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Trends in
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Review

# Peripheral neurovascular link: an overview of interactions and *in vitro* models

Afonso Malheiro,[1] Paul Wieringa,[1] and Lorenzo Moroni[1,*]

**Nerves and blood vessels (BVs) establish extensive arborized networks to innervate tissues and deliver oxygen/metabolic support. Developmental cues direct the formation of these intricate and often overlapping patterns, which reflect close interactions within the peripheral neurovascular system. Besides the mutual dependence to survive and function, nerves and BVs share several receptors and ligands, as well as principles of differentiation, growth and pathfinding. Neurovascular (NV) interactions are maintained in adult life and are essential for certain regenerative mechanisms, such as wound healing. In pathological situations (e.g., type 2 diabetes mellitus), the NV system can be severely perturbed and become dysfunctional. Unwanted neural growth and vascularization are also associated with the progression of some pathologies, such as cancer and endometriosis. In this review, we describe the fundamental NV interactions in development, highlighting the similarities between both networks and wiring mechanisms. We also describe the NV contribution to regenerative processes and potential pathological dysfunctions. Finally, we provide an overview of current *in vitro* models used to replicate and investigate the NV ecosystem, addressing present limitations and future perspectives.**

## Peripheral NV system

With the evolution of ever-larger multicellular organisms, distinct tissues began to form with increasing complexity, eventually creating distinct organs that were capable of performing highly specialized tasks. Throughout this process, the development of the vascular and nervous systems was key. The vascular network covered the organs, providing oxygen and nutrient supply and removing the metabolic waste. Similarly, the nervous system extended branches that connected target organs to the central nervous system (CNS), establishing communication and permitting coordination of tasks [1,2]. The result was the formation of extensive and arborized patterns of nerves and BVs, with significant overlap between the two networks (Figure 1A–C). The mutual dependence of nerves and BVs also contributed to this NV alignment, since nerves require vascularization to ensure oxygen and metabolic support (Figure 1D), whereas large BVs need innervation to regulate vasodilation and vasoconstriction [3].

This review covers the peripheral NV system, highlighting developmental aspects, interactions during regeneration and NV dysfunction in pathological situations. Finally, current *in vitro* models of peripheral NV units will be discussed.

## Reaching the target

The formation of both the vascular and nervous system is tightly controlled by a series of developmental cues that ensure the formation of a complex and highly stereotypical mature network. In vertebrates, BVs arose later than nerves, but coadopted the same architectural principals and molecular mechanisms as those responsible to wire up the nervous system [4]. As a consequence, both tissues share several signaling pathways and principles of growth,

## Highlights

The peripheral NV system comprises a collection of peripheral nerves and BVs wired throughout the body in a highly stereotypical and congruent configuration.

Several ligands and receptors are shared between nerves and BVs, denoting the existence of similar biological mechanisms that emerged and were coadopted during the evolutionary history.

The development of the NV system is dictated by a myriad of cues that in soluble or bound (matrix- or cell-bounded) form, act on both nerves and BVs to influence their growth and organization, exerting either tissue attraction or repulsion.

The anatomical convergence between neural and vascular networks results partly from their mutual dependence and direct influence on each other, but also from the action of other tissues acting as central mediators.

Several pathologies can produce NV morphological aberrations and dysfunctions. For instance, patients with type 2 diabetes mellitus can develop myelin abnormalities and lower nerve conduction velocities, as well as BV leakage and reduced blood flow.

Neural ingrowth and vascularization are also associated with the onset and progression of certain pathologies such as some forms of cancer and endometriosis.

The NV system plays a vital role in the regeneration of some organs. In skin, the success and efficiency of wound healing is dependent on the epidermal nerve fiber secretome and in neovascularization.

*In vitro* models have been developed to recreate the PN environment and offer


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differentiation, organization and pathfinding [4,5]. During embryonic development, vasculature is formed at an earlier stage than nerves, when mesoderm-derived angioblasts differentiate and coalesce to form the primary vascular plexus, in a process termed vasculogenesis [6]. Subsequent vessel formation occurs mainly via sprouting angiogenesis, as the vascular network extends and remodels to cover avascular regions [7]. The peripheral nervous system (PNS) arises from the trunk neural crest, as neurons of different subtypes send neurite projections in a spatially and temporally orchestrated manner [8]. Arising from the CNS, parasympathetic nerve fibers project towards most organs to innervate them. From the dorsal root ganglia and sympathetic ganglia, sensory and sympathetic neurons, respectively, extend axons towards their targets, while motor neurons from the ventral spinal cord send projections to the periphery [9]. To vascularize and innervate tissues, BVs and nerves make use of similar pathfinding mechanisms. Sprouting BVs designate a specialized endothelial cell as a 'tip cell' to sense the environment and pave the way, while trailing 'stalk cells' proliferate and form capillary lumens to allow blood flow. In a similar fashion, neuron growth cones project numerous filopodia that actively extend and retract in response to the environment [5]. Both tissues manage to travel over long distances to far away targets by dividing their path into smaller segments, bounded by intermediate targets, thus simplifying the navigational task [1,4]. To help in this, and to promote growth and survival, there is a plethora of signals and respective receptors that are shared between BVs and nerves (Table 1). These signals can act at a short range when matrix-bound or at a long range when freely soluble, and can either provide an attractive or a repulsive cue [2,10] (Figure 2). As an example of common signals, nerve growth factor (NGF) is a known neurotrophic factor but can also exert a positive influence on endothelial cell (EC) proliferation, survival, and migration [11,12]. Similarly, the vascular endothelial growth factor (VEGF) family is a well-characterized inducer of vasculogenesis and angiogenesis, but evidence has shown it can also promote neurogenesis, neuroprotection, and neural growth [13]. Furthermore, four families of classic axonal guidance cues – ephrins, netrins, slits, and semaphorins – were discovered to induce vessel guidance as well (Table 1). These cues can stimulate attraction or repulsion, depending on receptor configuration or activity of secondary messengers [1,5,7]. For this, the cell leading front contains a pool of mRNAs encoding for receptors and intracellular signaling proteins that regulate the cytoskeleton dynamics. Attractive cues induce actin polymerization towards the cue direction, while repulsive cues signal actin depolymerization and cause the cell to steer away. The expression of these surface receptors is also dynamic and is changed at 'choice points', in a precisely orchestrated process [14].

Besides this developmental stereotypical configuration of larger BVs and nerves, target tissues also regulate vessel sprouting and axonal arborization. Hypoxic tissues secrete VEGF to recruit vascular supply, whereas target tissues devoid of synaptic input secrete neurotrophic factors, such as NGF, to attract innervation. In both cases, once the target tissue is sufficiently supplied with oxygen or electrically stimulated, the production of growth factors (GFs) by the target tissue subsides [1].

## NV wiring

From embryonic development to adulthood, NV networks maintain an ordered configuration, where nerves and BVs often follow a convergent path. To arrive at this composition, two distinct mechanisms are known to be in place. One is the existence of a central mediator that attracts and directs nerves and BVs organization [15]. This is observed in the mouse whisker pad, which displays a double ring structure around each follicle, composed of nerves (inner ring) and BVs (outer ring). In mutant mice, lacking trigeminal neurons and thus lacking nerve rings, vessels rings still form normally. Similarly, in mice with deformed vessel rings, nerve rings are able to form normally. These findings indicate the existence of a central and independent patterning

a research platform that minimizes the use of animals and permits the investigation of biological interactions in a more direct, reproducible and simple manner. To date, current models are yet to show a level of biomimicry and tissue functionality that fully translates the *in vivo* complexity.

Future developments in the fabrication of NV *in vitro* platforms will contribute to an improvement of biological phenomena understanding as well as discovery of new therapies.

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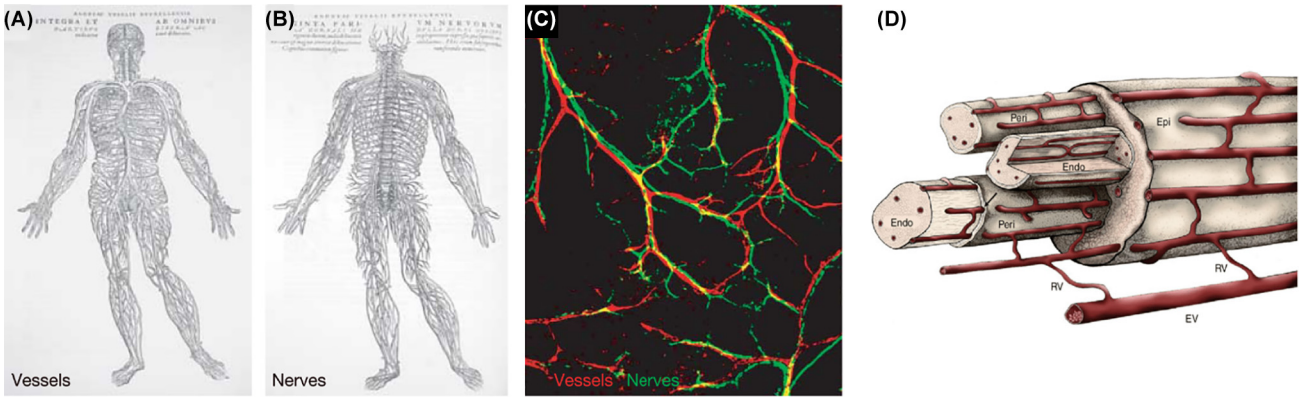



Figure 1. The neurovascular network. Illustrations of the vessel (A) and nerve (B) network drawn by Andreas Vesalius, a 16th century Flemish anatomist and physician. (C) Example of vessels (red) and nerves (green) following a similar path. (D) Illustration of the vasa nervorum, showing how vessels cover the peripheral nerves. (a–c) were extracted from Carmeliet *et al*. [1] (2005) and (d) from Mizisin *et al*. (2011) [110].

mechanism [16]. The other patterning mechanism results from the direct influence of a tissue on the other. A well-characterized example of this occurs in the developing limb skin, where cutaneous nerves invade the primary capillary plexus and induce nerve–vessel alignment. The recruitment of vessels to the vicinity of nerves, occurs via secretion of Cxcl12 by Schwann cells (SCs) and neurons, which attracts Cxcr4$^{+}$ ECs [17]. Following NV alignment, VEGF-A secreted by the SCs and neurons induces arteriogenesis, marked by upregulation of ephrinB2 and neuropilin (Nrp_1. Nrp1 upregulation further leads to increased sensitivity to VEGF-A, which helps to maintain this nerve-artery congruency (Figure 3A–D, F–M). In mutant mice lacking sensory and motor nerves, the vessels exhibited an altered pattern and defective arterial differentiation. Furthermore, in mutants with abnormal nerve patterns, remodeled arteries aligned with these nerves and could undergo arterial differentiation [9]. Taken together, these observations indicate a direct influence of nerves on BVs. Conversely, a direct influence of BVs on nerve patterning also occurs. This is the case for sympathetic nerve alignment with arteries, as observed in the skin. Smooth muscle cells cover the arteries and secrete artemin, which acts

Table 1. Common signals and receptors shared by nerves and blood vessels and their influence on pathfinding (attraction or repulsion)

| Ligand | Form | Receptor | Type of cue | |
|---|---|---|---|---|
| | | | Nerves | Blood vessels |
| *Ephrin* | Cell membrane-bound | ■ Eph | Attractant [93]/ repellent [94] | Attractant [95]/ repellent [96] |
| *Netrin* | Matrix-bound | ■ DCC<br>■ Unc5 | Attractant [97,98]/ repellent [97–99] | Attractant [100]/ repellent [101] |
| *Slit* | Matrix-bound | ■ Robo | Attractant [102]/ repellent [103,104] | Attractant [105]/ repellent [102] |
| *Semaphorin* | Diffusible | ■ Plexin<br>■ Nrp | Attractant [106]/ repellent [106] | Attractant [107]/ Repellent [107,108] |
| *VEGF-A* | Diffusible/Matrix-bound | ■ VEGFR2<br>■ Nrp1 | Attractant [13] | Attractant [2] |
| *NGF* | Diffusible | ■ TrkA, p75NTR | Attractant [1] | Attractant [11,12] |

Abbreviations: DCC, deleted in colorectal cancer; Nrp1, neuropilin1; p75NTR, p75 neurotrophin receptor; Robo, roundabout; TrkA, tropomyosin receptor kinase A; Unc5, uncoordinated 5; VEGFR2, VEGF receptor 2.

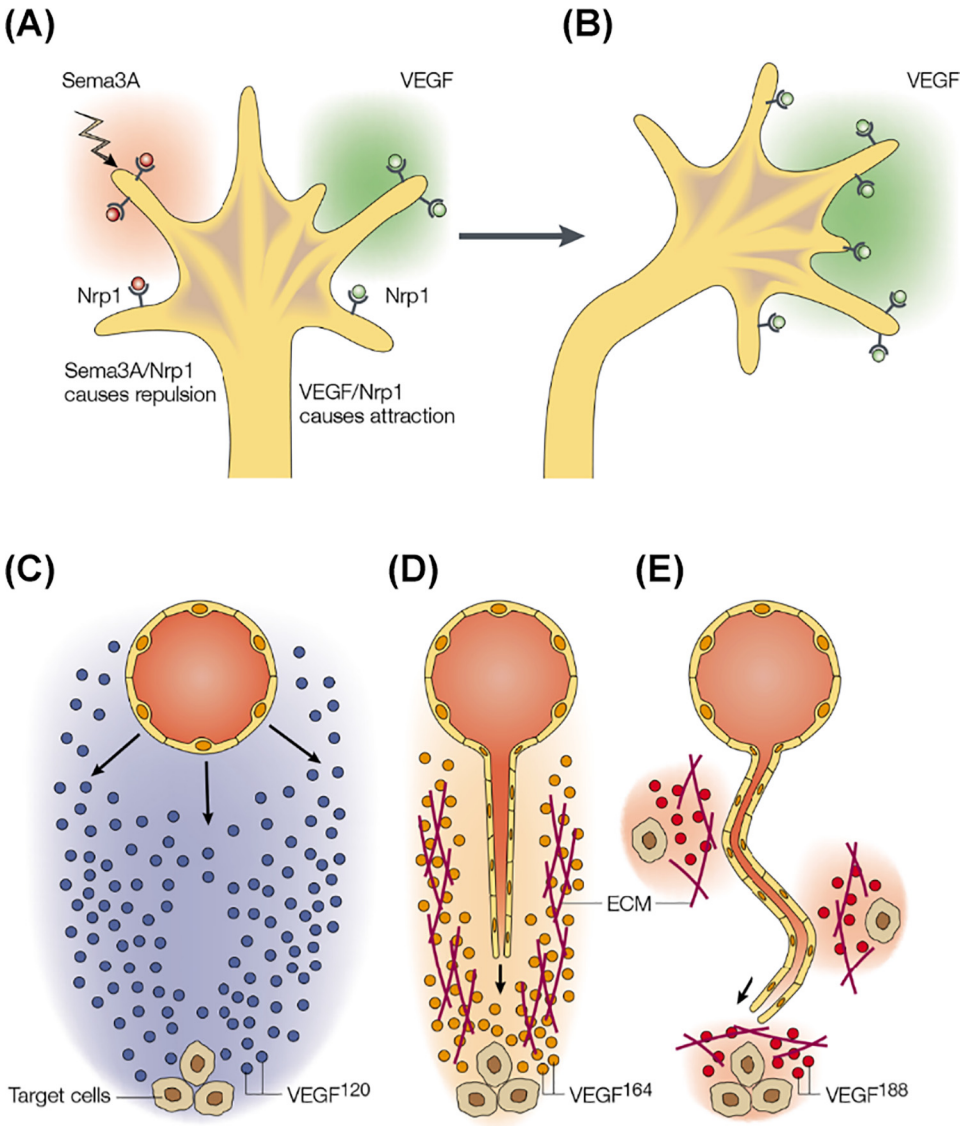



Figure 2. Pathfinding mechanisms of nerves and blood vessels. (A,B) Attractive and repulsive cues determine the direction of axons and sprouting vessels. (A) Both axons and endothelial cells (ECs) express neuropilin1 (Nrp1) at their filopodia, to which Sema3A and vascular endothelial growth factor (VEGF) bind. Sema3A causes repulsion whereas VEGF attracts the filopodia. (B) As a result, axons or ECs move towards the VEGF gradient. (C–E) Role of VEGF signaling in vessel branching. (C) The soluble VEGF120 does not bind to the matrix and consequently fails to provide long-range guidance. (D) The VEGF164 isoform provides both short-range matrix guideposts and long-range attraction gradients that allow the sprouting vessel to reach the target efficiently. (E) The VEGF188 isoform binds only to matrix and thus does not provide any long-range attraction, causing the sprout to be misguided over short distances. (A–E) were extracted, with permission, from Carmeliet *et al.* (2003) [2].

as a neurotropic factor, attracting sympathetic fibers to ride along the arterial (and sensory fiber) template (Figure 3E). Because artemin secretion gradually shifts distally, a local growth factor gradient is perpetuated to guide sympathetic fibers as they grow towards the target organ, resulting in NV alignment [2,9,18].

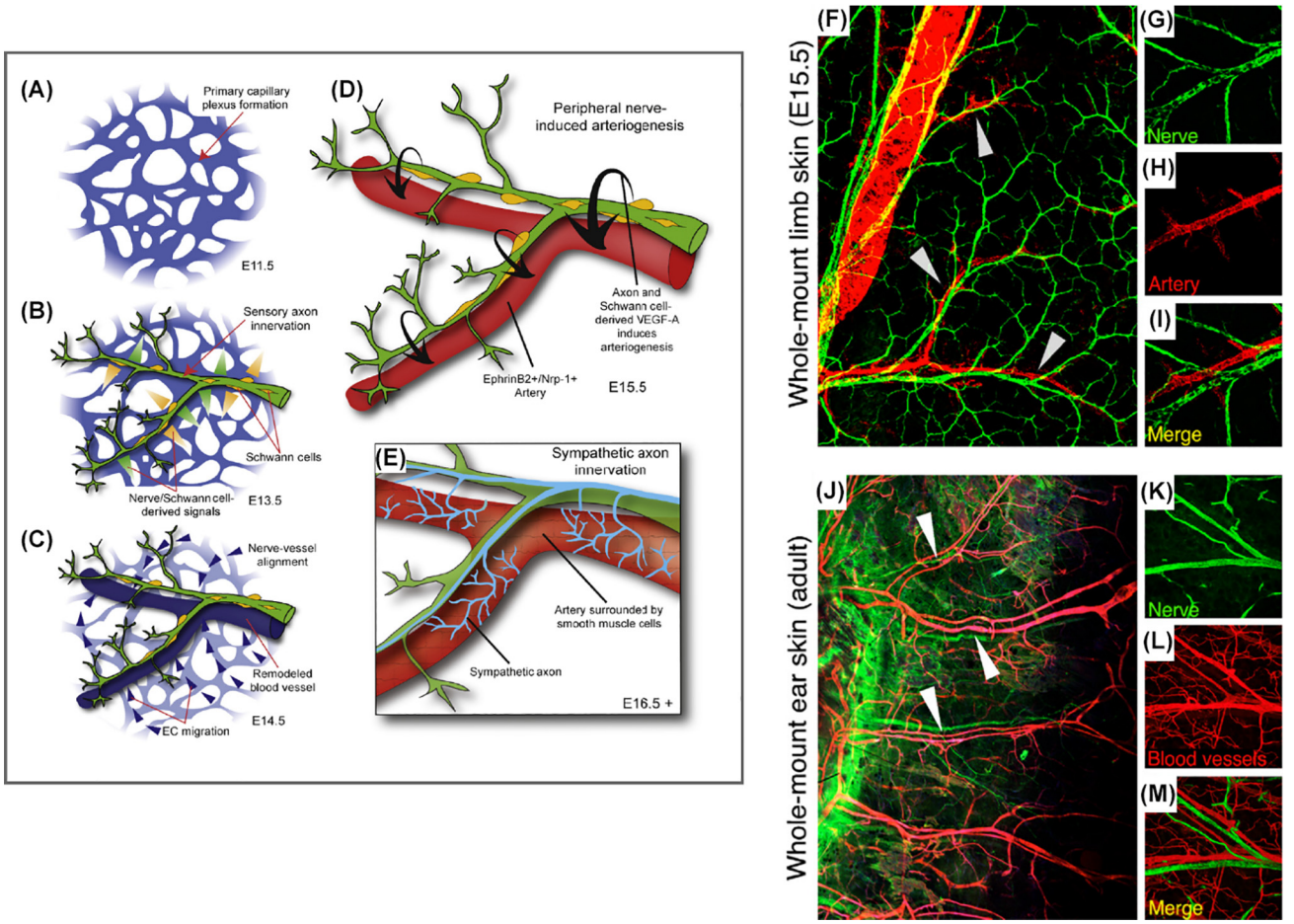



Figure 3. (A–E) Mechanisms of neurovascular (NV) patterning within the developing limb skin. (A) Formation of a primary capillary plexus via vasculogenesis at E11.5. (B) Sensory axons accompanied by Schwann cells invade the vascular plexus at E13.5 and secrete signals (e.g., Cxcl12) to pattern the vessels. (C) In response to those signals, the vessels align with the nerves. (D) Blood vessels (BVs) aligned with the nerves undergo arteriogenesis in response to nerve and Schwann-cell-derived vascular endothelial growth factor (VEGF)-A, upregulating ephrinB2 and neuropilin (Nrp)-1. (E) Sympathetic axons innervate the limb skin, using the BVs and sensory nerves as a template for migration. Sympathetic innervation of the arteries is necessary to regulate vessel tone. (F–M) NV congruency in the embryonic and adult skin. (F–I) NV alignment on E15.5 mouse embryo skin. Whole-mount immunofluorescence micrograph showing the alignment of nerves (green) and arteries (red). (J–M) NV alignment is maintained in the adult phase. Whole-mount ear skin preparations from an adult mouse, showing nerves (green) and BVs (red) following parallel paths. All images were extracted, with permission, from James *et al*. (2011) [9].

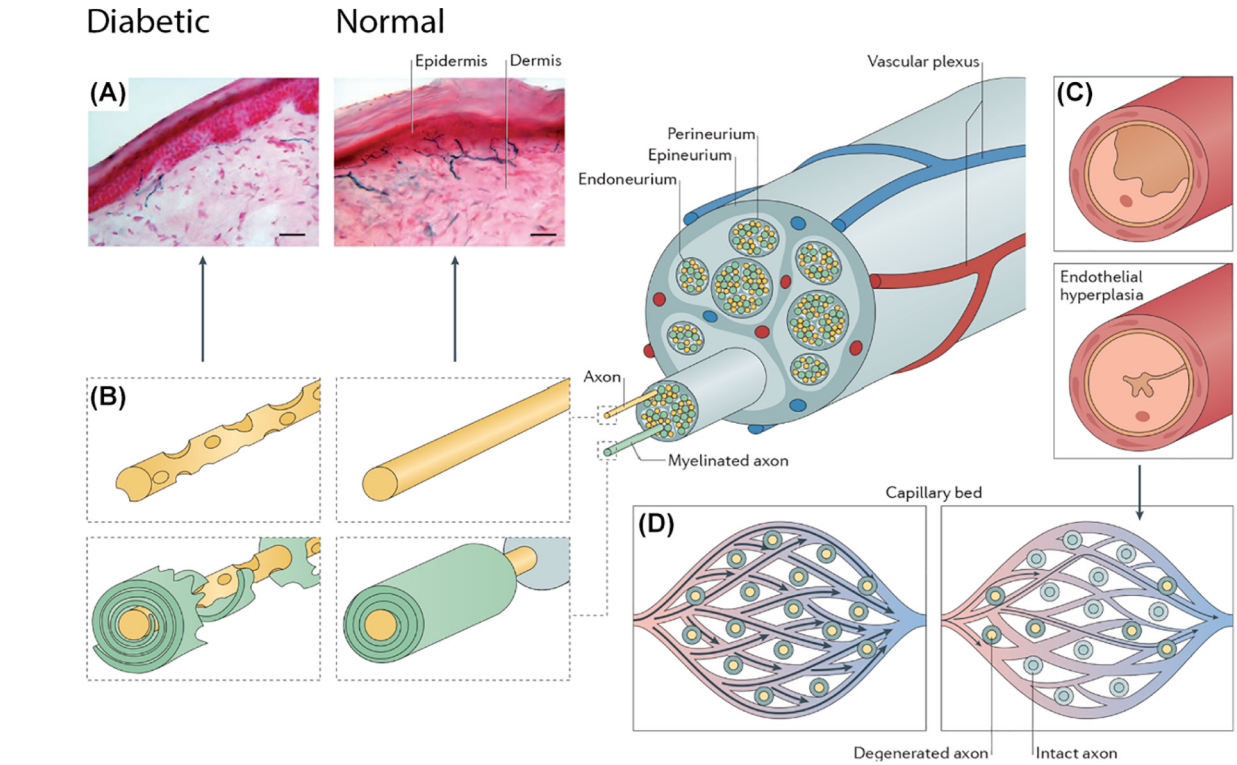
## NV dysfunctions

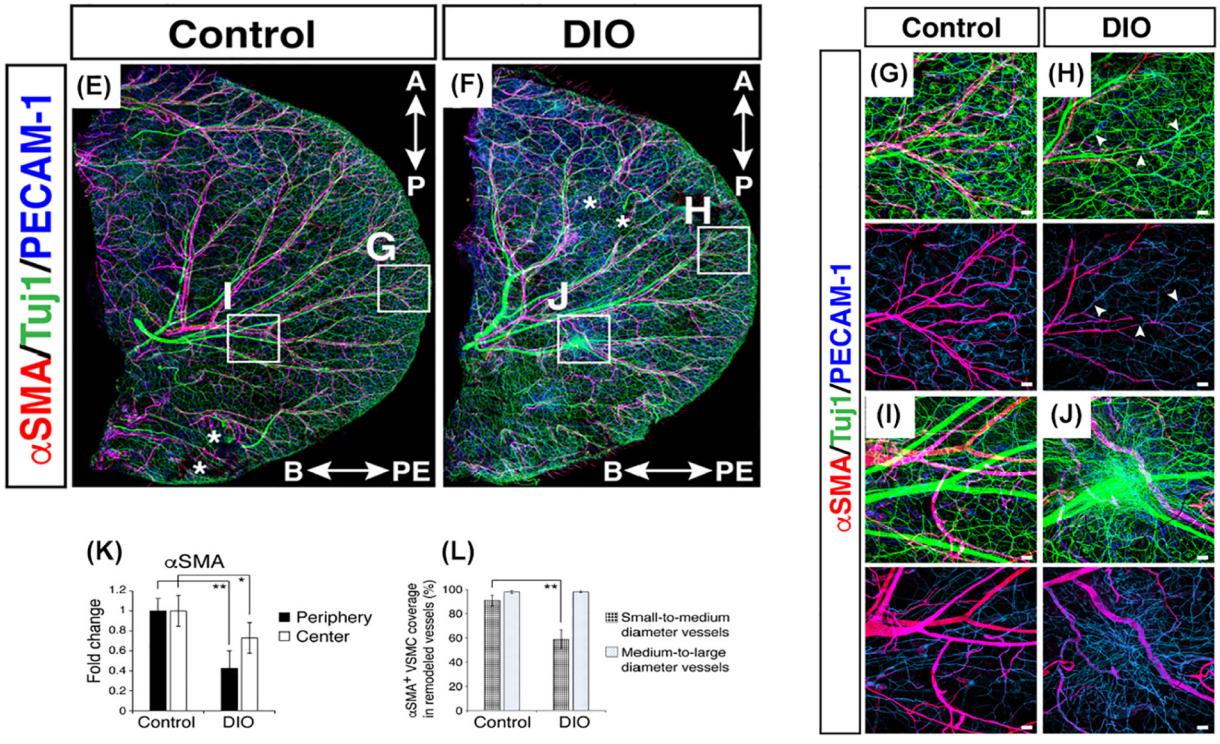
Damage to the PN tissue can occur in diverse situations, from localized traumatic insults (nerve crush, compression, stretching, or transection) [19], to systemic disorders, such as in Guillain–Barré syndrome or type 2 diabetes mellitus [20]. Because of the close interlink between nerves and BVs, peripheral neuropathies often result in NV dysfunctions.

The PNS contains an abundant and redundant vascular supply (Figure 1D). Thus, if the blood flow is partially interrupted due to an artery block (e.g., atherosclerosis or thrombosis), ischemia can still be prevented [21]. However, in the eventuality of complete blood flow interruption, ischemia ensues and can provoke axonal conduction block and distal axonal degeneration. Ischemia can also arise after nerve damage if regeneration is incomplete, resulting in chronically denervated nerve stumps. In these situations, blood flow is typically reduced as a result of poor vascular

regeneration due to an unfavorable environment (e.g., scarring or fibrosis) or due to lower metabolic demands because of axonal loss [21]. Traumatic injuries, however, account only for a small fraction of neuropathy cases. In diabetes mellitus, 50% of individuals develop neuropathy within time [20]. Diabetes mellitus can lead to profound alterations in the vascular, glial, and neuronal components, with potential severe consequences affecting not only the PNs but also other organs such as kidneys (diabetic nephropathy), eyes (diabetic retinopathy), and skin (Figure 4A) [22]. The most common pathogenic insults are dyslipidemia, hypertension, impaired insulin signaling, and most frequently hyperglycemia [22,23]. Increased intracellular glucose levels activate the polyol pathway, which converts glucose to sorbitol via aldose reductase activity. Sorbitol accumulation leads to elevated osmotic pressure, oxidative stress, and mitochondrial dysfunction that results in cellular damage [23,24]. Myelinating SCs express aldose reductase and are consequently affected by hyperglycemia, which is manifested by dedifferentiation to an immature phenotype, denoted by reduced myelin protein synthesis. Due to this, the myelin layer can reduce in thickness (demyelination) and develop morphological aberrations, such as infoldings, outfoldings and layer decompaction (Figure 4C), ultimately leading to lower nerve conduction velocities [24,25]. Furthermore, SC dysfunction also includes reduced secretion of trophic factors, such as ciliary neurotrophic factor (CNTF) and desert hedgehog, which support neurons and ECs [23]. Similarly, ECs also express aldose reductase and suffer behavioral alterations. Most notably, the polyol pathway flux activates the proinflammatory and prothrombotic pathways, potentially leading to capillary membrane thickening and pericyte/smooth muscle cell (SMC) dysfunctions that can result in loss of BV coverage (Figure 4E–L) [23,26]. The end result is vessel leakage and reduced blood flow, which decreases the trophic and oxygen support to neurons and SCs, causing cellular damage.

Neural and vascular growth can also be directly associated to pathologies and provoke unwanted consequences. A major example is the role that vascularization and innervation play in the onset and development of tumors. The dependence of vascularization for tumor growth has been known for long, because as in any other tissue, cells that are farther than 200 μm from a BV will suffer hypoxia. Therefore, for tumors to growth beyond 1–2 mm, they need to recruit BVs, which is done through the secretion of angiogenic factors, such as VEGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF) [27]. There are several mechanisms for tumor vascularization, including not only the classical sprouting angiogenesis, but also intussusception, vessel co-option, vasculogenesis, vasculogenic mimicry, and lymphangiogenesis [28]. The vascular network that is formed within tumors is different from the one present in normal tissues or the one formed during wound healing. Normal BVs display an ordered, hierarchical and uniform architecture and appropriate mural cell coverage. Conversely, BVs in tumors exhibit a tortuous and irregular shape and their density is heterogeneous. Moreover, these BVs are leaky and lack appropriate pericytes/SMC coverage [6,27]. To prevent tumor growth, several strategies have been deployed, but most research has focused in inhibiting VEGF signaling [27,28]. To this end, anti-VEGF antibodies (e.g., bevacizumab) have been used clinically in combination with chemotherapy or cytokine therapy, but have produced only a small increase in patient's survival [6,27]. This resistance to VEGF inhibitors stems from the adaptation of tumors to other forms of vascularization, such as vasculogenic mimicry and vessel co-option from surrounding tissues [27]. Additionally, other nontargeted angiogenic factors such as FGF and HGF can also attract BV ingrowth [6]. More surprisingly, nerves were also discovered to play a preponderant role in tumor initiation and progression. In various examples, such as in prostate, gastric, and pancreatic cancer, nerves were found to stimulate tumor development, and tumor denervation via surgical or pharmacological techniques was effective in suppressing further growth [29]. This influence of nerves derives from the release of neuropeptides (e.g., substance P; SP) and neurotransmitters (e.g., acetylcholine)

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from nerve fibers surrounding the tumor and in the tumor, which are able to modulate metastatic cascades [30]. Another important aspect of the nerve–cancer relationship is that tumor cells also release neurotrophic factors. These signals lead to the activation of cancer cell survival, proliferation, and invasion, but also stimulate the outgrowth of nerves into the tumor [29]. Secretion of NGF, either in final or precursor (proNGF) form, has been detected in various forms of cancer (e.g., breast and prostate). Because of this, tumor suppression strategies using anti-NGF antibodies have been explored, with success in preventing tumor growth, metastasis and angiogenesis in breast cancer [31]. Furthermore, the expression of axon guidance molecules, including different semaphorin and slit isoforms have also been detected in solid tumors, although their role in axonogenesis is still unclear.

Cancer is not the only pathology whose progression and symptoms are directly linked to NV tissue development. In endometriosis, an inflammatory condition affecting 5–10% of women in reproductive age, whose exact origin is still unknown, a heavily vascularized and innervated ectopic tissue resembling the endometrium, develops in pelvic area sites (e.g., ovaries, ligaments, and peritoneal surfaces). The formation of this tissue can lead to severe pain, the most common symptom [32]. Similarly to tumor vascularization, endometrial implant vascularization is proportionally related to its size and is triggered by an angiogenic switch from its hypoxic cell mass [33]. This angiogenic switch is denoted by the large release of angiogenic factors, such as VEGF, FGF, and angiopoietin by the endometrial implant but also by recruited activated macrophages and other inflammatory cells, including neutrophils, dendritic cells, and regulatory T cells [34]. Hormone stimulation, namely estrogen, is also responsible to stimulate vascularization. Again, sprouting angiogenesis is not the only vascularization mechanism, since vasculogenesis from circulating endothelial progenitor cells (EPCs) and inosculation of pre-established vascular networks can also provide a vascular supply to endometriotic lesions [34]. In women without endometriosis, the functional layer of endometrium is absent of innervation. However, endometriotic lesions display a rich innervation of mainly sympathetic and sensory fibers, particularly nociceptors expressing the transient receptor vanilloid subtype 1 [3,35]. Both the endometrial cells and the immune cells – macrophages, mast cells, and neutrophils – establish an inflammatory environment rich in neurotrophic factors, containing NGF, brain-derived growth factor (BDNF), and neurotrophin-3 that attract nerve fibers. These fibers are thought to contribute to the inflammatory environment by secreting proinflammatory molecules. At the same time, nociceptor fibers surrounding and infiltrated in the endometriotic implant become sensitized by this inflammatory milieu and convey this information to the CNS, where a pain sensation will be perceived [3,36]. To alleviate pain, treatments must focus target and arrest NV tissue development. Currently, there is no effective therapy for endometriosis-associated pain, and most research has focused on inhibiting angiogenesis, via VEGF blockers and receptor tyrosine kinase inhibitors [3].

Figure 4. Neurovascular (NV) dysfunctions in diabetes mellitus (DM). (A–D) Illustration of the different types of damage in diabetic neuropathies, involving axonopathy, schwannopathy, and microvasculopathy. (A) Skin section showing epidermal nerve fiber loss in a diabetic patient (left panel) compared to a healthy one (right panel). Scale bar is 40 μm. (B) Axonal and myelin degeneration resulting in nerve fiber loss. (C) Endoneural capillaries from patients with DM. Top panel shows a capillary from a patient without DM neuropathy, and bottom panel shows a capillary from a patient with DM, in which endothelial cell hyperplasia and basement membrane thickening led to capillary lumen size reduction. (D) Narrowing of individual capillaries might not prevent blood from passing through the endoneural capillary bed *per se*, but the resulting increase in velocity of blood prevents efficient oxygen extraction, causing hypoxia. (E–J) NV abnormalities in the ear skin of an adult mice with DM. Whole-mount immunostaining to BVs (blue, PECAM-1), axons (green, Tuj1) and vascular smooth muscle cells (αSMA) on control (E) and diabetic (F) mice. (G–J) Magnification of the box regions shown in (E, F), showing axonal abnormalities and lack of vascular smooth muscle cell coverage in diabetic mice. Asterisks indicate skin damage from the dissection and/or staining procedure and scale bar is 100 μm. (K, L) Quantification of αSMA presence shows reduced mural cell cover in diabetic specimens. (A–D) were extracted and modified from Gonçalves *et al*. (2017) [23] and (E–J) were extracted from Yamazaki *et al*. (2018) [26], with permission.

A summary of the above-mentioned pathologies and consequential NV dysfunctions is given in Table 2.

## NV interactions in regeneration

Both the neural and vascular systems possess the ability to react to damage and engage in a repair process to restore functionality. By sensing the environment, neural and vascular cells can alter their phenotype and enter in a regenerative mode that encompasses remodeling and regrowth until homeostasis is achieved. Understanding these repair mechanisms is critical for the development of smart therapies that can harness and potentiate the natural healing ability of tissues.

The events following a PN injury have been thoroughly investigated, and it is now clear that BVs play a crucial role in the success of PN regeneration. After a traumatic injury, damaged axons secrete the neuropeptides SP and calcitonin gene-related peptide (CGRP) that promote enlargement of intraneural BVs [37]. Vasodilation coupled with the release of monocyte chemoattractant protein-1 by SCs prompts the recruitment of resident and circulating macrophages [38]. The arrival and differentiation of these cells is crucial for PN regeneration, as activated macrophages phagocyte the axonal and myelin debris that inhibit axonal regrowth (Wallerian degeneration) [39]. Moreover, macrophages also secrete VEGF-A in response to hypoxia to induce neovessel formation, and secrete IL-1 to stimulate SC secretion of NGF and SC proliferation [40]. The newly formed vessels are critical to ensure oxygen and metabolic support to proliferating SCs that organize in cellular tracks, termed bands of Büngner, to act as conduits for regrowing axons [37]. In the situation of full nerve transection, BVs can also act as templates for Bands of Büngner. In a study by Cattin *et al*. [41], the authors discovered that macrophages invade the regenerating bridge, formed between the nerve stumps, and secrete VEGF-A in response to hypoxia. The latter is responsible for EC attraction and formation of a network of anisotropic vessels that span the bridge. Following this, SCs use these vessels as tracks to proliferate and cross the bridge, taking regrowing axons along. In mice with an absent or compromised PN vasculature, nerve repair was compromised, confirming the dependence of nerve growth on vasculature.

Table 2. Overview of the most prominent pathologies that result in NV dysfunctions

| Pathology | Cause | Neural dysfunction | Vascular dysfunction | Refs |
|---|---|---|---|---|
| Peripheral neuropathy | Trauma | • Conduction block<br>• Function loss<br>• Demyelination<br>• Axonal damage<br>• Endoneurium/perineurium damage<br>• Loss of nerve continuity<br>• Necrosis | • Increased vessel permeability<br>• Ischemia<br>• More complete blood flow interruption<br>• Hemorrhage | [19,109] |
| | DM | • Axonal degeneration<br>• Myelin degeneration<br>• Demyelination<br>• Function loss<br>• Reduced/blocked conduction | • Vessel leakage<br>• Wall thickening and weakening<br>• Reduced vessel density<br>• Reduced blood flow<br>• Pericyte/SMC coverage loss<br>• EC hyperplasia | [20,22–26] |
| Cancer | Various | • Tumor innervation<br>• Release of neurotransmitters that module cancer metastatic cascades | • Defective/irregular BV network<br>• Vessel leakage<br>• Reduced pericyte/SMC coverage | [27–31] |
| Endometriosis | Unknown | • Release of proinflammatory molecules<br>• Innervation of the ectopic implant<br>• Nociceptor sensitization | • Vascularization of the ectopic endometrial tissue | [32–36] |

These discoveries have propelled the development of new therapies that can improve the PN regeneration potential. Hobson *et al.* have demonstrated that supplementation of VEGF in a silicon nerve guide enhances vascularization, axonal regeneration and SC migration, in a dose-dependent manner [42]. In a different strategy, Fang *et al*. [43] used a gene-delivery system to introduce plasmid DNA encoding for NGF and VEGF in rats with a crushed sciatic nerve, and observed a synergistic effect of both GFs compared to individual dosages. Furthermore, VEGF-only doses led to higher sciatic function index and thicker myelin sheaths than NGF-only doses. Because VEGF is also a neurotropic factor, it is not clear whether the improvement of neural regeneration seen in both studies is a direct consequence of neovessel presence or VEGF stimulation. Nevertheless, the pre-establishment of vascularization prior to nerve growth is now regarded as a critical and necessary step for effective PN repair. Due to this, several nerve grafts have been designed to stimulate vascularization [44]. Earlier strategies included the use of nerve grafts with intact vasculature [45] or the use of artificial guides that were prevascularized *in vivo* [46]. However, such strategies are limited due to the need of autologous transplantation. To overcome this, some tissue engineering strategies have used biomaterial nerve guides seeded with endothelial cells (and other cells such as SCs or fibroblasts) and shown successful vascular and neural regeneration [47]. Bioprinting has also been used to fabricate fully cellular nerve grafts that are able to encourage nerve repair in animal models [48]. However, to date, bioprinting of prevascularized nerve guides is yet to be demonstrated.

Whether the nerve guide is prevascularized or permits a rapid *in vivo* vascularization, future repair strategies are now regarding vascularization as a central concern for efficient PN regeneration.

NV regeneration is not only important in the context of PN repair, but also to restore function to other organs. In the wound healing process, the precise and regulated participation of BVs and nerves is essential for an appropriate tissue regeneration. The first phase of this process is hemostasis, where BVs become constricted and a fibrin clot is formed in order to stop bleeding. The clot and the surrounding wound tissue releases proinflammatory cytokines and GFs, such as FGF, EGF, and transforming growth factor (TGF)-β to attract inflammatory cells, thus commencing the inflammatory phase. During this phase, infiltrating neutrophils, lymphocytes and macrophages are responsible for clearing wound debris and inducing a reparative state that promotes vascularization, extracellular matrix (ECM) formation and re-epithelialization [49]. In the hypoxic wound site, ECM fragments and activated macrophages release a myriad of angiogenic GFs, including VEGF, FGF (1 and 2), and TGF-β that trigger an angiogenic response [50]. Recruited ECs become activated and secrete proteolytic enzymes (e.g., matrix metalloproteinases) that degrade the surrounding ECM in order to proliferate, migrate, and form new capillaries [51,52]. Following this, BVs are stabilized via mural cell recruitment and ECM deposition. Once the oxygen levels are restored, a remodeling phase begins to promote vessel regression and replacement of the provisional ECM with a collagen type I-rich matrix [50,52].

The participation of nerves is also determinant for a successful and quick wound healing process. The skin is richly innervated by sensory and sympathetic fibers. Defective innervation in neuropathies, such as spinal cord injury or diabetes mellitus, can result in impaired wound healing [10]. Both the sensory and sympathetic fibers secrete several neurotransmitters and neuropeptides that have been shown to play vital roles and enhance the wound healing process [53]. For instance, the vasoactive intestinal peptide (VIP) and NGF stimulate re-epithelialization and angiogenesis; while the first promotes collagen deposition, the second promotes collagen maturation and remodeling [54]. Sensory neurons, in particular, have been shown to accelerate skin re-epithelization in a wound healing model, via SP mediation [55]. In animal models denervated of sensory fibers, wound healing was delayed and incomplete [54,56]. SCs also aid

in the regenerative process by differentiating to a repair phenotype and providing trophic support via paracrine signaling to non-neural cells at the wound site [57]. The secretome of nerve fibers can directly influence skin cells, for instance, by promoting proliferation of keratinocytes and fibroblasts. However, it can also indirectly contribute to the wound healing, by enhancing the angiogenesis process. As an example, SP stimulates vasodilation, vascular permeability and EC proliferation. Similarly, CGRP is a potent vasodilator and stimulates EC proliferation and wound contraction [10]. In summary, nerves and BVs interact with each other and directly with skin tissue to drive the wound healing mechanisms.

Contrary to amphibians, the ability to regenerate limbs is absent in adult mammals, with the exception of the distal digit [58,59]. If the digit tip is removed distal to the nail bed, tissue regeneration is still possible in a process deeply influenced by nerves. Using a murine digit tip regeneration model, Johnson *et al.* [58] proposed that after damage, axons degenerate and their associated SCs undergo dedifferentiation. Dedifferentiated SCs localize to the regenerating blastema and start secreting GFs, particularly oncostatin M and PDGF-AA, which promote the proliferation of the blastema mesenchymal precursor cells. Blastema cell expansion is essential for the regeneration of tissues such as bone and dermis, and in the absence or dysregulation of SC activity, blastema expansion is insufficient to drive full multi-tissue regeneration. Once regeneration is complete, axons regrow into the newly formed tissue and associate with dedifferentiated SCs, which either differentiate to a mature phenotype or maintain their regenerative program.

In some situations, often related to disease-induced neuropathies, NV regeneration is not possible and the damage can be irreversible. If the underlying cause of neuropathy is not removed, spontaneous regeneration cannot occur and treatments are rendered futile [60]. Despite the worldwide prevalence of diabetic neuropathy (DN), leading to nerve function loss, there is yet no cure for this disease [60]. In DN, the skin is often affected and shows reduced density of epidermal nerve fibers (ENFs), which causes loss of sensorial ability and impaired wound healing [61,62]. Some research has focused in promoting functional recovery of denervated skin by stimulating the regrowth of ENFs. For this, therapies aim to protect intact neurons and induce the regrowth of previously pruned ENFs towards the right targets [60]. Due to the chance of off-targets, systemic drug delivery systems have been abandoned in favor of localized therapies. For instance, local insulin treatments have shown improved skin innervation and conduction velocities in diabetic rodents [63,64]. Similarly, transdermal delivery of GFs have also demonstrated potential in promoting re-innervation [65,66].

## *In vitro* NV models

Current understanding of NV interactions in healthy and pathological conditions is still limited. Contributing to this and hampering new developments, is the lack of *in vitro* models that are representative of the native milieu and at the same time, accessible and cost-effective. To date, most research has relied on the use of animal models, mainly rodents, which offer a great tool to observe the intricate NV patterns in a normal state [9] (Figure 3) and their dysfunctions in a disease context [26] (Figure 4).

However, animal models are complex tools that are difficult to access, have high costs and bear several ethical concerns. Moreover, their innate divergence from human anatomy/physiology limits the translational potential of the findings [67,68]. *In vitro* models that reproduce the NV ecosystem may be able to shed light on current enigmas, by providing a human-based, tailor-made, and biologically/physiologically defined research platform. For the generation of an NV *in vitro* model, several criteria must be met, of note. (i) Appropriate dimensionality: 2D models

fail to recapitulate native cell–cell interactions and do not allow volumetric tissue growth and organization, essential for the formation of capillaries with lumens and appropriate innervation [69]. Thus, the exclusive use of 3D models is critical. (ii) Presence of ECM-like materials: the use of biocompatible, adhesive, tunable, and degradable matrices of natural (e.g., collagen and fibrin) [70] or synthetic origin (e.g., polyethylene-glycol-based hydrogels) [71] that provide a correct mechanical environment and permit cell–material interactions for cell modulation and tissue development. (iii) Appropriate cell model: primary ECs may be harvested and isolated, but their expansion potential is limited, while human primary neurons are not an option [69]. To overcome this, the differentiation of stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can provide a sustainable and unlimited source of neurons [72,73] and ECs [74] with defined and representative phenotypes. (iv) Presence of support cells: SCs are essential for neuronal maturation, by ensheathing axons with myelin for electrical insulation (in myelinated nerve fibers). Similarly, BVs require mural cells (pericytes and smooth muscle cells) for vessel stabilization and maturation [6]. Other cell types, such as fibroblasts [75,76] and mesenchymal stem cells [77] have also been reported to promote and maintain vasculature *in vitro*. Additionally, support cells also participate in regeneration mechanisms and are affected in diverse pathologies. Again, the differentiation of stem cells into support cells (e.g., iPSC-derived SCs [78]) can provide a sustainable and easily accessible cell stock. (v) Biomimetic architecture: for nerves this entails having a neuron population organized in soma clusters with anisotropic axonal projections, thus imitating the dorsal root ganglion (DRG) morphology. BVs should be arranged in an interconnected, multiscale and open network, exhibiting spatial congruency with the nerves (NV alignment).

At present, most NV platforms have focused in recreating the blood–brain barrier within the CNS [79–81], and only a few peripheral NV models have been reported (Table 3). Grasman *et al*. [82] established a 2D coculture of rat/chicken DRGs with human umbilical vein endothelial cells (HUVECs), and showed that EC-derived BDNF enhances axonal growth (Figure 5A). In a different study [83], the same authors reported that a HUVEC-lined channel within a collagen gel was able to attract axons from a chicken DRG, enhance their length, and induce co-alignment. A similar effect could be replicated in hollow BDNF-loaded channels. Because HUVECs originate from the umbilical cord, a noninnervated tissue, their relevance in NV models is limited. Yuan *et al*. [84] described a 2D model containing human microvascular endothelial cells (HMVECs) as a vascular population instead. These cells originate from the skin, a richly innervated tissue, and

Table 3. Summary of the state-of-the-art in *in vitro* peripheral NV models

| Authors | Dimension | Neurons | ECs | Materials | Comment |
|---|---|---|---|---|---|
| Grasman *et al*. [82] | 2D | DRG (rat or chicken) | HUVECs | Poly-D-L-lysine-coated polystyrene | • ECs stimulate axonal growth via BDNF secretion |
| Yuan *et al*. [84] | 2D | DRG (rat) | HMVECs | Collagen | • Co-cultures showed greater cell viability and mRNA/protein expression of VEGF and NGF than single cultures |
| Osaki *et al*. [86] | 3D | ESC motor neurons (human) | HUVECs/ iPSCs (human) | Collagen in a polydimethylsiloxane (DPMS) microfluidic platform | • ECs improved neuronal length, differentiation and activity<br>• Vessel perfusion accelerated synapse formation and synapse elimination<br>• Neurons promoted vessel enlargement |
| Kannan *et al*. [85] | 2D/3D | ESC sensory neurons (human) | ESC ECs (human) | Collagen | • Sensory neurons and neural crest stem cells induced vessel-like formation<br>• NV alignment in the 3D model |
| Grasman *et al*. [83] | 3D | DRG (chicken) | HUVECs | Collagen with a channel | • ECs attracted axons, enhance their length and induced co-alignment<br>• BDNF-loaded channel replicates EC activity |

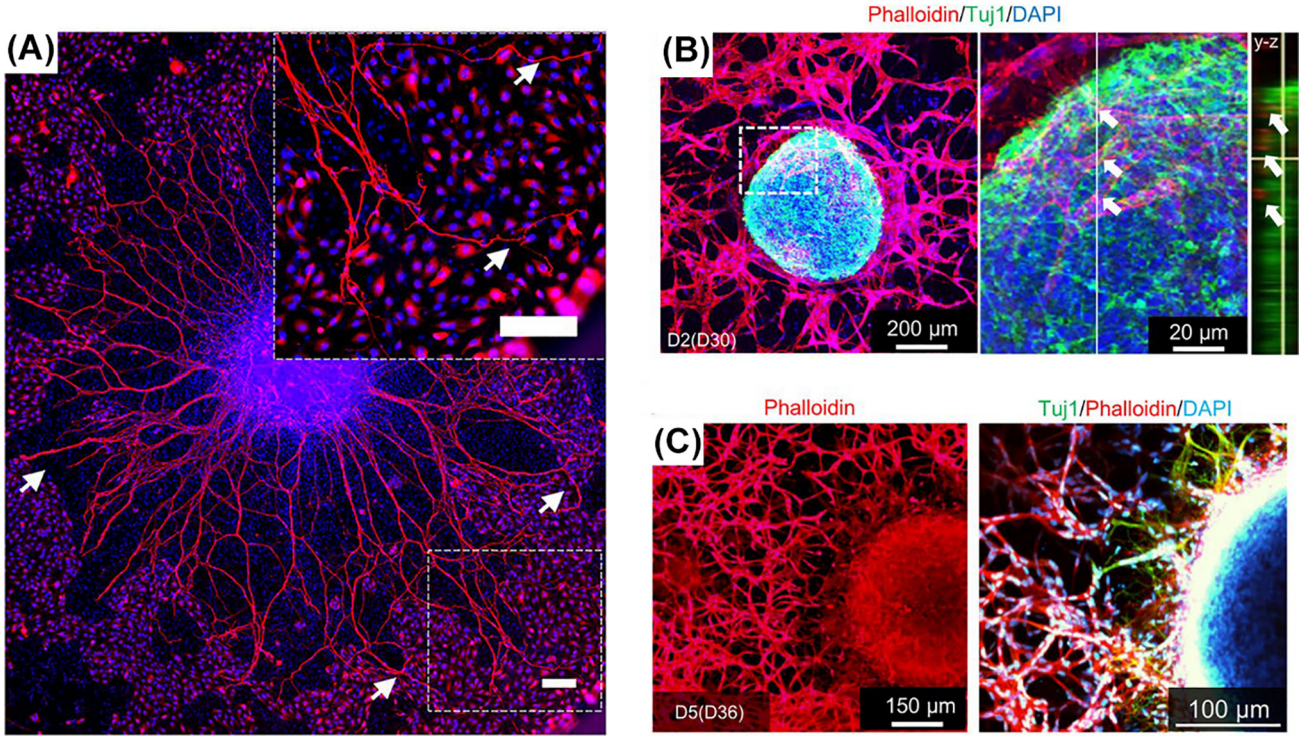


Figure 5. *In vitro* models containing neurovascular (NV) tissue. (A) 2D coculture of human umbilical vein endothelial cells (HUVECs) and a dorsal root ganglion. Axonal elongation was enhanced in the presence of HUVECs compared to samples without the vascular component. Immunostaining to Tuj1 (red). DAPI is shown in blue. Scale bar is 200 μM. Image extracted, with permission, from Grasman *et al.* [82]. (B) 3D coculture of embryonic stem cells derived motor neurons (ESC-MNs) and HUVECs in a collagen gel. (B) After 2 days of coculture, the HUVECs formed well-connected microvascular networks surrounding the spheroid. (C) After 5 days of culture, neurites began to elongate and establish connections with the existing vasculature. Immunostaining to Tuj1 (green). Phalloidin is shown in red and DAPI in blue. (B,C) were extracted, with permission, from Osaki *et* al. [86].

thus constitute a more relevant cell source. The authors reported that DRG/HMVEC cocultures led to higher overall cell viability, higher mRNA levels, and higher secretion of VEGF and NGF, compared to single cultures. However, the planar cocultures cannot achieve the architectural complexity of an NV axis. Aiming to address these limitations, Kannan *et al*. [85] developed a 3D model containing human ESC-derived sensory neurons and ECs in a collagen gel. The vascular cells could form vessel-like structures and aligned with neurons. However, the exhibited vessels did not establish interconnected networks and displayed an immature morphology. Additionally, the neuron population was dissociated and scattered rather than clustered and sending axonal projections as in the PNS. Thus, it did not replicate the native form of NV communication which occurs between ECs and axons, while the neuron cell bodies remain physically distant. In a more biomimetic approach, Osaki *et al.* [86] described a NV microfluidic platform containing ESC-derived motor neuron spheroids and iPSC-derived ECs within a collagen gel (Figure 5B,C). The presence of ECs improved neuronal differentiation, length and activity (measured by $Ca^{2+}$ oscillation). Analogously, neurons were able to influence vascular networks and promote vessel enlargement. Still, this model presents some limitations, mainly regarding the lack of glial and mural cells, which precludes the formation of mature NV tissue. In addition, the miniature dimension of the generated tissue leads to a mismatch of physical forces/parameters (e.g., oxygen tension) between this model and human-sized tissue.

Future efforts should focus in tackling current issues with the aim of generating biomimetic, functional and mature NV tissue that can be conveniently probed in a controlled scenario. For this, the presence of support cells is key. SCs have been extensively shown to improve neural tissue in *in vitro* models [87] and even being indispensable for target innervation [78]. BV maturation and long-term stabilization are also dependent on the presence of mural cells, which not only enhance lumen formation but also prevent/minimize fluid leaking [88]. Tissue functionality must also be shown by the presence of electrically active neurons, preferentially with on-demand stimulation (e.g., optogenetically [89]), and BVs that can be perfused to deliver nutrients and drugs, ideally at physiological fluid rates. Engineering of biomimetic tissue architectures is also crucial for accurate modelling of a NV unit.

With ever increasing complexity and the necessity to harmoniously combine multiple tissues into a single unit, several challenges arise. To address this, biofabrication technologies such as 3D bioprinting have been developed to provide the ability to precisely and automatically have spatio-temporal control over cells, biomaterials and biomolecules, for the design of biomimetic tissue [90]. With respect to vasculature, this is particularly important to generate perfusable networks with a macro- to microscale [91]. Despite permitting excellent tissue representations, biofabrication approaches are limited by the lack of control over physicochemical parameters, such as oxygen tension, shear stress, and drug concentration gradients. Moreover, current tissue analysis methods are invasive, disruptive and cumbersome, whereas automated noninvasive capability is highly desired for reproducible and long-term monitoring [92]. To this end, micro/macrofluidic technologies can be coupled with biofabrication methods to generate organ-on-a-chip devices that allow precise environmental control and in-line parameter detection [92]. Such platforms also permit facile tissue compartmentalization that is necessary for multi-tissue arrangement and allow high-throughput compound testing [89]. Finally, due to the fine control that organ-on-chips offer, pathologies with multiple physiological imbalances such as DM can be better investigated through the individual assessment of each parameters' influence.

## Concluding remarks

Nerves and BVs establish intricate and convergent networks throughout the body to support the function of most organs, as well as aiding in their regeneration. The integrity of these networks can be compromised in the eventuality of pathophysiological imbalances, which may cause severe consequences. To investigate these pathologies and develop new therapies, there is a need for functional humanized and biomimetic *in vitro* NV models. However, much work is still needed to fully replicate the complexity of the native tissues (see Outstanding questions).

## Outstanding questions

Certain ligands are able to induce opposite effects, namely, attraction or repulsion, on neurons and endothelial cells based on receptor configuration and activity of other biological players. How are these interactions controlled? Are there other signals that we are unaware of and have a preponderant role in NV network regulation? Could we harness these regulatory mechanisms to control NV tissue formation?

Network convergence in the NV system arises partly as a result of the interactions established between nerves and BVs. While we now understand how these interactions unfold within the developing limb skin, NV wiring mechanisms in most organs are yet to be characterized.

The NV system plays a major role in the cascade of events occurring during skin regeneration. Can we improve the regeneration process by targeting and modulating the NV components? What other organs benefit from NV input?

Neural and vascular growth can produce unwanted consequences such as tumor growth and tumor metastasis. Will it be possible to halt tumor progression by targeting the NV system without causing systemic damage?

NV integrity can be affected in certain pathological scenarios. Which tools can we use to at least mitigate the occurrence of dysfunctions while the primary cause of disease persists?

Will we be able to develop *in vitro* models that recreate the peripheral NV environment with sufficient accuracy so that these can replace animal models entirely?

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### Declaration of interests

The authors have no interests to declare.

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