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Chapter 2.8

Innervation mapping of the hind paw of the rat using Evans Blue extravasation, Optical Surface Mapping and CASAM.

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ABSTRACT

Background: Although numerous studies investigate sensory regeneration and reinnervation of the hind paw of the rat after nerve damage, no comprehensive overview of its normal innervation is present in literature. The Evans Blue extravasation technique is a well-known technique to study patterns of skin innervation. This technique has been performed differently by various groups but was never used to study the entire skin innervation in rats' hind paw including all three branches of the sciatic nerve and the saphenous nerve in detail.

Methods: In this paper, we have used the Evans Blue extravasation technique to chart the skin areas innervated by the sural, peroneal, tibial and/or saphenous nerves, which together innervate the entire hind paw of the rat, and use a new technique to analyze the distribution, overlap and variability of the results. The technique is based on analysis of whole hind paws using Optical Surface Mapping (OSM) in combination with the Computer Assisted Surgical Anatomy Mapping (CASAM) technology.

Results: While the plantar hind paw is mainly innervated by the tibial nerve, the dorsal hind paw is supplied by the sural, peroneal and the saphenous nerve.

Comparison with existint methods: Although our results basically concur with the general nerve-specific innervation of the rat hind paw, they show considerable detail in their areas of overlap as well as in the amount of variability between animals.

Conclusions: These results will be invaluable to study and evaluate patterns of innervation and reinnervation of intact and damaged nerve fibers.

INTRODUCTION

The past decade has seen an increased interest in the patterns of skin innervation by individual nerves in both naïve and pathological situations, such as neuropathic pain and itch in humans as well as in animal models. The reason for this growing interest is the possibility to analyze different classes of sensory skin fibers for diagnostic purposes.[1, 2] The glabrous skin of rat's hind paw has become a commonly used tissue to investigate the pathological changes of sensory skin fibers, especially when examining sensory denervation and reinnervation after peripheral nerve injury [3-6].

In order to properly evaluate these studies it is crucial to understand the pattern of innervation by individual nerves and the areas of overlap found between separate nerves as well as to the intra-individual variability in these patterns, as they might vary in different pathological conditions.

Previous studies have mapped the innervation of rats' hind paw by a recognized technique called 'Evans Blue extravasation' [7-20]. After intravenous injection of the Evans Blue dye the peripheral nerve of interest is electrically stimulated to cause plasma extravasation, which will be visible as blue staining on the skin. Since plasma extravasation is caused by sympathetic nerve fibers, namely C-fibers [21, 22], this technique offers a valuable approach to study the innervation areas of C-fiber afferents in the skin. Indeed, both in normal and pathologic conditions the areas with extravasated dye have been shown to correspond with the cutaneous innervation area of the investigated nerve[23]. Although plasma extravasation has been used in multiple studies, it is mostly evaluated by either a translation of the staining pattern to a diagram or extraction of the dye from the skin sample. The translating technique is sufficient for a general localization of the extravasation, but as size and anatomy varies between rats and interpretations may vary between individuals, the translation to a diagram is always based on subjective interpretation, which may cause high variability in results[8, 13, 16]. Extracting the Evans Blue dye for photospectrometry is used when studying quantity of the dye[10, 12]. However, apart from increased vascular permeability, electric stimulation eventually may also cause vasodilatation, which enhances the blood flow and thereby increases the Evans Blue extravasation in the skin after electrical stimulation. Therefore, the results of innervation areas by extracting the dye could be overestimated. Moreover, when extracting the dye from the skin the localization of the dye is lost. This prevents a description of the overlap between extravasation areas or to provide an overview of the entire pattern of extravasation areas. Yet, although the Evans Blue extravasation is known to be

a valid method for visualizing skin innervation areas, there is no comprehensive study describing and quantifying the detailed innervation areas, overlap areas and variability of innervation patterns by the nerves that innervate the rat's hind paw.

The aim of the present study is to use a novel technique to describe and analyze the cutaneous region of the hind paw that is innervated by a particular nerve. This technique enables a merging of measures of localization, quantity, and overlap. This is accomplished by combining a standardized Evans Blue extravasation technique, Optical Surface Mapping (OSM) and computer-assisted surgical anatomy mapping (CASAM) technologies[24-26].

METHODS

Animals and anaesthesia

Experiments were performed on adult female Lewis rats (n=37). All experiments were approved by the Dutch Ethical Committee at Erasmus MC Rotterdam on Animal Welfare (DEC) and all procedures adhered to the European guidelines for the care and use of laboratory animals (Council Directive 86/6009/EEC). During the experiments 3 animals were excluded due to lack of extravasation after 10 minutes of stimulation. Possible reasons for the lack of extravasation could have been a potential break of the anodal or cathodal wire of the stimulation cuff. The final experimental group contained 28 rats, which were subjected to stimulation of either the tibial (n=7), sural (n=7), peroneal (n=7) or saphenous nerve (n=7). Six additional animals were used in a control study.

The animals were anesthetized with 3% isoflurane, weighted and subsequently the paw was depilated using depilatory-crème 'Veet' to acquire a clear image of the skin.

Surgical procedure

An incision was made on the lateral or medial side of the hind limb depending on the choice of nerve to be investigated. The sciatic branches were approached by a 4 cm incision over the intramuscular septum between the vastus lateralis and the biceps femoris muscles, whereas the saphenous nerve was exposed by a 5 cm incision over the inner thigh. Subsequently, the nerve of interest was microsurgically dissected from its environment and crushed proximal to the stimulation site in order to prevent central propagation of the stimulus[11, 16]. The surgical procedure and placement of the stimulation cuffs were performed under microscopic guidance (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) to prevent damage to the nerve of interest and adjacent tissue.

Evans blue extravasation procedure

The nerve was inserted into a handmade silicone stimulation cuff (diam. 3 mm, length 6 mm) in which the anodal and cathodal electrode were embedded (Figure 1). These cuffs were developed to restrict stimulation of surrounding tissue. A solution of Evans Blue (2% EB solved in 0.9% saline, 4ml/kg body mass) was injected slowly into the tongue vein of the rat. Because of discrepancies noted in literature[11, 16, 20], we determined the optimal EB concentration in several trial experiments using subjective evaluation of the extravasation contrast.

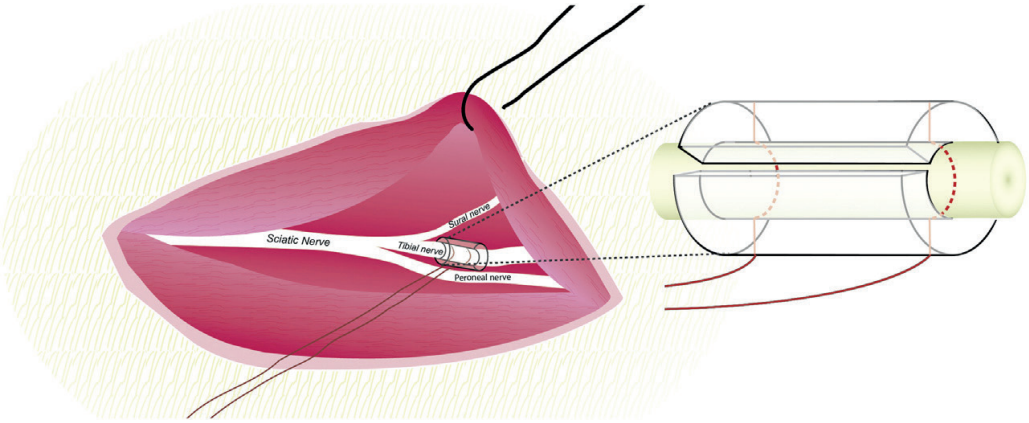

Figure 1. Cuff stimulation of nerves.
The stimulation cuff is placed over the nerve of interest (n. peroneous in this figure). Proximal to this site a crush lesion is made to avoid central transmission of the stimulation. The cuff is made of a silicone tube with both anodal (+) and cathodal (-) wires (visible in red) placed in it. The wire's insulation is removed in the cuff but remains intact outside the cuff to avoid stimulation of the surrounding structures.

Stimulation of the nerves started 5 minutes after the Evans Blue injection and lasted for 10 minutes using a continuous 10Hz, 0,5ms pulse width and 12mA current stimulation (Viking stimulator, Nicolet Biomedical IES405-2). Parameters were chosen based on trials and resembled those in literature[11, 12, 14]. During stimulation a slow blue coloring of a part of the hind paw was observed which was attributed to extravasation of the dye. Often, additional extravasation was seen for several minutes after terminating the stimulation[12]. Therefore, a 6-minute interval in which the animals were not handled was incorporated before sacrificing the animals by an overdose of pentobarbital (100mg/kg ip). Subsequently, the hind paws of all animals were cut at the level of the heel joint. Then the hind paws were fixed in an identical position using insect needles. Both

the tendons and the skin was pulled back to obtain a neutral hind paw position in animals; without flexion of the toes and enough web space between the toes for further imaging of the paw.

Control experiments

In three additional rats the correlation between the Evans Blue extravasation in the skin and the epidermal innervation was verified. In this animal the sciatic nerve was transected and ligated proximally to prevent regeneration of this nerve. Five weeks later, to ensure complete degeneration of the sciatic nerve, the Evans Blue experiment (see section 2.3) was performed by stimulation of the unaffected saphenous nerve, which resulted in extravasation of the medial side of the paw. After sacrificing the animal the paw was photographed. Using microscopical guidance, a superficial epidermal cut was made with a scalpel at the border of the extravasated and non-extravasated skin. A subsequent large skin biopsy of the transition area was taken, fixated in 2% paraformaldehyde-lysin-periodate (PLP) for 24 hours at 4˚C. The skin was embedded in 10% gelatine, hardened and sectioned at 40 µm with a freezing microtome. Sections were processed to visualize nerve fibers using PGP9.5 immunohistochemistry according to Duraku et al.[4, 5] Briefly, free-floating sections were rinsed in phosphate-buffered saline (PBS), pre-treated with hydrogen peroxide to reduce background, heated to 80˚C in citrate buffer (pH 8.75) to unmask immunoreactivity, incubated in rabbit anti-PGP9.5 (48 h at 4˚C in PBS with 0.5% Triton X-100; dilution 1/10.000: Enzo). After subsequent incubations with biotinylated goat-anti rabbit (1.5h at room temperature, RT; dilution 1/200: Biotine) and Vectastain ABC-Elite™ (1.5h at RT: Vector, Burlingame, CA) followed by additional signal amplification using biotin tyramide 27 for 12 minutes, the antigenic sites were revealed by diaminobenzidine (DAB) histochemistry. Sections were counterstained with thionine, which faded the epidermis blue as a result clear border between epidermis and dermis was visible. Finally, the slides were dehydrated and coverslipped with Permount (Fisher, Hampton, NH). The labelled nerve fiber terminals within the epidermial region of 64 mm² (400µm x 160µm) at the level of the epidermal cut were quantified using an Olympus BH microscope equipped with a Lucivid™ miniature monitor and Neurolucida™ software (MicroBrightField, Inc., Colchester, VT) with a 20x objective.

OSM and CASAM

An optical projection tomography (OPT) scanner [28, 29], which is a commonly used technique for investigating small specimens to visualize aspects of anatomy

and gene expression, was used to produce a 3D image of the surface of rats' hind paw in a novel procedure called Optical Surface Mapping (OSM). This method allows an accurate quantification of the paw in which even the areas between the toes can be taken in consideration in contrast to a mere 2D-analysis. Furthermore, we have developed a systematic way of recording and quantifying extravasation making use of a 360° view of the paw by combining the UV sensitive quality of the Evans Blue dye and the UV Filter in the Bioptics OPT scanner For determining statistical differences of the surface areas, the one way-ANOVA with a Tukey post hoc test was used for intergroup comparisons to calculate the average staining in each group. Errors in variations were determined as standard error of the mean (SEM), and $p < 0.05$ was taken as significant.

In the CASAM analysis single pixels within the circumference of the landmarks were either assigned the label "stained" or "non-stained". Pixels with identical incidences (ranging from stained in 1 animal to stained in all 7 cases) were grouped and two way t-test with a Tukey post hoc test was used to determine the threshold for significance ($p<0.05$) between groups. Subsequently overlap was divined as groups of individual significantly "stained" pixels with no significant difference between the compared nerves. [28, 29] Subsequently, the variability in nerve innervations patterns between various individuals was assessed by applying the CASAM technology[30], which is a new anatomy mapping tool used in clinic to improve description of the variability of complex anatomical regions. Using the combination of Evans Blue extravasation, OSM and CASAM allows us to present, for the first time, an anatomically correct impression of the average innervations pattern and indication of intra-animal variability as well as assessment of the areas of overlap between the innervation territories of different nerves.

Analysis

The Bioptronics OSM Scanner 3001 was set up as described in the manual. However, the scan medium was changed from benzyl alcohol benzyl benzoate to demineralised water to avoid unwanted interaction with the specimen or the dye. The scans were made using UV light and a Cys3 filter (Cys3 fluorescence, 545nm/30nm exciter, 610/75nm emitter)[31, 32]. In our experiments bright-field images were used. Every specimen was scanned using a 1.8° angle increase per frame, resulting in 200 frames per scan. Every paw was recorded in two series because their sizes exceeded the 21 mm frame of the OSM scanner in the vertical position. Both series were merged whilst overlap between recordings was digitally excluded using based on the vertical position of the specimen in the scanner.

Quantification of the extravasation was performed using a custom-developed labVIEW™ (NationalInstruments) application. The percentage of staining per view was calculated using different thresholds to distinguish between background, unstained skin and stained skin. The thresholds were adjusted every 18° (10 frames) to compensate for the varying lightning due to the irregular surface of the paw. The 0°point was determined as the largest surface area (as measured by the total number of stained plus unstained pixels containing skin) on the plantar side of the paw.

The extent of extravasated skin area and the overlap between areas innervated by different nerves were determined using the CASAM technology[30]. Four frames in total, at 0° (plantar view), 90° (lateral view), 180° (dorsal view) and 270° (medial view) were selected to show the average extravasation, intra-animal variability of extravasation per nerve and overlap of extravasation for the studied nerves. Matching frames were selected from every scan and characteristic landmarks such as on each toe and at the base of the paw were placed, resulting in 30 to 50 landmarks per frame depending on the shape and variability of the paws for that specific frame. From these landmarks an average size and shape of the hind paw was computed and all paws were morphed to match this average. Subsequently, the area of extravasation for each animal was manually selected onto these average paws. Merging and comparing the resulting images in Adobe Photoshop enabled determination and quantification of both intra-animal variability of extravasation area per nerve as well as of the areas of overlap between two nerves.

Statistical analysis

For determining statistical differences of the surface areas, the one way-ANOVA with a Tukey post hoc test was used for intergroup comparisons to calculate the average staining in each group. Errors in variations were determined as standard error of the mean (SEM), and $p < 0.05$ was taken as significant.

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RESULTS

All nerve stimulations resulted in a blue coloring of a part of the skin of the hind paw. Several controls were conducted to verify that Evans Blue extravasation was positively correlated with the innervation of the skin innervated by the stimulated nerve. First, lesion of the nerve distal to stimulation site 10 weeks prior to the Evans Blue experiment failed to result in blue coloring of any part of the skin (not shown). In addition, in three rats a lesion of the sciatic nerve was performed five week previous to stimulation of the saphenous nerve which resulted in a blue colored extravasation of the medial part of the paw (Figure 2). The transition area was indicated by a shallow epidermal cut which was subsequently excised and processed for PGP9.5 immuohistochemistry. Although the Evans Blue coloring disappeared during the immunohistochemical procedure, the results clearly demonstrate that at the formerly extravasated side of the transition line (i.e. medial of the cut) PGP9.5-positive fibers of the stimulated saphenous nerve are present (Figure 2C), which could not be discerned at the formerly non-extravasated area (Figure 2D), lateral of the cut, which had been denervated by the sciatic lesion. Quantification of the epidermal nerve fibers was performed in all three animals. Average of 980 ± 16 PGP9.5 positive epidermal nerve fibers per mm² were counted on the formerly extravasated side. The few (28 ± 7 per mm²) remaining fibers, which are mainly present in the upper dermis, at the non-extravasated area are very thin (arrows in Figure 2D) and could reflect sprouting fibers.

These results exclude any possible blue staining of the skin due to diffusion of the dye and show a positive correlation between the extravasation by Evans Blue and the innervation of the epidermis.

Finally, two control animals were transcardially perfused with saline followed by 4% paraformaldehyde in order to determine if the wash-out of Evans Blue from the blood vessels interfered with the OSM measurements. Because no difference was observed between the perfused and non-perfused animals we chose to standardly use non-perfused animals in the current study

Evans Blue extravasation areas of the individual nerves

Stimulation of the four selected nerves resulted invariably in characteristic patterns of extravasation as evidenced by a dark blue staining of the skin. Stained areas always formed a consistent and continuous region with well-defined borders. Remarkably, no staining was observed on the footpads while less staining was seen on the callosities of the toes in all cases (Figure 3 and supplementary material S1). The extravasation of the innervated areas by individual nerves were

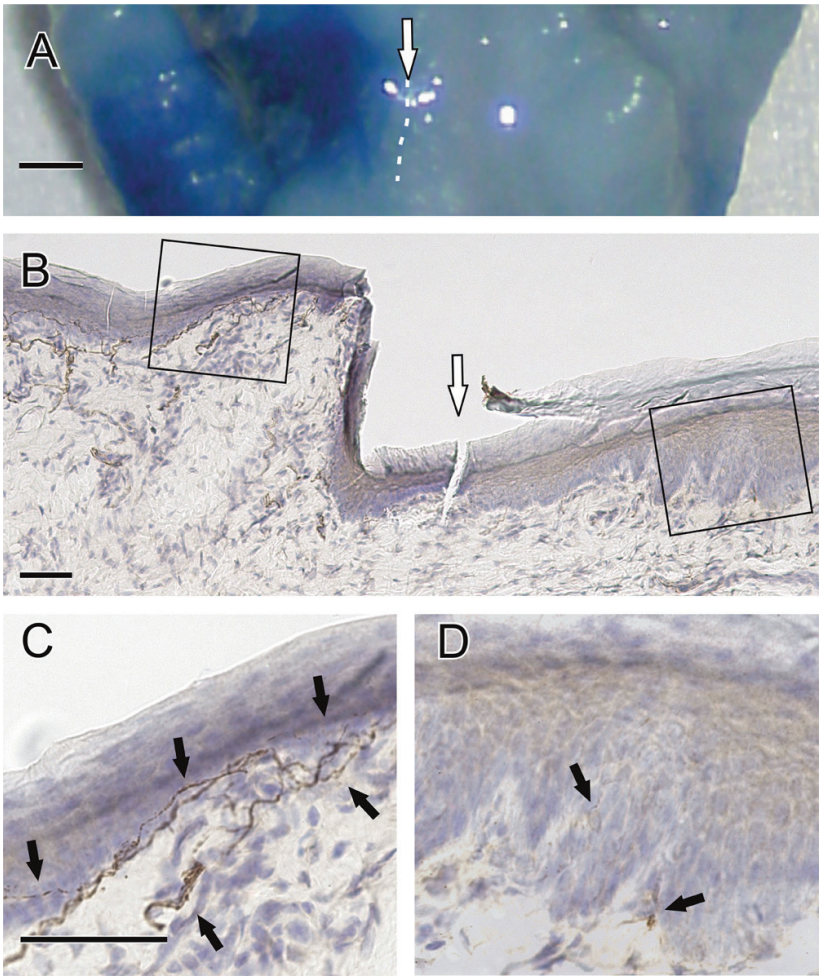

Figure 2. Correlation between Evans Blue extravasation and the innervation of the skin.
A) View of a part of the left plantar hind paw of the rat. After ligation of the sciatic nerve, stimulation of the healthy saphenous nerve showed blue coloring of the medial part of the plantar hind paw. The border between the extravasated and non-extravasated skin was indicated by a scalpel incision visible as white dotted line (white arrow).
B) PGP9.5 staining of the glabrous skin of rats' hind paw, in which an epidermal cut (white arrow) is made to localize the border between extravasated (left) side and non-extravasated (right) side. PGP9.5-positive nerve fibers are observed in the extravasated area medial to the incision.
C) Magnification of the Evans Blue extravasated side of the skin. The black arrows show the brown PGP9.5 positive nerve fibers.
D) Magnification of the non-extravasated side of the skin. The black arrows show some light staining in the upper dermis of the skin. These thin fiber-like structures could reflect sprouting fibers. Scale bar A) 1mm B-D) 50µm. E = epidermis, UD = upper dermis.

quantified and are represented by different colours (i.e. tibial nerve pink, sural nerve red, peroneal nerve green and saphenous nerve blue) (Figure3). In addition, different shadings of the colours pink, red, green and blue from bright to dark were used to represents the incidence (i.e. one to seven) of extravasation resulting from the stimulation of a particular nerve in the group of 7 animals (Figure 3).

In order to obtain accurate quantification of the extravasation areas in the paw, a 3D movie of the staining was acquired using the 200 frames (supplementary material S1). In addition, the percentage of staining per individual case was calculated from these 200 views in which the unstained hind paw from the most proximal footpad until the tip of the toes (including the footpads and the callosities) was automatically determined as 100% of the area that was analyzed by labVIEW. Subsequently, the average overall staining of the hind paw for the individual nerves and in each view of the 360° rotation was calculated over all 7 cases in each group (Figure 4). A general description of the 3D staining pattern and the average percentage staining for the individual nerves is given below.

Tibial nerve

Stimulation of the tibial nerve typically caused staining of the glabrous skin, extending to both the lateral and the medial sides of the paw (Figure 3). Maximal coverage of the paw with extravasated skin (up to 66,7% ± 5.0) after tibial nerve stimulation was seen on the plantar side of the hind paw at 0-45° and 315-360° (Figure 4A and S1). Note that the cycle starts with the full plantar view. Since the footpads, that do not show extravasation, are included in this view a lower than maximal percentage of extravasation was obtained. The dorsal view also shows some extravasation due to the innervation of the skin areas between the toes (Figure 4A and S1). The average overall extravasation area of the skin of the rats' hind paw after tibial nerve stimulation is 44.4% (±2,3) (Figure 4E).

Sural nerve

Stimulation of the sural nerve resulted in a dark blue staining on the lateral side of the paw, extending to the 4th and 5th digit on the dorsal side and partially the 5th digit on the plantar side (Figure 3). Although the sural nerve extravasation area is narrow proximally, it becomes wider towards the toes (Figure 3). This typical narrow elongated band of staining on the lateral part of the hind paw is represented by the low rather narrow peak that was present around 100° reaching 71.3% ± 6.3 (Figure 4B and S1). Lowest coverage (7.1%± 0.1) is seen in the medioplantar view of the paw between 270° and 315° (Fig. 4B and S1). Stimulation of the sural nerve caused an overall extravasation of 28.8% (±2.7) in

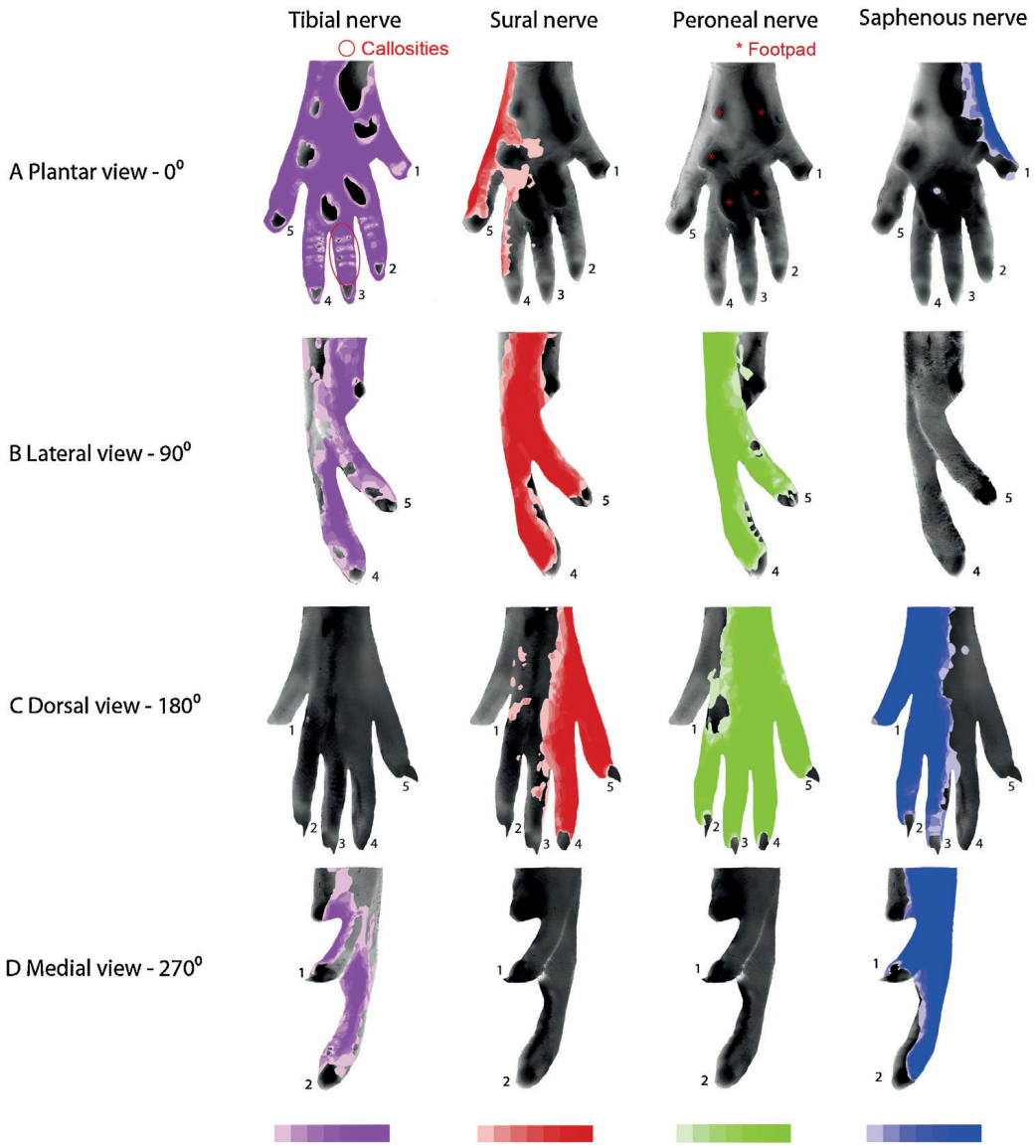

Figure 3. Intra-animal variability in nerve specific extravasation patterns.
The figure shows examples of the nerve specific extravasation pattern results from CASAM protocol after stimulation of the tibial, sural, peroneal and saphenous nerve in four views: A) 0̊ for the plantar view, B) 90̊ for the lateral view, C) 180̊ for the dorsal view and D) 270̊ for the medial view. All grey blank hind paws represent the views without extravasation. The pink (tibial nerve), red (sural nerve), green (peroneal nerve) and blue (saphenous nerve) colours indicate nerve specific extravasation ranging in incidence from light (1 animal) to dark (all 7 animals) as shown in the colour legend at the bottom of the figure. A) Footpads are visible as red asterisks and callosities as red circles.

rats' hind paw, which represents the lowest overall Evans Blue extravasation of the four nerves supplying the hind paw (Figure 4E).

Peroneal nerve

The skin area coloured by peroneal nerve stimulation shows remarkable correspondence with the proximal part of the area of extravasation by sural nerve stimulation. However, peroneal nerve-induced extravasation extends far wider distally than the sural nerve and covers all digits except digit 1 (Figure 3) with a wide high peak of 88.0% (± 3.3) in the dorsolateral view of the paw at 131° (Fig. 4C and S1). Note that both slopes of the peroneal nerve and the sural nerve are initially very much alike (Figure 4B,C and S1). Like the sural nerve, the least amount of extravasation after peroneal nerve stimulation was found in the medioplantar view of the paw between 270° and 315° reaching a minimum of 11,4% (±1.5) (Figure 4 and S1). The average extravasation after peroneal nerve stimulation is 40,9% (±1.3) (Figure 4E).

Saphenous nerve

Saphenous nerve stimulation typically resulted in extravasation of the medial-most side of the paw, covering the best part of the 1st, 2nd and 3rd digit and extending till the medial footpads on the plantar side of the paw (Figure 3). Similar to the stimulation of the peroneal nerve, the saphenous nerve has a broad extravasation area causing a high wide peak covering 78.6% (±3.0) of the dorsomedial paw at 225° (Figure 4D and S1). Minimal extravasation (9.7% ± 1.2) was found on the plantar view of the paw. Overall, stimulation of the sapheneous nerve caused 36.6% (±1.7) of the skin to demonstrate extravasation (Figure 4E).

In summary, the average extravasation area of the skin in rats' hind paw after tibial nerve stimulation is 44.4% (±2.3), whereas the sural nerve shows 28.8% (±2.7) extravasation, the peroneal nerve 40.9% (±1.3) and the saphenous nerve 36.6% (±1.7) extravasation (Figure 4e). From this first analysis, which is based on the summed projections of individual frames, it is determined that the sum of all averaged extravasation areas in the skin of rats' hind paw contributed to 150.7% (±16.0), which indicates a considerable overlap of the extravasation areas induced by stimulation of the individual nerves.

Analysis of variability and overlap

Although stimulation of a particular nerve generally resulted in extravasation of a specific part of the skin, small differences were observed between animals. As these differences may reflect intra-animal variability in the extent of the areas

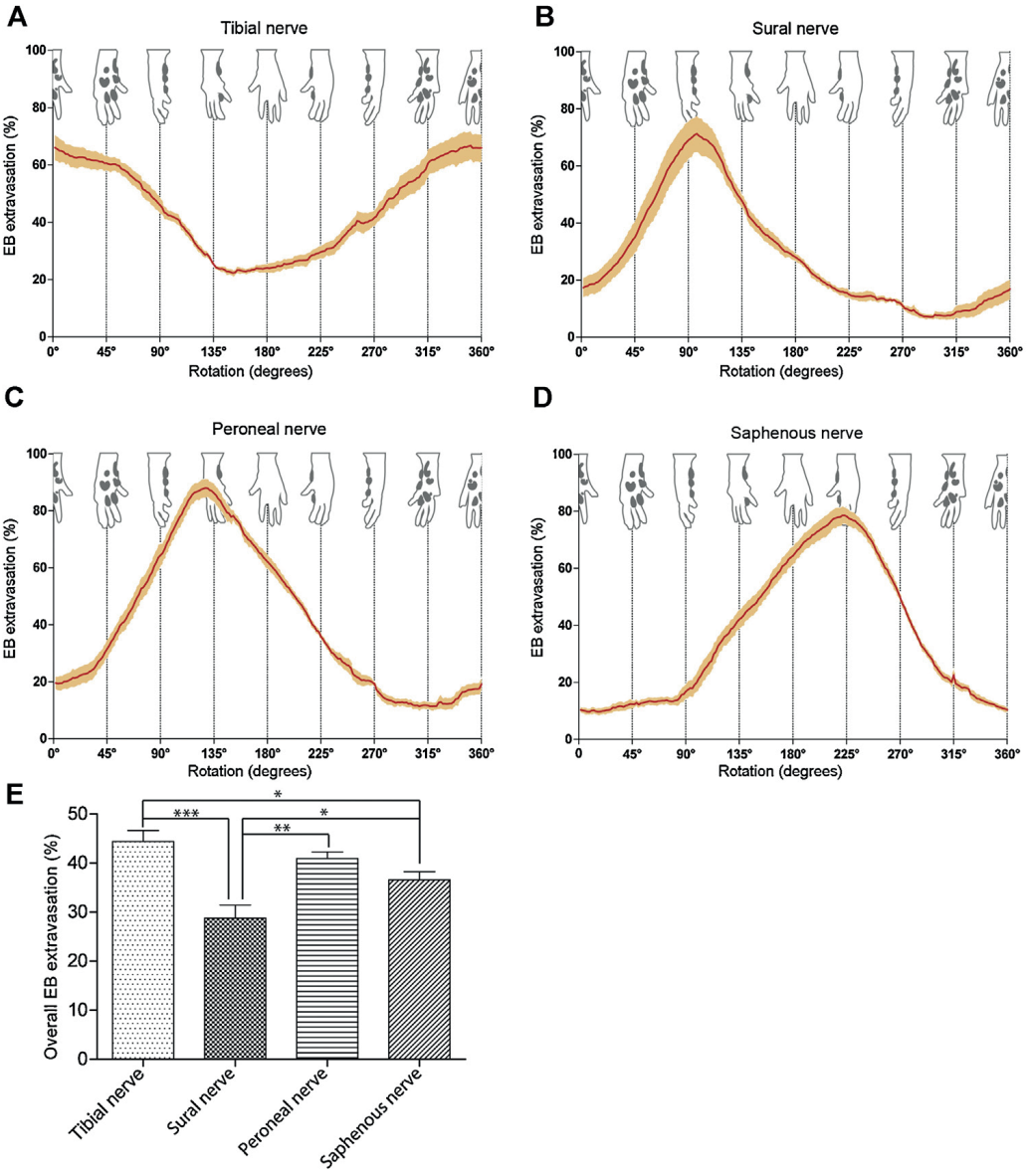

Figure 4. Percentage average extravasation per nerve.
A-D) show the average extravasation as a function of rotation after stimulation of the tibial nerve, the sural nerve, the peroneal nerve and the saphenous nerve subsequently. The yellow band around the red line indicates SEM's in figures. E) shows the average extravasation calculated for tibial, sural, peroneal and saphenous nerve over all 200 frames made by the OSM-scanner. In this figure the location is not taken into consideration. Note the significant differences in average percentage extravasation between the different nerves and that these percentages add up to 155%. Analysis by two-tailed t-test. * ($p<0.05$) ** ($p<0.01$) *** ($p<0.001$).

innervated by a single nerve, we have analyzed the intra-animal variability further using the CASAM technique 24, which entails superimposing the extravasated areas on an averaged hind paw (Figure 3). In addition, the staining areas of two adjacent nerves were merged to gain a better understanding of the location of the considerable overlap areas as found in our previous average staining calculations (Figure 5,6,7,8 and 9). The intra-animal variability within the four groups of seven animals has been taken into account by using different shades of the colour that represents the specific cutaneous nerve extravasation ranging from bright to dark as described previously. After merging the stained areas for two nerves, it was possible to create a new 49 (7x7) grid of colour combinations that represents the incidence that a particular surface area is extravasated by stimulation of either or both of the nerves (Figure 5B, 6B, 7B, 8B). Subsequently, the areas that showed significant overlap in extravasation by both nerves (i.e. at least four of the seven animals showed extravasation in this area in both groups) were extracted (Figure 5C, 6C, 7C, 8C). Using this analysis we can now describe which skin areas show systematic overlap in extravasation due to stimulation of different nerves.

Plantar View

As shown in figure 3A and visible in figure 5, parts of the plantar aspect of the paw are extravasated by the saphenous, tibial and sural nerves. The plantar skin of the paw shows most consistent staining after stimulation of the tibial nerve (Figure5A). Colour coding indicates the consistency of the results. Dark pink indicates that all seven studied animals showed extravasation of the tibial nerve in the same region whereas bright pink to white regions depict areas with extravasation in only one of the rats. Note that in all animals the footpads appear as black islands since they never show extravasation of the skin (Figure 5). Variety in shape and position of footpads caused some lower densities around the footpad areas. The callosities on the plantar side of the toes also demonstrate a lower incidence of coloring.

Both the sural and the saphenous nerves have a minimal area of extravasation in the plantar view as compared to the dorsal view. Moreover, these small plantar areas also show some variability as indicated by the amount of bright colours (i.e. bright red in case of the sural nerve in figure 5A.3). Intra-animal variability in the extent of extravasation area for the saphenous nerve is further represented by the white shade proximal to the first digit next to the first proximal footpad (Figure 5A.1).

In addition, Figure 5C shows the consistency in areas of overlap between the tibial and saphenous nerve (Figure 5C-1.2) and between the tibial and sural nerves

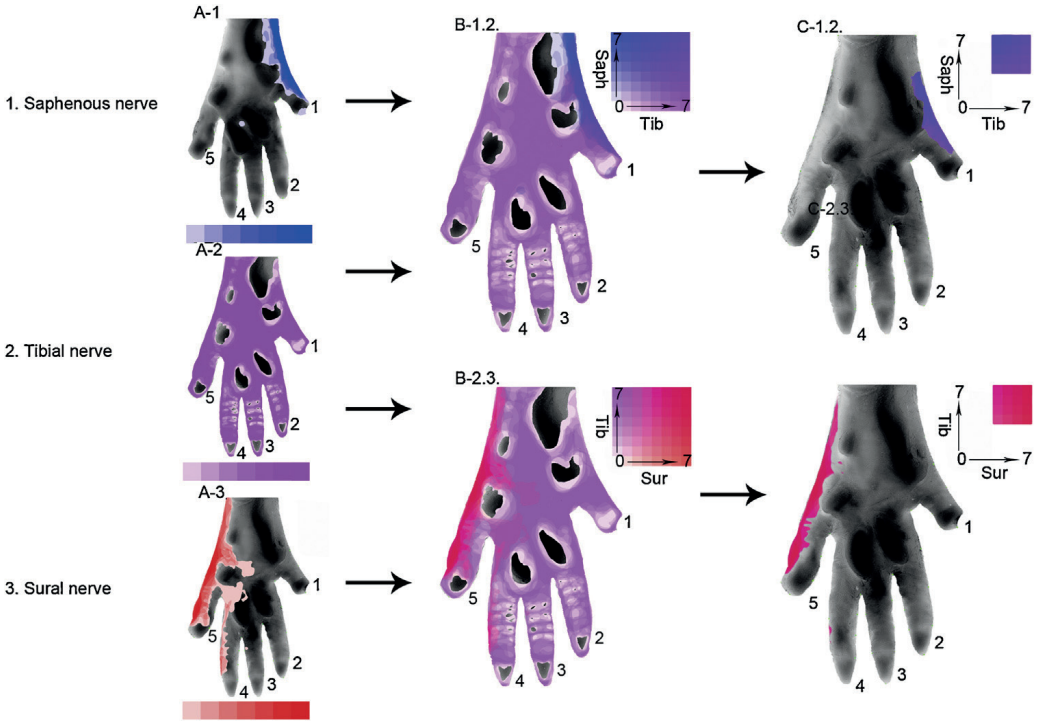

Figure 5. Plantar overlap analysis.
A) The intra-animal variability of the extravasation is shown individually for saphenous (blue), tibial (pink) and sural (red) nerve on the plantar side of the paw ranging in incidence from light (1 animal) to dark (all 7 animals). Note the lack of extravasation by the peroneal nerve on the plantar view as shown in the colour legend below the hind paw. B) Combined extravasation areas showing overlap. The combined figures of the saphenous and the tibial nerve (B-1.2) as well as the combined figures of tibial and the sural nerve (B-2.3) showed a small blue area on the medial and red area on the lateral part of the paw showing overlap with tibial nerve extravasation. C) Extraction of significant overlap areas between the n. saphenous and the n. tibialis (C-1.2) shows a small area of overlap on the medioproximal part of the plantar side while the overlap between n. tibialis and n. suralis (C-2.3) is seen on the lateral part of the paw. B) The 7x7 grid of colour combinations is represented to show the incidence that a particular surface area is extravasated by stimulation of either or both of the nerves as given in the x and the y axis. C) 4x4 grid of colour in overlap areas of two nerves in at least four of the seven animals in each group. Saph= saphenous nerve, Tib= tibial nerve, Sur = sural nerve.

(Figure 5C-2.3). Indeed, small regions of consistent overlap in extravasation by these nerves are noted. The overlap region of the saphenous nerve and the tibial nerve starts at the medial base of the proximal-most footpad and ends on the 1st digit (Figure 5C-1.2). Lack of overlap at the base of the first digit next to the most proximal footpad, visible as a white curve surrounding the medial side of the footpads, can be observed (Figure 5C-1.2). This is related to absence of extravasation by tibial nerve stimulation on this part of the glabrous skin (Figure 5A-2).

The overlap between the saphenous and tibial nerves at the medial edge of the plantar view is mimicked laterally by the overlap between the tibial and the sural nerves (Figure 5C-1.2, 5C-2.3). The area of overlap covers almost the entire lateral edge of the paw including the 4th digit (Figure 5C-2.3: fuchsia area). However, it should be noted that this overlap region is more consistently involved after tibial nerve stimulation as compared to sural nerve stimulation.

Lateral view

The lateral view of the hind paw incorporates a view of both the 4th and 5th digit and the separation of dorsal and glabrous skin on the lateral side of the paw (Figure 3B). The saphenous nerve is the only nerve that does not contribute to the extravasation of this view while the sural nerve provided the most consistent extravasation (Figure 3B). Peroneal and tibial nerves, to varying degrees, also contribute to the extravasation of the lateral aspect, both resulting in considerable overlap with the territory of the sural nerve (Figure 6A, B). However, note that, in contrast to the tibial nerve, both sural and peroneal nerve stimulation predominantly resulted in extravasation of the dorsal edge of the paw.

Although, the extravasation area by the peroneal nerve shows overlap with extravasation areas of tibial as well as sural nerves, more prominent interaction was seen with the latter. This is illustrated by figure 6C-1.2 where the large dark red/dark green areas indicate consistency of overlap in the extravasation areas between the peroneal and sural nerve without any dominance for either nerve in this area. Less overlap was seen between the sural and tibial nerves (fuchsia area in Figure 6C-2.3), which were mostly restricted to the border between the glabrous skin and non-glabrous skin at the lateral view. However, least overlap was found between the peroneal and tibial nerve (Figure 6-C3.4).

Dorsal view

All animals showed extravasation of at least some parts of the skin at the dorsal aspect of the paw after stimulation of either the saphenous, peroneal or

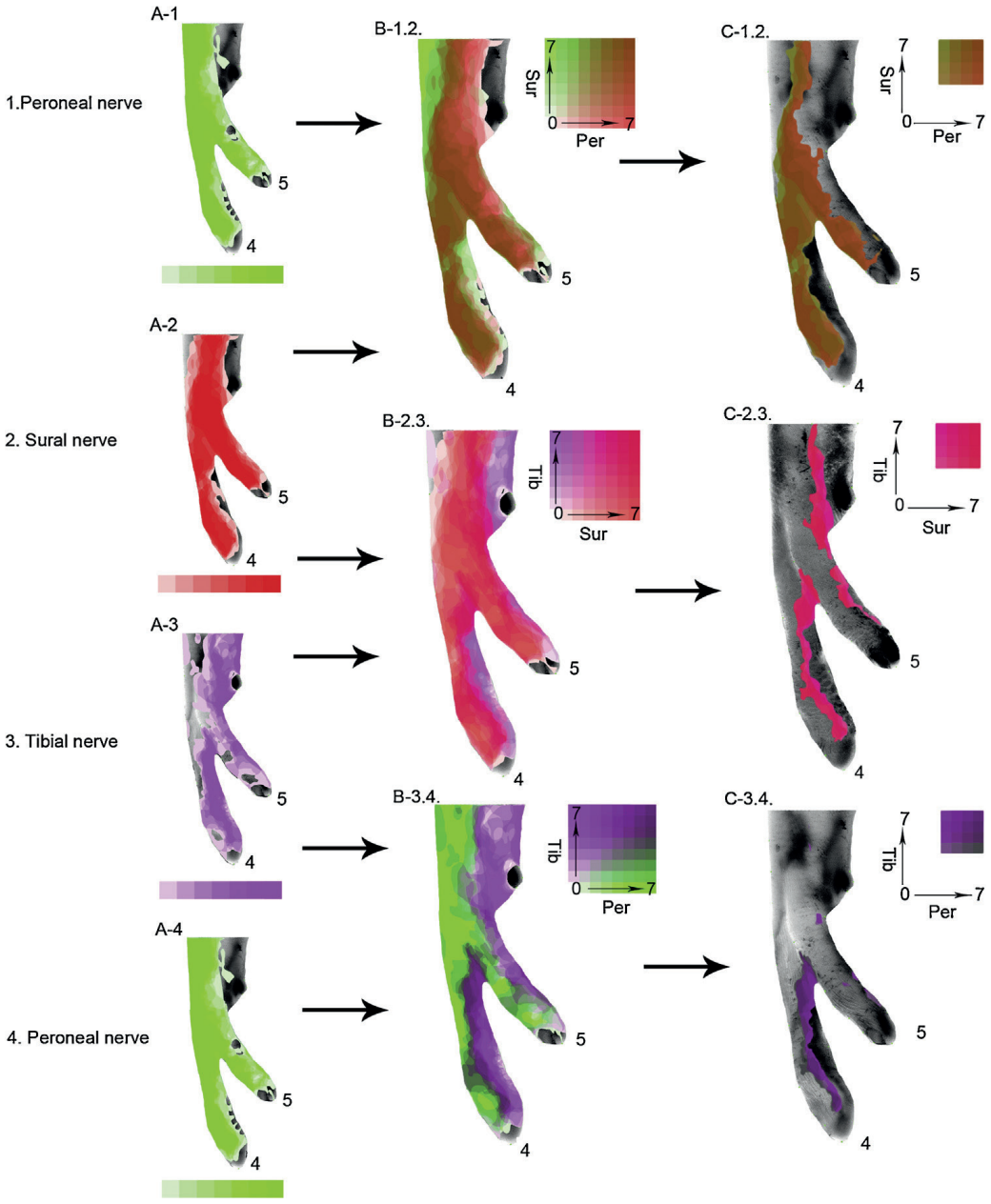
1.Peroneal nerve
2. Sural nerve
3. Tibial nerve
4. Peroneal nerve
A-1
A-2
A-3
A-4
B-1.2.
B-2.3.
B-3.4.
C-1.2.
C-2.3.
C-3.4.
Sur
Per
Tib
0
7
4
5

Figure 6. (left page) Lateral overlap analysis.
A) The intra-animal variability of the extravasation is shown individually for the peroneal (green), sural (red) en tibial (pink) nerves on the lateral side of the paw ranging in incidence from light (1 animal) to dark (all 7 animals) as shown in the colour legend below the hind paw. In the lateral view there is overlap between three nerves, therefore in (A) the peroneal nerve was displayed twice. B) Combined extravasation figures show comparable extravasation areas while after correction for anatomical variety three different overlapping areas are seen (C). Relatively large significant overlap was seen between the peroneal and sural nerve (C-1.2), whereas the tibial and the sural nerves show typically overlap in a vertical line on the lateral border between the glabrous and hairy skin (C-2.3). The peroneal and the tibial nerve present overlap mostly on the 4th digit of the lateral hind paw (C-3.4). B) The 7x7 grid of colour combinations is represented to show the incidence that a particular surface area is extravasated by stimulation of either or both of the nerves as given in the x and the y axis. C) 4x4 grid of colour in overlap areas of two nerves in at least four of the seven animals in each group. Sur = sural nerve, Per= peroneal, Tib= tibial nerve.

sural nerves. Tibial nerve stimulation resulted only in some animals in some spots of extravasation between the digits (S1). Since these spots did not represent a significant overlap they were excluded in the analysis of overlap with other nerves.

Stimulation of the sural nerve caused extravasation of the dorsolateral part of the paw, covering the 5th and the 4th digit and in 3 out of 7 cases some spots were noticeable on the 3rd digit as well (Figure 3C, 7A). Saphenous stimulation, on the other hand, resulted in coloring of the dorsomedial aspect of the paw, usually including the 3rd digit. Peroneal-induced extravasation is located centrolaterally at the dorsal view of the paw and incorporating the 2nd -5th digits in all 7 cases. As such, some overlap with both the sural nerve laterally and the saphenous nerve medially exists (Figure 3C, 7A).

From the dorsal extravasation figures it can be appreciated that the territories extravasated by the saphenous and peroneal nerve stimulation cover almost the entire dorsal aspect of the paw (Figure 7B-2.3). Both areas meet each other proximally in the centre of the hind paw and generally demonstrated a shared extravasation of the skin of the 2nd and 3rd digits. Comparing the extravasation representations of the peroneal and sural nerve in the lateral view indicates that the sural nerve area is systematically and almost completely overlapped by that of the peroneal nerve. The overlap area is mostly dark red showing an even administration of the extravasation with a dark green line at the centre of the hind paw due to dominance of the peroneal nerve in this overlap area (Figure7C-1.2).

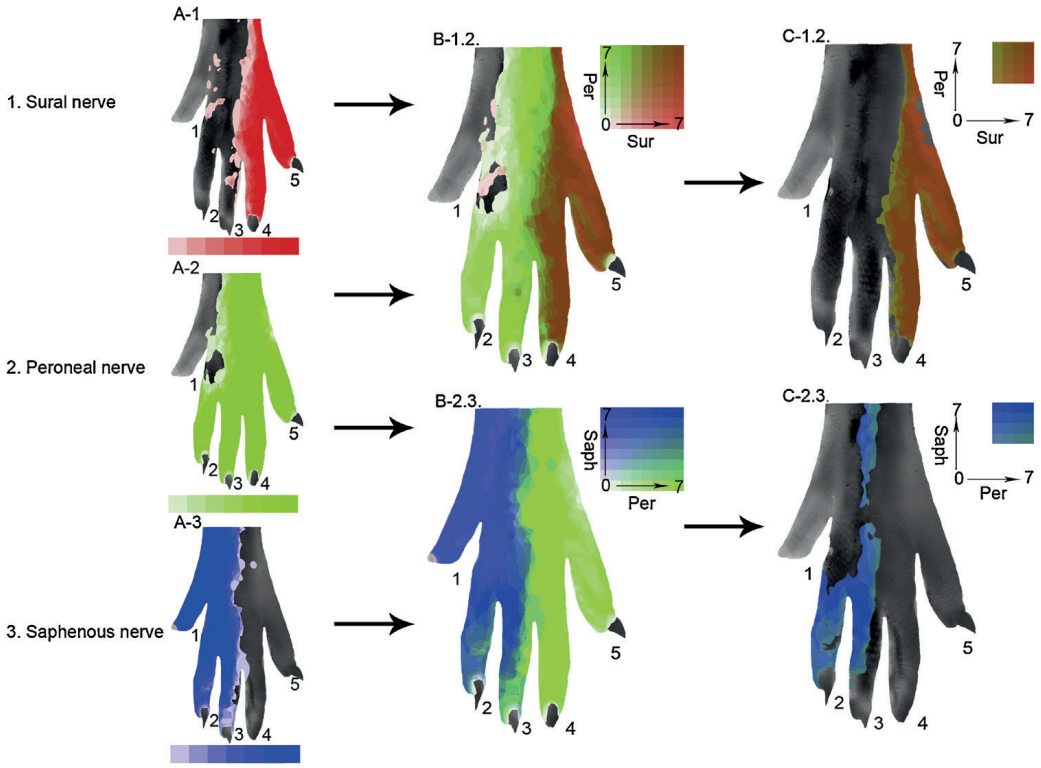

Figure 7. Dorsal overlap analysis.
A) The intra-animal variability of the extravasation is shown individually for the sural (red), peroneal (green) and saphenous (blue) nerves on the dorsal side of the paw ranging in incidence from light (1 animal) to dark (all 7 animals) as shown in the colour legend below the hind paw. B) Combined extravasation areas showing overlap (B-1.2.) Note that the area of combined overlap of the peroneal and the sural nerve is very alike the extravasation figure of peroneal nerve (A-2) (B-2.3) The combined extravasation areas of the saphenous and the peroneal nerve show a complete innervation of the dorsal skin of the hind paw. C) The significant overlap between the saphenous and the peroneal nerve (C-1.2) and between the peroneal and sural nerve (C-2.3) are shown. B) The 7x7 grid of colour combinations is represented to show the incidence that a particular surface area is extravasated by stimulation of either or both of the nerves as given in the x and the y axis. C) 4x4 grid of colour in overlap areas of two nerves in at least four of the seven animals in each group. Per= peroneal, Sur = sural nerve, Saph= saphenous nerve.

Medial view

The medial view specifically incorporated the 1st and 2nd digits and the separation of dorsal and glabrous skin at the medial side of the paw (Figure 3D, 8A). The saphenous nerve caused extravasation of almost the entire medial view of the paw while extravasation by the tibial nerve was observed at the medioplantar side of the paw (Figure 8A). Stimulation of the tibial nerve resulted in coloring of more selective and rather variable spots while extravasation by

the saphenous nerve showed more consistent and elaborate staining. Note again that also in this view no extravasation of the footpads was noted (Figure 8). Furthermore, the saphenous nerve (blue) dominates the mediodorsal side, while the tibial nerve (pink) dominates the medioplantar side (Figure 3D, 8A). Similar to the lateral view of overlap (Figure 6C-2.3), the overlap at the medial aspect is mostly restricted to the border between the glabrous skin and non-glabrous skin at the medial view (Figure 8C-1.2).

All significant extravasation areas by individual nerves and all significant overlap areas are summarized in the four views shown in Figure 9. This Figure clearly illustrates the dominant appearance of the tibial nerve at the plantar side of the paw, whereas the dorsal side is covered by saphenous, peroneal and sural nerves and demonstrates the significant overlap in innervation areas of these nerves.

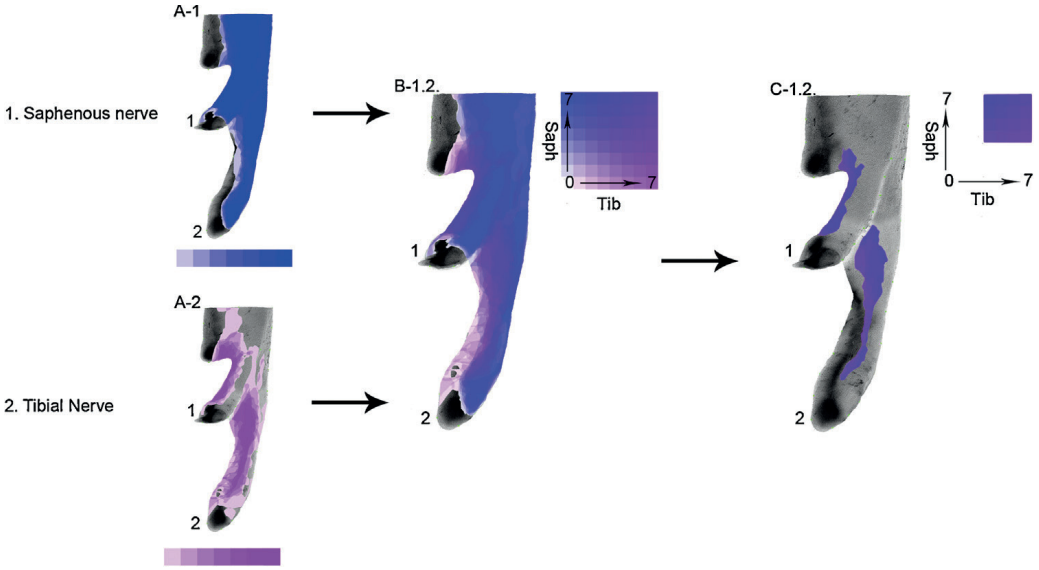

Figure 8. Medial overlap analysis.
A) The intra-animal variability of the extravasation is shown individually for the saphenous and tibial nerves on the medial side of the paw ranging in incidence from light (1 animal) to dark (all 7 animals) as shown in the colour legend below the hind paw. B) The combined extravasation areas of the saphenous and tibial nerves shows extravasation of all skin areas of the medial view except the footpads. This area consist mostly of the colours blue (saphenous nerve) on the dorsal side and and pink (tibial nerve) on the plantar side of the medial view, suggesting little overlap. C) Indeed the overlap figure shows a small area of significant overlap between saphenous and tibial nerve. B) The 7x7 grid of colour combinations is represented to show the incidence that a particular surface area is extravasated by stimulation of either or both of the nerves as given in the x and the y axis. C) 4x4 grid of colour in overlap areas of two nerves in at least four of the seven animals in each group. Saph= saphenous nerve, Tib= tibial nerve.

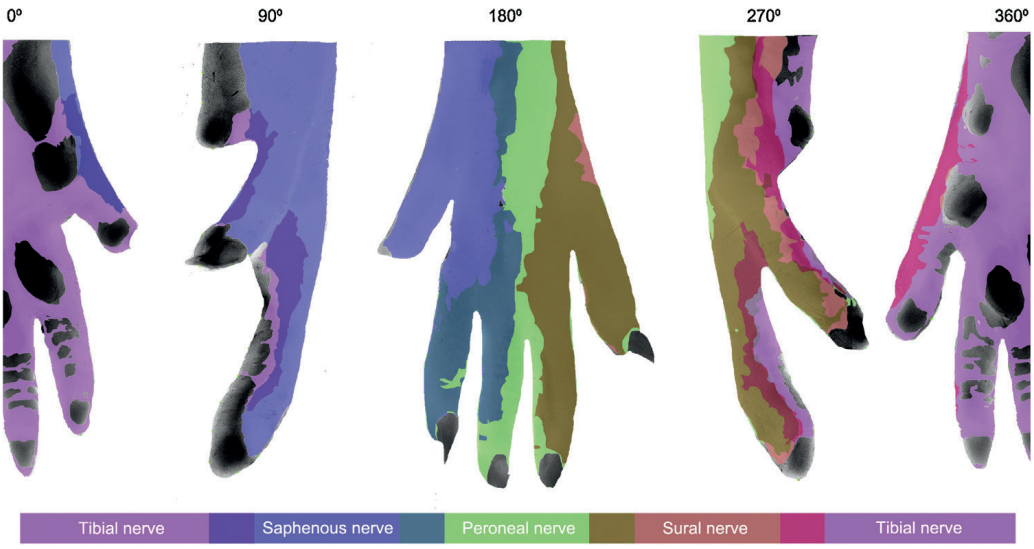

Figure 9. Complete extravasation areas of rats' hind paw.
All extravasation areas of rats' hind paw are shown in plantar (0º), lateral (90º), dorsal (180º) and medial (270º) view by tibial (pink), saphenous (blue), peroneal (green) and sural (orange) nerve. Each significant overlap area between the two nerves is indicated by different coulours: overlap between tibial and saphenous nerve is dark purple, between saphenous and peroneal nerve dark green, between peroneal and sural nerve brown and between sural and tibial nerve fuchsia. This figure serves as a summary of both individual extravasation as well as overlap in extravasation areas of rats' hind paw.

DISCUSSION

Methodological considerations combining Evans Blue extravasation, OSM scanner and CASAM technology

Sensory nerve fibers are important in perception of the environmental stimuli in order to allow interaction but also to avoid traumatic forces and extreme thermal exposure[33]. Individual fibers are characterized by well-defined receptive fields, which may partly overlap with that of other fibers. Many studies have been devoted to the analysis of changes in sensory skin fibers after peripheral nerve injury. The hind paw of the rat has become a commonly used model in studying these changes resulting from different kinds of trauma[3, 5].

In order to better evaluate and understand the various processes that take place during degeneration and regeneration of cutaneous nerves we have noted that a more precise description of the innervation areas of the hind paw would be invaluable. Although several studies have used the well-known technique based

on vascular labeling in order to examine the cutaneous regions of innervation by particular nerves,[7-20], their results are prone to subjective interpretations or are very cumbersome to obtain; i.e. subjective translation of staining patterns and extraction of dye from skin samples, respectively . The OSM scanning technique enabled a new and objective analysis of the area of extravasation by using a full 360° circle of views. Furthermore, it should be noted that the results of extraction studies are based on the assumption that the extracted amount of the extravasated dye is proportional to the number of nerve endings present in the skin[10]. However, this assumption seems less reliable since studies indicated that the extent of plasma extravasation depends on stimulation parameters such as frequency and time[11, 12]. These parameters have been used differently in literature, which makes comparison of evaluated dye extraction between studies difficult.

The stimulation parameters used in the present study were chosen with utmost care. Our stimulation frequency of 10 Hz was based on that of earlier results from which it could be deduced that changing stimulation frequency from 4 to 8 Hz (for 10 min) resulted in a more prominent plasma extravasation in the event of 8 Hz[11, 12, 14]. Furthermore, a trial study was performed from which the stimulation parameters were determined to ensure an optimal plasma extravasation without loss of information. Although some studies have used guanethidine (a vasodilator), at varying concentrations in order to enhance both blood flow and extravasation[12, 13, 16], this was not deemed necessary in the present study , as we have used isoflurane as an anesthetic, which has been demonstrated to also have a vasodilatory action[34].

After Evans Blue experiment the hind paw was morphed to an average size using CASAM technology. However, the heel was excluded in these calculations since no anatomic landmarks, which are required to morph the hind paw, are present in the heel. In addition, the heel is partly innervated by the gastrocnemius nerve, which is not included in this manuscript[35]. Therefore, the hind paw from the most proximal footpad until the tip of the toes was chosen as the area of interest to maintain consistent and accurate CASAM measurements for each paw and to show the innervation areas of the four (tibial, sural, peroneal, and saphenous) nerves supplying this area of the hind paw. The glabrous skin containing footpads is an important area in nerve regeneration and neuropathic pain studies in animals; behavioral studies to determine mechanical sensitivity using von Frey filaments is performed between the footpads[36-38], while thermal sensitivity (cold- and hot plate)[39, 40] is measured in the footpads. Furthermore, the four nerves that supply this area are stained in immunohistochemical studies, in which the sensory nerves in the footpads and/or in between the footpads are visualized and quantified[3, 41, 42].

Although accurate and exact results were obtained by combining the Evans Blue extravasation technique and the 360° imaging using OSM scanning technique, it was not possible to determine the overlap between the extravasation areas of different nerves within the same animal. Knowledge about the extent of overlap of innervation areas is crucial when studying the short and long-term effects of peripheral nerve injury. In addition, collateral sprouting of undamaged fibers following nerve injury may induce or enlarge areas of overlap in innervation area, which may be important when evaluating the behavior of regenerating nerves.

By using extravasation of two different dyes (Evans Blue and colloid silver) Dux et al.[20] already used the plasma extravasation technique in order to study the overlap areas of cutaneous innervation. Staining of the skin by both dyes visualized skin areas with Evans Blue, areas with colloid silver and areas with both Evans Blue and colloid silver staining that indicates for the overlap territory. However, these authors used an interval of 60 minutes between the stimulation of the two nerves in the same specimen to exclude any skin staining by the previous dye and waited another 30 minutes after the last nerve stimulation. It is known that Evans Blue is a fluorescent dye[43], which faded in time in our trial study (data not shown). In our view, this may have caused an underrepresentation of the Evans Blue extravasation areas in the study by Dux et al.[20], which could explain the smaller overlap in extravasation areas in comparison to our results. Moreover, the intra-animal variability of the individual extravasation areas was not taken in consideration in their translation studies. As the CASAM technology employed in our study uses many landmarks in order to determine an average hind paw, an accurate representation and subsequent comparison of the individual extravasation areas was possible[24]. Subsequently, the individual representations were marked and an overlay was made to quantify the area of overlap between two adjacent extravasation areas. It is important to note that in order to prevent dispersion in average paw size calculation, all animals had to be from the same strain, sex and of a comparable body weight. Although the hind paws were scanned and analyzed in 200 frames to cover 360° to determine the extravasation and overlap areas, only four views are shown in the present study as they cover the complete hind paw (apart from the 3rd inter-digital space).

Results of extravasation in comparison with previous studies

Previously it has been shown that antidromic stimulation of the autonomic nervous system can lead to an increase in skin blood flow due to activation of Aδ-fibers[21], whereas the C-fiber activation causes both vasodilatation and plasma extravasation of Evans Blue by release of neuropeptides[22]. Therefore,

the interpretation of the results from Evans Blue extravasation is based on the assumption that the stained skin corresponds with the afferent C-fiber innervation area of the stimulated nerve, as could be verified with electrophysiological techniques[23]. Consistent with this evidence, preliminary results from our laboratory using the pan-neuronal marker PGP9.5 confirmed that the extravasation areas correspond exactly to the innervation area of the stimulated nerve (see control study). From these findings we can conclude that the Evans Blue extravasation results in an indirect but accurate representation of C-fiber innervated skin regions of a particular nerve.

Swett and Woolf were one of the first to describe the size and location of cutaneous innervation of rats' hind paw by electrophysiological recording techniques and labeling[44]. Consistent with our findings, they show that the plantar side of the paw is mainly innervated by the tibial nerve (Figures 5, 9). Moreover, the boundaries between the sural and saphenous nerves with the tibial nerve corresponded closely with the hair line margin of the plantar skin, which is similar to our results (Figures 5B). Interestingly, over time different groups studying nerve regeneration have been referring to the classic work of Swett and Woolf when considering the plantar innervation area of rats' hind paw[45, 46]. It seems however that these studies described the plantar innervation of the hind paw differently: in three equal parts in which the medial part (including the entire 1st and parts of the 2nd digit) would be innervated by the saphenous nerve, the central part by tibial nerve and the lateral part (including parts of the 4th digit and the entire 5th digit) by sural nerve. In the light of the present findings and the initial paper of Swett and Woolf we think that this representation is incorrect and should be interpreted attentively and with caution.

The results from this study indicated that the intra-animal variability in extravasation areas for individual nerves is rather low (Figure 3). This is visible by small areas of bright-discolored (low incidence) hind paw (Figure 3) and small SEM values for average extravasation calculation (Figure 4). Hence, the stained areas were consistent and continuous in all animals showing characteristic patterns of extravasation. As described by other studies, the skin of the footpads never show any sign of extravasation (Figures.3 and S1)[13, 18]. Moreover, we found that the callosities also show less extravasation in several cases (Figures.3 and S1), while it is shown that the footpads are innervated[47]. These findings are previously ascribed to the thickening of the skin in the footpads . However, recent studies in our lab by Duraku et al.[4], showed that the density of peptidergic nerve fibers in the epidermis of the footpads was considerably lower in comparison to the surrounding non-footpad area of rats' hind paw. After stimulation of the

nerve, peptidergic neuropeptides are excreted and cause the extravasation of the dye[12, 48]. Moreover, the epidermis of the footpads is almost three times as thick as the surrounding skin. Taken together, these properties, at least partly, could explain the generally poor extravasation results of the foot pads.

Although our extravasation areas are highly consistent, they are generally somewhat larger than those observed in in other studies[8, 9, 18] . A control experiment was performed to exclude that possible diffusion of the Evans Blue dye could cause the larger extravasation area. The results of this control experiment showed a positive correlation between the Evans Blue extravasation and the innervation of the skin by PGP9.6 positive nerve fibers (Figure 2). In our view the larger extravasation area can be ascribed to the usage of the hook electrodes that could keep the nerve under tension and may cause damage to the nerve. Although mineral oil and liquid paraffin was used, relatively long stimulation time (ranging from 5 to 90 minutes) might have caused drying of the nerve[8, 9, 18].

Furthermore, some studies have mapped the innervation of the hind paw of the rat in which both hind paws (ipsi- and contralateral) were simultaneously stimulated. Although comparable stimulation intensity as in the recent study was applied, these studies showed smaller extravasation areas that could be assigned to bilateral stimulation of the nerve[9, 10]. This assumption is confirmed by a study in which subsequently two nerves are stimulated to show overlap areas: when saphenous nerve is stimulated first followed by the peroneal nerve the dorsal 5th digit, which is stained completely in the recent study due to peroneal nerve stimulation (Figure 7A-2), does not show any extravasation. However, when the condition is reversed and the peroneal nerve is stimulated first almost the entire 5th digit shows extravasation[18]. From these findings we can conclude that bilateral or subsequent stimulation of two or more nerves shows less plasma extravasation, which, apparently, may result in incomplete extravasation areas. In addition, it seems possible that by allowing central transmission, stimulation of a nerve also influence extravasation not only of the contralateral paw but potentially in the ipsilateral paw as well. Therefore, in the present study unilateral stimulation was performed with a crush lesion proximal from the stimulation site to obtain a complete and detailed extravasation.

One of the most remarkable findings from this study is the dorsal innervation of the paw by the peroneal and saphenous nerve. These nerves intersect at the center of the dorsal hind paw causing a complete extravasation of the skin with a small area of overlap (Figure 7B-2.3). In contrast, the combination of the sural and peroneal nerve extravasation areas showed exclusively overlap of the entire area of the sural nerve on the dorsal view (Figure 7B-2.3). Moreover, it is

interesting to note that larger areas of overlap were seen on the dorsal part of the paw, whereas the overlap on the plantar side was minimal[18]. In our view these differences in overlap between the dorsal and the plantar surface could have implications for regeneration processes of the relevant nerves.

Results from this study could be used as control to compare the denervation and reinnervation areas of specific nerves in the skin after nerve injury. Since sprouting plays a major role once denervation takes place, it is important to acknowledge the correct and detailed innervation areas and the amount of overlap in healthy skin when studying nerve regeneration. In our view the use of the Evans Blue technology in combination with OSM and CASAM analysis represents an excellent tool to examine the origin of the nerves that sprout and contribute to the hypersensitivity in neuropathic pain animals. This could help answering the question why and where neuropathic pain syndromes occur.

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