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The multifaceted role of nerves in animal regeneration

Chiara Sinigaglia and Michalis Averof

Address: Institut de Génomique Fonctionnelle de Lyon (IGFL), Ecole Normale Supérieure de Lyon and Centre National de la Recherche Scientifique (CNRS), 32 avenue Tony Garnier, 69007 Lyon, France

Corresponding author: Sinigaglia, Chiara (chi.sinigaglia@gmail.com)

The discovery that the nervous system plays a critical role in salamander limb regeneration, in 1823, provided the first mechanistic insights into regenerative phenomena and stimulated a long quest for molecular regulators. A role for nerves in the context of regeneration has been suggested for most vertebrate and invertebrate groups, thus offering a possible shared mechanism for the regulation of regenerative processes among animals. Methodological differences and technical limitations, especially in invertebrate groups, have so far hampered broad comparisons and the search for common principles on the role of nerves. This review considers both old and recent work in this topic and provides a broad perspective on the roles of nerves during regeneration. Nerves are found consistently to have important roles in regeneration, but their mode of action varies across species. The ongoing technological developments in a broad range of invertebrate models are now paving the way for the discovery of the shared and unique roles of nerves in animal regeneration.

Introduction

The discovery that salamander limb regeneration depends on elements of the nervous system [1**] provided one of the first insights into the regulation of regenerative processes. Analogous influences of the nervous system were subsequently found in planarians, annelids, echinoderms, arthropods and many other animal groups, suggesting that nerve-dependency might represent a conserved feature of animal regeneration [2,3].

Research on the topic has focused on vertebrates, particularly on amphibians, which have become the paradigm for understanding the mechanisms of nerve-dependent regeneration (extensively reviewed in Ref. [4]). In other animals, experimental approaches have been largely limited to surgical and pharmacological manipulations, to demonstrate the active role of nerves in regeneration. Technical limitations have hampered our understanding of molecular and cellular mechanisms that underpin this phenomenon in most animals, thus limiting cross-species comparisons and a broad evolutionary understanding [5**,6*]. Recent discoveries on the shared molecular mechanisms governing the development and physiology of nervous systems in animals (e.g. [7].), and the ongoing efforts to apply -omics, functional genetics and imaging approaches to a broader array of species [8] are now paving the way for re-addressing long-standing questions in this field.

What is nerve-dependency?

The term 'nerve-dependency' has been employed to refer to diverse phenomena. In its broadest sense, nerve-dependency would encompass any case where the presence of a nerve is needed for the correct unfolding of the regenerative process. The nerves could provide spatial or temporal signals. They could act directly, or indirectly through other cell types. Their effects could be all-or-none or graded, permissive or instructive.

There are three broad ways, in which the nervous system has been shown to contribute to regeneration (Figure 1):

a) Source of local signals: Nerve-dependency can be directly mediated through the local action of the nerve terminals, which release signaling molecules. In axolotl limbs, the growth factor FGF-2 produced by the nerve promotes the formation of the Apical Ectodermal Cap (AEC). The subsequent interactions between nerves and the AEC drive the regenerative process [9].

b) Support for other cell types: Nerves can also contribute by serving as a scaffold for other cell types with important roles, such as cells that provide essential signals or serve as progenitors during regeneration. These may be cells that are closely associated with nerves even in non-regenerative conditions, such as the Schwann cells and their precursors, which provide essential signals for regeneration in salamander limbs [10**,11] and in mouse digit tips [5**], or mesenchymal progenitors that contribute to bone formation during digit tip regeneration [12**]. Equally, nerves can support other cell types that are not usually present in nerves but become transiently associated with them during regeneration. An example is the granule cell, a crinoid-specific cell type, which migrate along nerves and release essential growth factors [13]. Such transiently associated migratory cells also include progenitor cells, such as annelid neoblasts migrating along the ventral nerve cord to the wound site [14], or the scattered cells described in some sipunculid species, which leave the ventral nerve cord to generate epidermis and nervous system at the wound site [15].

c) Source of systemic signals: The nervous system may also perform systemic functions, essential for regenerative success - for example by releasing neurohormones in the circulatory system. This mode of action has been suggested for caudal regeneration of several annelid worms, which is impaired by brain ablation even in the presence of nerves at the wound site [16].

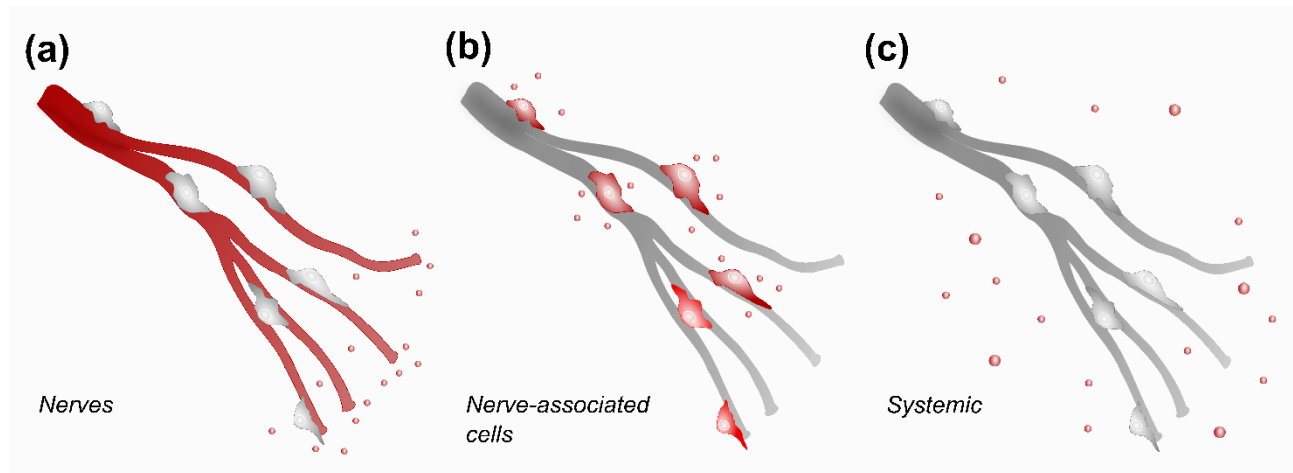


Figure 1: Modes of action of the nervous system during regeneration. The nervous system has been shown to contribute to regenerative processes in different ways, across animal groups. (a) Nerves can directly act as a source of local signals. (b) They can act as a support for other cell types, which provide signals and/or act as progenitors in the regenerating tissues. (c) Systemic signals originating from the central nervous system can also influence the regenerative process.

While nerves are not implicated in many aspects of embryonic development, they are known to play a role in specific cases. In development, as in regeneration, nerves can serve as a source of local instructive signals, a support or scaffold for other cell types, or a source of neuroendocrine signals that control development, metamorphosis and growth in a systemic fashion (e.g. [17]).

Phylogenetic and methodological disparity

Nearly two centuries of research on nerve-dependent regeneration produced a vast amount of disparate data, encompassing most animal groups (Figure 2, and Appendix). Vertebrate appendages (reviewed in Refs. [18,19]), echinoderm appendages (reviewed in Ref. [20]) and annelid bodies (reviewed in Ref. [21]) were extensively studied. Other taxa or regenerative processes received less attention, but nevertheless featured clear cases of nerve-dependent regeneration. For example, the presence of the nervous system was shown to be essential for body regeneration in a nemertean worm [22] and a holothurian [23].

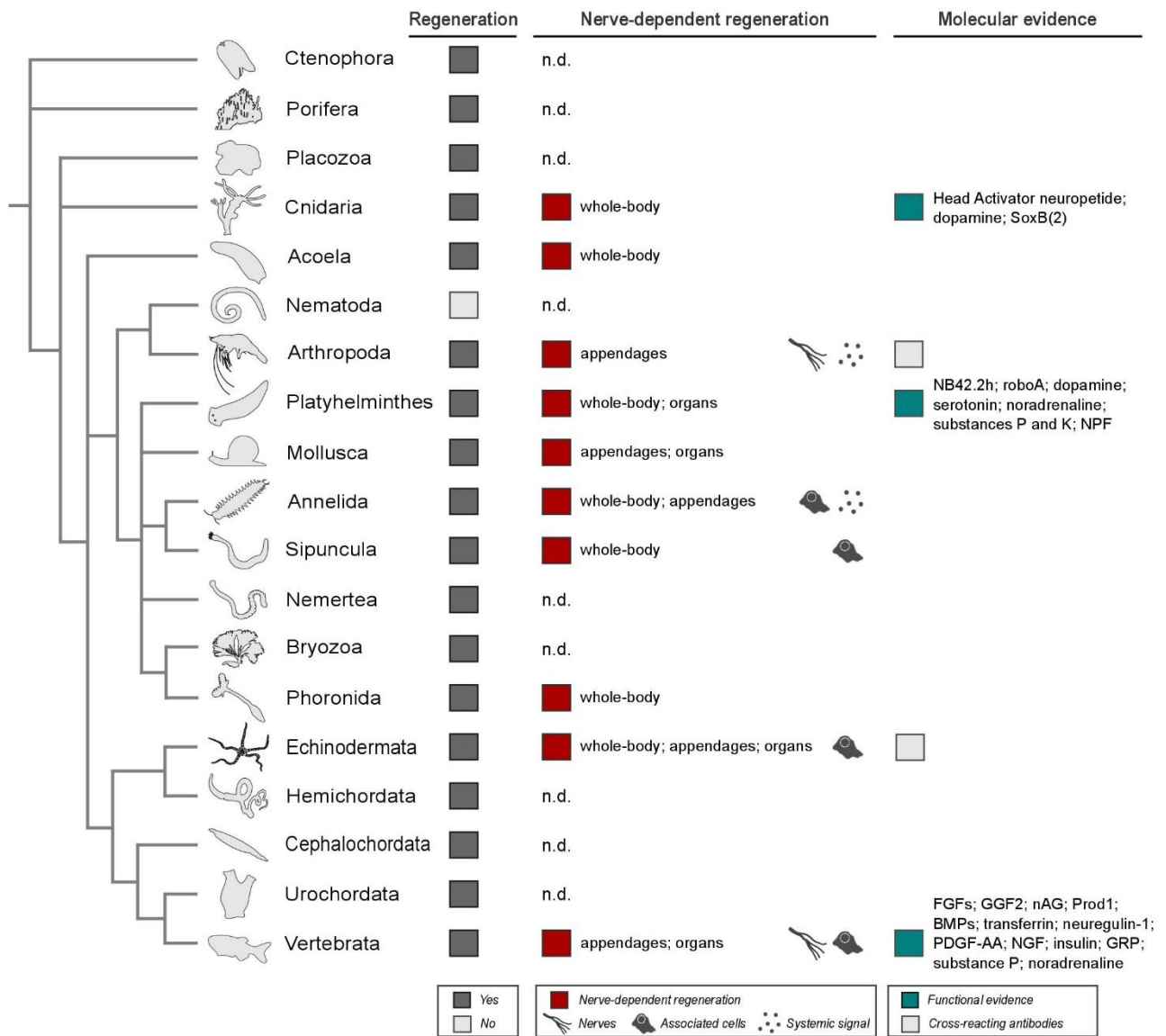


Figure 2: Distribution of nerve-dependent regeneration in animals. Most animal phyla include members with the ability to regenerate complex body parts (dark grey boxes). An influence of the nervous system during regeneration has been found in numerous instances (red boxes), while the molecular players involved in nerve-dependent regeneration (identified by pharmacological assays, immunological or genetic approaches) have been identified in some cases. Lack of evidence does not imply absence of nerve-dependent regeneration. n.d., not determined. Silhouettes adapted from PhyloPic.org, further information can be found in the **Appendix**.

The methodological approaches used to investigate the role of nerves have been very diverse, influenced by the history of technological developments and by the specific opportunities and constraints presented by each species. The purely surgical approaches employed in the early experiments, featuring burning needles to ablate the nervous system (e.g. classic study on a brittle star [24*]) or transplantation to re-combine regenerating tissues with nerves (as extensively applied to annelids, e.g. [25]), provided solid proof of the influence of the nervous system on regeneration, identifying nerve-dependent and nerve-independent phases [26**]. Later work focused on ‘inducing’ or ‘trophic’ factors mediating the effect of nerves. Biochemical analyses of body and nerve extracts identified candidate active substances, such as the *organisines* thought to be

released by regenerating planarian brains [27], or the mitogenic glial growth factor detected in regenerating newt limbs [28]. Immunological and pharmacological experiments allowed to detect and to test the function of specific compounds, in vivo and on cell cultures, identifying for example the mitogenic function of transferrin in amphibian limb regeneration [29]. Gene-oriented approaches provided molecular insights into the interactions regulating nerve-dependency [30]. RNAi screens on planarians identified several genes that are expressed in the nervous system and promote regeneration, including *NB42.2h* and *roboA* [31*,32]. Differential expression analyses and a yeast-two hybrid screen allowed the identification of the Prod1 receptor and its ligand nAG, which mediate the interaction between Schwann cells and the AEC in regenerating salamander limbs [10*,33].

The multiple contributions of nerves

Forgotten studies, incomplete knowledge, and diverse – sometimes outdated – technical approaches represent a challenge for a thorough re-evaluation of nerve dependency in regeneration, particularly when searching for shared regulators. A systematic comparison across species offers nevertheless an interesting perspective over their multiple roles. Nerves can play an essential role at different stages of regeneration - wound healing, cell proliferation, cell differentiation, patterning and morphogenesis.

Wound closure does not appear to depend strictly on the presence of nerves, but mounting evidence suggests that the nervous system could play a facilitating role in this process. Re-epithelialization at the amputation plane in the absence of nerves has been reported for several species, including annelid worms [34], sea stars [35], zebrafish [36]. However, in several vertebrates, including mammals [37] and birds [38], denervation delays cutaneous wound healing. In amputated lobster limbs the migration of ectodermal cells appears more pronounced in regions that lie closer to nerves [39].

Severed nerves could facilitate wound healing by helping to detect injuries and to mount an immune response. Cut nerve terminals are among the first tissues to react after amputation, by retracting from the wound site and undergoing degeneration; examples are found in cephalopod molluscs [40], brittle stars [41], decapod crustaceans [42] and phoronids [43]. Injured nerves also stimulate macrophage recruitment [44,45]. Macrophages are necessary for cell proliferation at the amputation site; their depletion blocks regeneration in both salamander limbs [46] and zebrafish fins [47].

An increase of cell proliferation is a common regenerative response of most studied systems (reviewed in Ref. [48]). Proliferating cells may be broadly distributed (e.g. in asteroids), or localized close to the amputation plane, in a proliferating mass called blastema (e.g. in salamanders, planarians and crinoids) [49,50].

Nervous systems have been shown to promote cell proliferation in numerous regenerating species, regardless of their regenerative strategies. Denervation experiments caused a reduction in the number of mitoses and prevented the formation of a histologically detectable blastema in regenerating newt limbs/tails [51], and in the ear of a regenerating mouse strain [52]. The molecular mediators of this function have been characterized in few vertebrate systems. In salamander limbs [10*] nerves play an essential role in triggering/sustaining cell proliferation in the blastema by secreting nAG. This protein binds to a receptor, Prod1, identified in responding blastemal cells, and elegant experiments have shown that the effects of limb denervation can be

rescued by providing exogenous nAG, even if nerve and muscle tissues could not regenerate normally [10**]. Cholinergic nerves promote cardiomyocyte proliferation during heart regeneration in both mouse and zebrafish [53].

In invertebrates, molecular evidence for a role of nerves in inducing cell proliferation is overall lacking. Some descriptive evidence would be consistent with such a role, such as the close proximity of nerves to proliferating cells (e.g. in crustaceans [42], enteropneusts [54], echinoderms [41], and annelids [55]) and the fact that cell proliferation starts concomitantly with the regrowth of nerves into the blastema (e.g. in cuttlefish [40]). Growth defects or delays in regeneration have been observed following denervation, particularly among regenerating arthropod limbs [56*,57].

The influence of nerves on the differentiation of progenitor cells and on growth is even less well understood. In hydra, nerve cells secrete peptides controlling the spatial and temporal patterns of differentiation [58]. In the sea star, regeneration is entirely nerve-dependent, and denervation blocks the differentiation of progenitor cells, and the growth of the regenerating arm [35].

Innervation can also influence patterning and polarity. Clonal cell lineage tracing in mice revealed morphological defects in regenerating finger tips upon denervation, indicating that nerves affect tissue patterning in both ectodermal and mesodermal derivatives [59]. The nerve cord of annelids was shown to impart ventral identity to the regenerate (reviewed in Ref. [60]), while the central nervous system of planarians controls the polarity of regenerating body fragments by detecting the presence of cephalic structures [61]. Furthermore, the identity of regenerating elements might also depend on their nerve supply: in several crustaceans, the presence of the optic ganglion appears to be required for regenerating an eye and removal of that ganglion leads to regenerating an antenna-like structure [62].

Denervation commonly results in the formation of a stump that fails to regenerate, as classically shown after amputation of posterior body segments of annelids [63], echinoderm arms [24*], salamander limbs and fins of teleost fish [64]. In some instances, however, the removal of nerve induces a more dramatic response, where also tissues far from the wound degenerate. This phenomenon, observed initially in the denervated and amputated limbs of larval urodeles, has been termed the 'regression effect' [65,66]. In the denervated antennae of cockroaches, the amputated stump degenerated over several molts, but could regenerate once it was re-innervated [67*].

Re-routing nerves to a wounded area can also have a converse gain-of-function effect, inducing the ectopic formation of a regenerating structure. This effect has been best studied in axolotl limbs (termed the Accessory Limb Model, [68,69]), and also described during anterior regeneration in annelids [70].

Searching for common molecular mediators

The molecular regulation of nerve-dependent regeneration remains poorly understood, and most of the factors identified so far derive from vertebrate research (e.g. nAG and Prod1 [10**], FGFs [9,71], GGF2 [28], BMP [72], transferrin [29], and Neuregulin [6*] – reviewed in Refs. [19,21,73]).

Among the wide range of signaling molecules produced by nerves, neurotransmitters might represent the best candidates for conserved molecular mediators. Besides their well-known roles in neuronal communication, neurotransmitters have been implicated in development and shown to influence regeneration in several contexts [74]. In planarians dopamine levels vary significantly

during regeneration, and treatment with a dopamine antagonist reduces cell proliferation, delaying regeneration [75*]. Analogous results have been obtained in the regenerating arms of sea star [76] and in regenerating fragments of a sea anemone [77]. Significant fluctuations in serotonin concentration during arm regeneration have been detected in crinoids [78]. Drug treatments have shown that serotonin promotes regeneration in planarians and that this role is counterbalanced by acetylcholine signaling [79]. Similarly, noradrenaline has been found to promote regeneration in planarians [75*] and annelids [80] and has been detected in the regenerating limbs of newt, where its levels correlate with cAMP activity [81].

Neuropeptides are signaling molecules that mediate local interactions between neurons or act systemically as hormones [82,83]. Substance P and substance K (Neurokinin A), two neuropeptides of the tachykinin family, have been shown to have a pro-mitogenic effect on diverse tissues [84]. Treatment with substance P and K promotes neoblast proliferation in regenerating planarians [85]. Substance P was also detected in the proliferating blastema of newt limbs, where it similarly stimulates regeneration [86]. Neuropeptide F (NPF) stimulates mitotic activity of neoblasts and promotes pharyngeal regeneration in planarians [87]. Interestingly, a pro-mitogenic action of NPF was recently shown in the germ stem cells niche of *Drosophila* [88]. Hydra polyps regeneration depends on the Head Activator (HA) neuro-peptide, which promotes proliferation and differentiation of nerve and epithelial cells, via the cAMP pathway [58,89]. In mammals, HA promotes proliferation of nervous system or neuroendocrine-derived cells, during development [90]. Neuropeptides from cutaneous nerves contribute to wound healing in vertebrates: gastrin-releasing peptide (GRP, a homologue of amphibian bombesin) promotes wound healing in mammals by stimulating growth and spreading of keratinocytes [91].

Nerve-dependency and the evolution of animal regeneration

A widespread requirement for the presence of nerves emerges from the comparison of animal regenerative processes, suggesting that the nervous system could play a conserved role. Given the ongoing efforts to identify any unifying principles in the unevenly distributed regenerative capacities of animals, finding a shared role for the nervous system would have profound consequences for our understanding of the evolution of regenerative properties [92**].

The evidence reviewed here, however, highlights the diversity of the various processes classified as 'nerve- dependent regeneration'. The function of nerves can be mediated locally by the axons themselves, by cells variably associated with the nervous system, or systemically by circulating signals. Nerves can intervene at different stages of the regenerative process, with the control of proliferation being perhaps the most broadly shared feature. At present, it is uncertain which aspects could be considered homologous and compared across widely divergent groups, in terms of the mode of action, the cell types involved or the molecular mediators. Within vertebrates, the scaffolding/support that nerves provide for factor-secreting glial cells could be considered as a shared feature, but the molecular regulators only partially overlap between salamander limbs (the role of nAG is not well conserved), mouse digits [5**] or teleost fins [36]. The molecular mediators might evolve rapidly, even if the overall process is conserved.

The lack of comparable molecular data across animals represents an important obstacle for the emergence of a unified view. Cephalopod molluscs, despite their excellent regenerative abilities [93] and the extensive knowledge about the physiology of their nervous systems [94], appear to have been largely overlooked with regard to nerve- dependent regeneration. Similarly, no relevant molecular data are available for cephalochordates or ctenophores, two groups with high

regenerative capabilities and relevant phylogenetic positions [95,96]. The discovery of neuropeptide signaling in placozoans, a non-bilaterian group lacking a nervous system [82,97], opens the intriguing possibility that neuropeptides might be involved in regeneration even in the absence of a nervous system.

Tools are now becoming available to tackle the evolution of nerve dependent regeneration through a broader sampling of animal groups. Achieving denervation by means of precise laser ablation or inducible genetic cell ablation systems, instead of invasive surgery, now allow to spatially and temporally control the interactions between nerves and target cells. Transgenic reporters and *in vivo* cell tracking approaches provide more powerful/specific ways to assess the effects that denervation has on regeneration, for example by uncoupling the dynamics of axons and nerve-associated cells. These approaches could be applicable and particularly informative in arthropods, where parallels with vertebrate regeneration such as the role of satellite-like cells in regenerating muscles have already been established [98]. Classical ablation studies in cockroaches and crustaceans have thus far only revealed subtle effects of removing nerves during regeneration, possibly due to the fast regrowth of axons into the stump [99]. *In vivo* imaging of regeneration using transgenic reporters, in the crustacean *Parhyale* [100], would allow to effectively monitor the denervation process and to dissect the cellular events that follow.

Nerve-dependent regeneration is a complex process and understanding its evolution will be challenging. The phenomenon is so widespread that it could be ancestral, despite the high variability observed between and within animal groups. Alternatively, there may be no unifying principle; nerves may have been co-opted into the regenerative process multiple times with different roles. Investigating the evolution of nerve-dependent regeneration with a common approach in a broader sample of animals will allow for a better understanding of the evolution of regenerative developmental mechanisms.

Conflicts of interest statement

Nothing declared.

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Appendix

Supplementary Table. References related to Figure 2.

Nerve-dependent regeneration		
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