerative propensity of intact nociceptive axons of the sciatic nerve following the transection of the capsaicin-treated saphenous nerve. Indeed, in the chemodenervated skin area, a gradual and significant recovery in both intensity and the spatial extent of the mustard oil–evoked vasodilatatory response was observed following transection (and ligation) of the capsaicin-treated saphenous nerve. Since the saphenous nerve was prevented from regeneration, this finding indicates an invasion of adjacent intact sciatic nerve afferents into the denervated saphenous skin territory, reinnervating the denervated area by way of collateral sprouting, similarly to that observed after transection and ligation of the saphenous nerve [16]. This assumption was strongly supported by the elimination of the vasodilatatory response in skin areas served by the sciatic and by the saphenous nerve after perineural treatment of the sciatic nerve with capsaicin. This indicates that the vasodilatatory response observed in the saphenous skin area may be indeed attributed to activation of sciatic afferents which sprouted into the saphenous skin area.

The results obtained by making use of the phenomenon of mustard oil–induced vascular labeling to delineate innervation territories of peripheral nerves yielded similar results. Previous light microscopic studies demonstrated that skin areas exhibiting vascular labeling strictly coincide with skin areas of intact peptidergic sensory innervation [7]. Therefore, measurement of silver-stained skin regions furnishes reliable information on the functional integrity of cutaneous peptidergic nerves. In naïve rats, application of mustard oil onto the dorsal skin of the hind paw produced a brownish coloration of the skin, indicative of increased vascular permeability as a result of neurogenic inflammation. Perineural capsaicin treatment of the saphenous nerve resulted in the complete abolition of the mustard oil–induced brownish coloration of the skin area served by that nerve. Repeated measurements failed to indicate significant changes in silver-stained skin areas for at least 4 weeks. However, transection and ligation of the saphenous nerve resulted in gradual increase in the silver-stained skin area, indicating the recovery of function of the sensory nerves mediating the neurogenic inflammatory response. Since the saphenous nerve was transected and ligated, this may result from collateral sprouting of intact sciatic afferents serving adjacent skin areas. This conclusion is supported by the observation showing a complete abolition of silver-containing skin areas following perineural capsaicin treatment of the sciatic nerve.

Collectively, the present findings confirmed previous observations which demonstrated a lack of regeneration of afferent nerve fibers following perineural treatment with capsaicin and, importantly, the failure of the collateral sprouting of intact sensory fibers into the chemodenervated skin area [15]. Importantly, however, the present experiments demonstrated that transection and ligation of the nerve treated previously with capsaicin restores the propensity of intact nerve fibers for collateral sprouting.

These observations suggest a novel mechanism promoting peripheral nerve regeneration, in particular collateral sprouting of nociceptive afferents. However, the present experiments provide no clues as to the mechanism(s) of this permissive conditioning lesion effect promoting collateral sprouting. Mechanistically, non-degenerating capsaicin-sensitive and intact unmyelinated and myelinated nerve fibers which persist in the chemodenervated skin may inhibit the invasion of adjacent intact sciatic nerve fibers into a denervated skin area. Collateral sprouting of intact sensory axons is critically dependent on the production of trophic factors and/or cytokines in degenerating nerves [35] or denervated target tissue [36] triggered by degenerating axons. Moreover, discordant changes in the expression of cytokines and chemokines were demonstrated following peripheral nerve transection and perineural treatment with capsaicin [37]. The lack of Wallerian degeneration of myelinated and capsaicin-insensitive unmyelinated afferent and sympathetic axons following the vanilloid treatment of peripheral nerves [9] and consequent failure of the production of specific signaling molecules may be a likely explanation for the failure of the collateral sprouting of intact sciatic afferents. The induction, by transection of the nerve previously treated with capsaicin, of Wallerian degeneration of cutaneous nerve fibers spared by capsaicin-induced chemodenervation may lift this inhibitory influence by creating favorable tissue microenvironment for promoting invasion of afferent axons from neighboring skin of intact innervation. Further studies on the molecular mechanisms of this permissive conditioning lesion effect may promote our understanding of the regulation of axonal regenerative processes under neuropathic conditions characterized by the partial sparing of nerve fibers and target innervation, such as diabetic or toxic neuropathies.

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Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and Szandra Győrfi-Lakatos Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

Szeged, on 11.12.2023.

date

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Péter Sántha, MD, PhD

The publication(s) relevant to the applicant's thesis:

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