Do you have the nerves to regenerate? The importance of neural signalling in the regeneration process

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The importance of nerve-derived signalling for correct regeneration has been the topic of research for more than a hundred years, but we are just beginning to identify the underlying molecular pathways of this process. Within the current review, we attempt to provide an extensive overview of the neural influences during early and late phases of both vertebrate and invertebrate regeneration. In general, denervation impairs limb regeneration, but the presence of nerves is not essential for the regeneration of aneurogenic extremities. This observation led to the “neurotrophic factor(s) hypothesis”, which states that certain trophic factors produced by the nerves are necessary for proper regeneration. Possible neuron-derived factors which regulate regeneration as well as the denervation-affected processes are discussed.

1. Introduction

Regeneration is a diverse concept, defined differently depending on the context, covering processes from tissue repair to asexual reproduction. Basically, each type of regeneration can be described as the ability of an organism to repair and regrow lost or damaged tissues, structures and even entire extremities without the formation of scar tissue and with functional integration of the regenerate in the pre-existing tissues (Oviedo and Beane, 2009). Although the regeneration capacity of humans is limited to the repair of cuts in our skin, healing of broken bones, regeneration of lost digit tips and parts of our liver, regeneration is not an uncommon feature. In fact, it is a trait that is widely distributed in the animal kingdom, and virtually each animal class has at least one representative with good regeneration capacities (Pagan, 2014). A lot of organisms can regenerate, at least partly, during early life stages, but lose this ability due to metamorphosis or ageing (Seifert and Voss, 2013). Some organisms maintain excellent regenerative abilities throughout their lives. These animals, both vertebrate (f.e. Xenopus species, axolotl, salamanders, and zebrafish) and invertebrate organisms (f.e. Hydra, planarians), are commonly used as model organisms in regeneration research (Sanchez Alvarado, 2000; Brookes and Kumar, 2008; Fior, 2014; Gurtner et al., 2008; Li et al., 2015). They are crucial to acquire each piece of information concerning the regeneration process, from involved genetic responses to cellular signalling. It is only by doing this that regenerative medicine can be successfully achieved and new insights in various pathological conditions can be discovered, since regenerative tissues and organisms have the potential to overcome degenerative disorders (Alzheimers disease, Parkinsons disease or cancer (Stevens et al., 2015). Important features of the regeneration process identified in various of these model organisms include the time point(s) of the proliferation peaks, the origin and migration of involved progenitor cells, the importance of apoptosis for regeneration to proceed, and the genes and signalling pathways involved (Sanchez Alvarado, 2000; Carlson, 2007; Vriz et al., 2014). Among these factors, innervation comes forward as a crucial parameter in successful regeneration. However, its exact role and the underlying mechanisms and factors remain largely unknown. The reason for this lack of knowledge is the fact that most of the research on this topic was published in the 1950s up to the 70s, when the necessary molecular techniques were simply not available. Although important contributions on the role of the nervous system for successful regeneration were made in

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the mid 1900s, a lot of information is still missing. During the last years, research on this topic continued or is ready to be re-investigated in more molecular contexts. In this review, we represent the state of the art on the interconnection of innervation and (in)vertebrate regeneration. We give an overview of potential signalling factors and pathways, including the possible involvement of the cellular redox state.

2. The importance of innervation in vertebrate regeneration

2.1. A brief description of vertebrate extremity regeneration

The general process of appendage regeneration (such as regeneration of limbs, tails and fins) proceeds in distinct phases (Fig. 1) (Simoes et al., 2014; Stocum, 2011; Kumar and Brockes, 2012). After amputation, epithelial cells first reorganise and migrate to the wound site in order to form the wound epidermis (WE) and close the wound. Via cell migration, the WE acquires additional cell layers and eventually establishes a specialized epidermis called the apical epithelial cap (AEC) (Simoes et al., 2014; Stocum, 2011; Kumar and Brockes, 2012). Next, in the mesenchymal tissue beneath the AEC the extracellular matrix of the tissues is degraded by proteases, liberating stem cells as well as mononucleate myofiber fragments, chondrocytes, fibroblasts and Schwann cells, which all start to dedifferentiate, migrate to the amputation plane and re-enter the cell cycle to give rise to the blastema (an undifferentiated cell mass, which will start to differentiate and in which the missing structures will be formed) (Simoes et al., 2014; Stocum, 2011).

In a last phase, interactions between the AEC and the blastema ensure growth and patterning of the regenerate until the formation of the apical epithelial cap (AEC) (Simoes et al., 2014; Stocum, 2011). The establishment and outgrowth of the regenerate are under the control of many factors, including the presence of nerves at the wound site. Due to the damage caused by the injury, nerves degrade, after which sensory neurons rapidly regenerate in the AEC, while motor neurons regenerate between the subjacent blastema cells (Stocum, 2011; Kumar and Brockes, 2012; Salpeter, 1965; Lentz, 1967). Both of these processes seem to be crucial for proper regeneration to proceed, since denervation results in various regeneration defaults depending on the extent and time of denervation (Brockes and Kumar, 2008; Carlson, 2007; Simoes et al., 2014; Stocum, 2011; Kumar and Brockes, 2012; Kumar et al., 2007). In the following section, the different aspects of the nerve dependence of the regeneration process are discussed.

2.2. Regenerative capacities are affected by denervation

Although many model organisms have been used to investigate the regeneration process, the earliest and most intensively studied example which describes the necessity of existing nerves during regeneration is that of the salamander limb. Researchers have used this model system in past and ongoing research to illuminate the necessity of neuronal presence – the abundance of innervation and the time of denervation rather than the type or activity of the nerves – for successful regeneration (Fig. 2) (Carlson, 2007; Stocum, 2011; Thornton, 1970; Mullen et al., 1996; Seifert et al., 2012; Singer, 1952). In the following section, we discuss their most important contributions on this topic in a chronological manner.

Nerve dependence during salamander regeneration was described for the first time in the 1823 – even before the establishment of the cell theory – by Tweedy John Todd (Todd, 1823). He noticed that salamanders experienced regeneration defaults after transection of the sciatic nerve in their limbs. Denervation before amputation resulted in healing with the formation of scar tissue, while regeneration was completely inhibited or retarded if denervation was performed after wound healing. If the nerve was transected after blastema formation, the regenerate remained static or regressed. Although these “simple” experiments provided early proof for the importance of innervation during vertebrate regeneration, it was not until the early 1900s that research on this topic continued.

In the early 1940s, Butler and Schotté (1941) performed denervation experiments on larval urodele limbs (Ambystoma punctatum, Ambystoma opacum, and Triturus virideescens) and noticed
that denervation (through repeated resections of the brachial plexus) followed by amputation through the distal radius and ulna results in regression of the limb tissues. Next, an unstable mass of blastema cells is formed as the stump undergoes histolysis, but eventually the blastema cells stabilise as the axons regenerate back into the regressing limb tip and regeneration proceeds to completion. However, when reinnervation fails, the limb regresses completely and scar tissue is formed under the wound epidermis (Butler and Schotté, 1941; Schotté and Butler, 1941).

Important details on this topic were revealed by Marcus Singer, who performed a long series of experiments (between 1943 and 1978) on the importance of nerves in limb regeneration (Singer, 1943, 1952, 1954, 1959, 1961, 1965, 1978; Singer and Craven, 1948; Singer et al., 1957, 1967, 1976). Among others, he concluded that it is not the type of innervation (sensorial, motor, and sympathetic) but rather the amount of nerves that influences and supports regeneration (Singer, 1952; Singer et al., 1976). The innervation density – the number of nerve fibres or cross-sectional axoplasmic area per unit area of the amputation surface – has to exceed a certain threshold to continue the regeneration process (between 793 and 1298 axons for regeneration of the upper arm, to be precise) (Sidman and Singer, 1951). Threshold requirements vary at

Fig. 2. The different aspects of nerve dependence of regeneration. The dotted red and orange lines (representing different types of nerves) represent degrading neurons, while the full lines represent intact neurons. (A) Nerve dependence arises during development and limbs that have never been innervated (aneurogenic limbs, in blue) are not dependent on neural signalling for their regeneration. (B) A threshold of nerve number, independent of the cell type, is necessary for regeneration to proceed. Complete absence of nerves completely inhibits regeneration, while a reduction in the number of nerves results in diminished regeneration. (C) The time point in the regeneration process at which denervation is performed affects the outcome of regeneration. If denervation is performed before amputation, the regeneration process is completely inhibited. If denervation arises after proper formation of the wound epithelium (WE), regeneration is diminished. Denervation after the blastema is successfully formed does not affect the outgrowth of the regenerate, but can result in patterning defects.
the different proximodistal levels of the limb and a relationship exists between regeneration rate and the degree of innervation (Singer, 1952). However, evidence is equivocal, since nerve abundance seems to be positively correlated with the rate of regeneration in *Ambystoma* species (Young, 1983, Seifert, 2012), but does not influence the regeneration capacity in other amphibians such as the tree frog (*Hyla arborea japonica*) and the wood frog (*Rana sylvatica*). A recent study on zebrafish showed that, in complete absence of the nerves (via resection of the nerves at the level of the brachial plexus), the fins were unable to form a blastema, while in the presence of a reduced amount of nerves regeneration was affected but a residual blastema was still formed giving rise to smaller and abnormal fins (Simoes et al., 2014). These results thereby support the theory of Singer that a certain amount of nerves is required for successful limb/fin regeneration (Fig. 2B) (Simoes et al., 2014; Geraudie and Singer, 1977). There is no correlation between regeneration rate and nerve density, since regeneration rates might differ slightly between individuals of the same species (so far tested in *Xenopus laevis, Ambystoma maculatum*, and *Amphiuma tridactylum*), but these differences are not the result of dissimilarities in innervation (Seifert et al., 2012; Kurabuchi, 1990; Van Stone, 1964; Scadding, 1983).

Another variable in the nerve-regeneration interaction is the time at which denervation occurs. The impact of the denervation moment on the size of regenerate was initially described by Tweedy John Todd (in 1823) (Todd, 1823), and later confirmed and further investigated in several other studies (Fig. 2C) (Simoes et al., 2014; Singer, 1952; Singer and Craven, 1948; Singer, 1978). If denervation occurs before amputation/injury, the regeneration process is completely inhibited. On the other hand, if the limb/fin is denervated at later stages of regeneration (during blastema formation but after proper WE establishment), the outgrowth of the regenerate diminishes. When denervation is performed after blastema formation, no differences in the size of the regenerate are observed, although patterning is clearly affected (Fig. 2C). These data indicate that innervation is not only fundamental during the initial phases of regeneration, but also exerts an important role during tissue morphogenesis following regenerative outgrowth (Simoes et al., 2014; Singer, 1952, 1978; Singer and Craven, 1948).

A more detailed discussion on the affected mechanisms and processes is given in the following sections (“The affected molecular and cellular processes following denervation”).

Not only limbs, but also tails depend on the presence of nerves for successful regeneration. Studies on different species of salamanders and lizards (e.g. *Ambystoma punctatum*, *A. opacum*, and *Anolis carolinensis*) showed that if the spinal cord is injured or destroyed, tail regeneration is inhibited. The number of peripheral nerve fibres is too low to support the regeneration process on its own which makes the presence of the spinal cord necessary for regeneration to take place (Carlson, 2007; Holzer, 1956; Kamrin and Singer, 1955).

Although quite some research has been performed concerning the requirement and influence of the nervous system on regeneration in various fish, amphibian and reptile systems, the potential importance of nerves for tissue replacement in mammalian appendages is less clear. Recent studies on mice show that cellular turnover, replacement, and differentiation from tissue progenitor cells remain largely unaffected following denervation of the hind limbs (via sectioning of the sciatic and femoral nerves), although denervation retards regeneration of the digit tips and causes patterning defects in the bone and nail matrix (Rinkevich et al., 2014; Takeo et al., 2013; Mohammad and Neufeld, 2000). Interestingly, these nerve-dependent phenotypes in mice strongly resemble complications in response to spinal cord injury in humans. Studies show that patients suffering from a spinal cord injury develop dermal fibrosis, progressive skin thickening and nail hypertrophy on lower limbs/digits, suggesting a role of the nervous system not only on regeneration but also during maintenance of the skin and nail organ, during which progenitor cells need to replace damaged or worn-out cells. Moreover, the severity of these complications is progressive and directly correlates with the degree of the injury (Stover et al., 1994; Stover et al., 1994).

All the above-mentioned data eventually led to the hypothesis, proposed by Singer, that the nerves produce a neurotrophic factors (“factor X”) that need to be present in sufficient quantities to not only initiate the early stages, but also to guide the progression of the regeneration process (Singer, 1954, 1965). In the following section, we give an overview of the most common factors that have been proposed throughout the years to be the neurotrophic “factor X”, which is necessary for the initiation and proceeding of regeneration.

### 3. The neurotrophic hypothesis of regeneration

In a number of seminal experiments, Singer showed that the insertion of a sciatic nerve from the hind limb of a frog, an extremity very well capable of regenerating, under the skin of the forelimb improves the poor regeneration capacities of this forelimb. He showed that neither the motor neural impulses nor the production of the motor neurotransmitter acetylcholine are responsible for this improvement, but rather yet unknown neurotrophic substances (“factor X”), which has to be present in sufficient quantities. This hypothesis became known as the “neurotrophic factor hypothesis” (Singer, 1943, 1959, 1965; Sidman and Singer, 1951; Drachman and Singer, 1971). Unfortunately, the results of this experiment could not be reproduced in lizard and mammalian extremities (experiments performed on rats, oppossums and the lizard *Lytosoma laterale*) (Bar-Maor and Gitlin, 1961; Mizzell, 1968; Simpson, 1961). Probably, nerve-regeneration interdependence is much more complex in these animals than originally anticipated (Yntema, 1959; Brookes, 1987; Thornton and Thornton, 1970). Other studies in which the application of neural extracts promote proliferation of blastema cell cultures (Stocum, 2011; Albert and Boilly, 1988; Boilly and Albert, 1988; Boilly and Baudin, 1988) confirm the presence of the neural-produced “factor (s) X”. The neural rescuing factors of these extracts are proteins, since treatments with trypsin or heating, but not with RNase, abolished the activity of the extracts (Stocum, 2011; Choo et al., 1978).

Nerve dependence arises during development. Yntema (1959) used *Ambystoma* larvae to show that limbs which have never been innervated do not depend on nerves for regeneration (Yntema, 1959). To give a more specific example, urodele limb buds are able to regenerate in the absence of axons until the digital stages of development, in which the limb buds become heavily innervated and regeneration becomes nerve-dependent (Brookes, 1987). Thornton (1970) performed various experiments during which he grafted aneurogenic limbs on normal hosts of *Ambystoma maculatum*, thereby replacing the normally innervated limbs of the host larvae (Thornton and Thornton, 1970). In the beginning, these aneurogenic limbs were still able to regenerate. In a later stage, the branchial nerves of the host started to innervate the aneurogenic limb and after approximately two weeks of innervation, the ability of nerve-independent regeneration was lost (Fig. 3). The underlying hypothesis is the production of the so-called “factor(s) X”. The tissues of the aneurogenic limbs are able to produce these factors themselves, thus enabling regeneration. Once innervated, they depend on the nerves for the production of these trophic substances. Innervation probably suppresses the production of the “factor(s) X” by the peripheral tissues. Denervation of the newly innervated limb resulted in nerve-independent regeneration in
about half of the limbs (Fig. 3) (Thornton and Thornton, 1970). On the contrary, Tassava and Olsen-Winner (2003) recently showed that aneurogenic tissues do not promote the regeneration potential of normal, denervated limbs. They grafted aneurogenic limbs under the skin at the distal end of normal amputated forelimbs and amputated both limbs of *Ambystoma maculatum*.
Regeneration should be impaired after their removal. A lot of as the ability to substitute for the nerves to support regeneration. The presence should be reduced following denervation. The combination remains unidentified through the production of neurotrophic factors, as up to date it initial regeneration of the nerves is properly established (Brockes and Kumar, 2008). The neural control is thought to be executed through the production of neurotrophic factors, as up to date it remains unidentified (“factor X”). In the following section, we discuss the interactions between the AEC/blastaema and the innervating axons as well as the signaling factors possibly involved.

4. Neurotrophic compounds: what is factor x?

The neurotrophic hypothesis stated by Singer suggests that nerves exert their influence on regeneration through the production of one or more factors, which promote proliferation and differentiation of the progenitor cells. But before this neural signaling can be accomplished, the “factor(s) X”-producing axons need to reach the wound site and innervate the AEC, since they degrade rapidly following amputation (Fig. 1). The degraded axons regenerate to the wound site, thereby responding to signals from both the epithelial cells and the blastema which guide their patterning (Kumar and Brockes, 2012; Richmond and Pollack, 1983). Various neurotrophins that are expressed in the blastema and/or AEC were investigated, such as substance P, retinoic acid, neurotrophic factor-3, glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and hepatocyte growth factor/scatter factor (HGF/SCF). All of these factors support axonal growth in vitro (Tonge and Leclere, 2000). Administration of retinoic acid to salamander spinal cord cultures promotes not only the number of axons, but also the length of these axons. Exposure of cultured blastemas to a retinoic acid inhibitor reduced axon outgrowth (Prince and Carlone, 2003; Globus and Alles, 1990). In most cases, the reinnervation happens through axonal elongation, but for instance during tail regeneration of salamanders, new nerve cells are produced (Brockes and Kumar, 2008). Data from these and other studies clearly indicate that there is a reciprocal interaction between the neuronal cells and cells of the AEC and blastema. On the one hand do blastema cells guide neuronal regeneration and elongation, while on the other hand neural signaling is necessary for proper AEC and blastema formation. The latter is manifested by the production of the neurotrophic substrates (“factor(s) X”), which influence the behaviour of the proliferative and differentiating blastema cells. It is still to be elucidated whether these factors are continuously produced during normal nerve functioning, or whether it is produced as a direct response to amputation.

To be characterized as factor X, a number of criteria have to be fulfilled. These factors have to be present in the blastema and their presence should be reduced following denervation. The compounds have to cause a mitogenic effect on blastema cells, as well as the ability to substitute for the nerves to support regeneration. Regeneration should be impaired after their removal. A lot of research has been done to unravel which nerve-produced compounds are required for regeneration and in this section an overview is given of the most important candidates that have been proposed by previous investigations (Mullen et al., 1996; Globus and Alles, 1990; Mescher, 1996; Brockes and Kintner, 1986).

One of the substances which was conjectured to be factor X was the fibroblast growth factor (FGF), and more specifically FGF-1, FGF-2, FGF-8 and FGF-10 (Gospodarowicz et al., 1978; Gospodarowicz and Mescher, 1980; Gospodarowicz and Mescher, 1981). But not all the FGFs meet the above-mentioned criteria, since FGF8 and FGF10 are not expressed in the nerves that innervate the blastema (Christensen et al., 2001; Christensen et al., 2002). However, FGF2 as well as the glial growth factor 2 (GGF2) are nerve-derived factors that have been demonstrated to stimulate regeneration of denervated limbs (Mullen et al., 1996; Brockes and Kintner, 1986; Wang et al., 2000; Brockes, 1984). The exact in vivo roles of these FGFs during regeneration are still not clear, but one hypothesis is that the FGFs synergize with the anterior gradient protein (AGP) to perform their proliferation-promoting function or even need AGP for their synthesis (Stocum, 2011). Since several FGFs are produced in multiple tissues from multiple sources, it seems unlikely that axonal release of these additional mitogens leads to the dependence on nerves for blastema formation (Mescher, 1996).

Another molecule with “factor X” characteristics is the anterior gradient protein (AGP). This protein was recently identified as a secreted factor, the expression of which is regulated by the nerves. It is able to induce regeneration in denervated limbs of salamanders (with exception of the muscle tissues), although it does not seem to play a similar role during zebrafish fin regeneration (Simees et al., 2014; Kumar et al., 2007). AGP is a ligand that interacts with Prod-1 thereby determining the positional identity of the blastema cells (Kumar et al., 2007; Blassberg et al., 2011). In the beginning, the AGP is produced by the Schwann cells, followed by expression in both the Schwann cells and the gland cells of the wound epithelium. The recombinant AGP acts as a growth factor for cells dissociated from the blastema. Expression of this protein is abrogated in both locations following a cut in the nerve at the base of the limb.

Transferrin can also be classified as a neurotrophic factor necessary for correct regeneration, since this factor is abundant in (regenerating) peripheral nerves and released from the nerve terminals in regenerating limbs (Mescher, 1996; Kiffmeyer et al., 1991). Transferrin operates as an iron transporter. It transports two atoms of ferric iron, which are essential for proper cell proliferation as cofactors for the activity of ribonucleotide reductase, for the rate-limiting enzyme for DNA replication, and for synthesis of mitochondrial cytochromes (Mescher, 1996). Denervation reduces the transferrin content in the blastema up to 50% and in vitro experiments have shown that inhibition of the uptake of transferrin results in a rapid cell arrest in the S or G2 phase of the cell cycle (Kühn et al., 1990). Moreover, the blastema growth-promoting activity of brain tissue extracts, which is generally observed in vitro, is lost by removing either iron or transferrin. This loss is completely restored by their readdition (Mescher et al., 1997).

Insulin-like growth factor-1 (IGF-1) is also a potential candidate. IGF-1 is involved in the blastema formation and the time required for blastema formation is reduced following injections of IGF-1 in amputated salamander limbs (Fahmy RES, 1998). The exact role of IGF-1 remains unknown, although this factor might affect DNA synthesis during blastema formation and disturb mitosis during blastema growth (Stocum, 2011; Vethamany-Globus et al., 1978; Kesik et al., 1986).

A lot of progress has been made in the identification of the mysterious factor(s) X and the signalling pathways. However, crucial information is still missing since many of the rescue
experiments rarely result in normal regeneration (Carlson, 2007; Mescher, 1996). This made scientist think about a negative model of the neural influence on regeneration, where an inhibitory factor is released by degenerating axons and/or Schwann cells, eventually inhibiting regeneration (Simoes et al., 2014; Ferretti and Brockes, 1991). Nonetheless, the positive model on the promoting role of nerve-derived trophic factors in the regenerating tissues prevailed so far (Stocum, 2011; Singer, 1978). This model is also supported by the finding that the secretory activity of the neurons changes during the different phases of regeneration, thereby affecting the production and transport of the neurotrophic factors (Simoes et al., 2014). It is likely that not a single molecule, but a cocktail of nerve-derived signalling factors triggers and coordinates both the innervations and thus the general regeneration process (Carlson, 2007). This could explain why administration of a single factor rarely results in a positive outcome concerning the rescue of regeneration abilities.

5. The affected molecular and cellular processes following denervation

In contrast to the numerous studies on nerve-derived factors and their importance for successful regeneration, a myriad of aspects mediating the neural influence on regeneration, such as the nerve-dependent target cells, the affected cellular processes, and the underlying molecular mechanisms remain largely unexplored (Hamrick and Brian, 2007). It goes without saying that knowledge on these targets is needed, especially in applied research fields such as regenerative medicine. In the first section, the affected cell types and cellular processes caused by the neurotrophic factors are reviewed. In the second section, the underlying molecular responses are discussed.

5.1. Cellular responses following denervation

Grafting experiments performed by Steen and Thornton (1963) showed that the WE is the main target of the nerve signalling, rather than the underlying tissues. Although it is interesting to note that the WE can be formed independently of the nerves (Carlson, 2007; Steen and Thornton, 1963). They demonstrated that regeneration fails when the wound of an aneurogenic limb is covered with normal skin. However, when some aneurogenic skin remains at the end of the stump, the WE is derived from aneurogenic epidermis and regeneration proceeds. Similarly, an entire aneurogenic limb filled with the mesoderm of normal tissue can induce limb regeneration (Fig. 3) (Carlson, 2007; Steen and Thornton, 1963). The formation of the wound epithelium proceeds independently of the nerves, but the AEC cannot be maintained unless innervation occurs by the regenerating axons, which signal the WE and AEC cells. The fact that aneurogenic limbs are able to regenerate also indicates that the nerves are not involved in the physical guidance of the progenitor cells (Stocum, 2011; Steen and Thornton, 1963).

Similarly to the WE formation, the differentiation process is not nerve-dependent, but the proliferation of the progenitor cells is affected by denervation. More specifically, denervation inhibits the onset of the mitotic activity and the dedifferentiated progenitor cells fail to progress through the S-phase of the cell cycle. These data indicate that neurons produce proliferation-promoting factors (“factor(s) X”) (Mescher, 1996; Tassava et al., 1987). The presence of proliferation-promoting compounds in neural tissues has been demonstrated by the application of neural extracts to blastema cell cultures. Spinal cord extracts of regenerating axolotls stimulated proliferation of blastema cells in culture twice as much as the extracts of non-regenerating animals. Infusion of brain extracts into denervated salamander limb blastemas or their addition to blastema explants partially restored protein synthesis (Stocum, 2011; Albert and Boilly, 1988; Boilly and Albert, 1988; Boilly and Baudin, 1988). The production of “factor(s) X” and thus the proliferation of the early blastema depends on the nerves, but in the later phases of the regeneration processes the wound epithelium becomes the predominant influence instead of the nerves (Mescher, 1976). This change in driving factor may correlate with the observed shift in AGP expression from Schwann cells to the cells at the wound site (Brockes and Kumar, 2008; Kumar et al., 2007).

The influence of neural signalling at different time points of regeneration was observed in many experiments, which show that the time at which denervation is performed affects the extent of the regenerative impairment. If denervation is executed at an early stage of regeneration, the regeneration process is prevented resulting in a reduced blastema. This indicates that the initial innervation is crucial for early blastema formation. However, at a later stage of regeneration when the blastema formation has already been completed, denervation only has a minor effect on regeneration, although the stem cells still tend to be arrested in the G1 phase of the cell cycle, resulting in smaller tissues (Maden, 1979; Loyd and Connelly, 1981). Although innervation is necessary for successful blastema formation, it does not seem to be instructive concerning the pattern formation or morphogenesis of the regenerate, since the nature of the outgrowth is determined by the location of the insertion (Brockes and Kumar, 2008; Singer and Craven, 1948; Schotté and Butler, 1944; Powell, 1969). Transaction and insertion of a peripheral nerve into the skin wound will evoke the formation of a supernumerary limb (Brockes and Kumar, 2008; Edgar, 1988; Endo et al., 2004). However, this seems not to be the case in all organisms, since denervation results in the pattern malformation of fin rays in regenerating zebrafish (Simoes et al., 2014).

In conclusion, the above-mentioned studies imply that both the nerve and the AEC are required for proper formation of the blastema. The neural influence on the early phases of regeneration, including blastema formation, involves the production of growth factors which sustain the progress of the progenitor cells through the cell cycle, rather than mitogens that trigger cell cycle re-entry (Mescher, 1996).

5.2. Molecular responses following denervation

Multiple studies indicate that the effects of denervation start at the RNA level, which obviously affects protein synthesis and functioning. Denervation decreases protein synthesis by 50–70% at all stages of blastema development and growth due to reduced transcription, rather than changes in the amino acid precursor pool, rate of protein degradation, or rate of translation (Stocum, 2011; Dresden, 1969; Tsonis et al., 1992; Rao et al., 2009). Although RNA synthesis is initiated in denervated limbs, it quickly fades, and the total RNA synthesis is reduced by 75% in denervated adult salamander late bud blastemas (Stocum, 2011; Dresden, 1969; Kelly and Tassava, 1973). Microarray analysis showed that the transcription of genes with critical functions in wound healing are not deregulated by denervation. The transcription of proliferation-controlling genes was only affected during the formation of the accumulation blastema following denervation (Monaghan et al., 2009).

Other deregulated processes are signalling pathways which coordinate the formation and maintenance of the AEC, as observed in zebrafish fins (Simoes et al., 2014). Marker genes of epidermal cells (krt8, left1, and wnt5b) could still be detected, but the levels and domains of expression were abnormal, although formation of the WE proceeds normally in denervated appendages. Not only
AEC signalling is affected following denervation, but also signalling components (fgf20a, shh, mkk3, and fgfr1) in the mesenchymal cells which give rise to the blastema are impaired (Simoes et al., 2014). The FGF signalling cascades play important roles during wound healing, embryonic development and regeneration. The combined data of the above-mentioned study strongly suggest that the FGF signalling pathway is involved in the contribution of nerves to appendage (in this case fin) regeneration (Simoes et al., 2014).

Only recently, molecular-orientated research was initiated to elucidate the effect of neural signalling on important signalling pathways which regulate the regeneration process, such as the wnt and FGF cascades (Simoes et al., 2014; Mullen et al., 1996). Further details remain to be elucidated in future research to illuminate all factors that cause the observed cellular and phenotypical abnormalities following denervation.

6. The importance of innervation in invertebrate regeneration

The first studies on regeneration were detailed experiments on invertebrates such as Hydra, crustaceans and planarians in the 18th century. Today, we know that many traits of the regeneration process are widely conserved and very similar between vertebrates and invertebrates, including the dependence on nerves (Brockes and Kumar, 2008). However, invertebrates posses a large variety of nervous system organisation (Carlson, 2007). A lot of Echinoderms, from crinoids to sea cucumbers and star fish, have good regenerative capacities and the regeneration in all these taxa is nerve-dependent. For example, arm regeneration in the model species Asterina gibbosa cannot occur after the removal of the radial nerve (Candidia-Carnevali, 2006). Moreover, the neurotrophic action of the nervous system is needed throughout the entire regeneration process (Huet, 1975). Another example is the nervous system of annelids, which consists of a ventral nerve cord that contains ganglia in each body segment. In normal circumstances, when an injury occurs and part of an oligochaete worm is missing, the nerves will regenerate quickly and innervate the wound epidermis. If prior to this insult the nerve cord was removed far enough from the amputation site, innervation does not occur, resulting in failure of regeneration of the body segments. If the anterior end of the nerve connects with the lateral side of the animals, a supernumerary head is formed, while a connection of the posterior end of the deviated nerve induced the formation of a supernumerary tail (Carlson, 2007; Morgan, 1902). It seems that also in invertebrates the nerves are not instructive concerning the morphogenesis of the regenerate, since the nature of the outgrowth is determined by the location of the insertion (Carlson, 2007).

There are still a lot of ambiguities concerning the role of the nerves in the arthropod regeneration process. Nerve dependence is not studied to a great extent in arthropods, and so far, findings are inconsistent. A study showed that innervation is essential for successful muscle regeneration during molting of moths (Con souls and Levine, 1997), while another study stated that the nerves only play a partial role in the support of appendage regeneration in the American cockroach (Nuesch and Teutsch, 1968). More research on a variety of species is necessary to make more general conclusions on the function of nerve signalling in arthropod regeneration.

In the regeneration processes of Hydra and planarians, the involvement of the nervous system is not very clear. Some experiments show that proper regeneration requires neuronal signalling. Nerve-deprived hydras are still capable to regenerate and form buds, but it remains unclear whether the normal regeneration process in innervated hydras is totally nerve-independent (Brockes and Kumar, 2008; Galliot et al., 2006; Marcum and Campbell, 1978). In a recent study investigating anterior regeneration in Hydra, a delay in regeneration following the downregulation of cnox-2 (a homeotic marker that is a specific marker for bipotential neuronal progenitors) was observed, which disrupted de novo neurogenesis (Mijlkovic-Licina et al., 2007). In planarians, five genes were identified so far, which are expressed in the central nervous system during regeneration and which inhibit regeneration when silenced (Reddien and Sanchez Alvarado, 2004; Reddien et al., 2005). Other research on these animals also demonstrates the necessity of the ventral nerve cords in the coordination of proper regeneration. Oviedo and colleagues showed that modulation of gap junction-dependent signalling (trough the GJ blocker, octanol) and neural signalling (via disruption of the ventral nerve cords) specifically induces the ectopic formation of anterior blastemas in posterior and lateral wounds, which form new brains that establish permanent primary axes (Oviedo et al., 2010). Via interference techniques (e.g. EGR or ERK silencing, inhibition of ROS production) researchers observed that a reduction of the brain ganglia is often accompanied with the impairment of blastema formation, indicating that there might be a link between nerve presence and planarian regeneration (Fraguas et al., 2014; Tasaki et al., 2011; Pirotte et al., 2015). However, direct links between brain formation and regeneration in planarians remain uninvestigated. Agata proposed a theory in which he suggests that the nerves influence the planarian regeneration process through the transcription and transportation of hedgehog (hh), a well-investigated regulator of the wnt signalling pathway (Agata et al., 2014). But this theory still needs to be confirmed.

In summary, the regeneration process is an evolutionary trait that has been widely conserved in the animal kingdom and many similarities are found between the effects of neuronal signalling on both vertebrate and invertebrate regeneration. Different vertebrate and invertebrate organisms fail to regenerate following denervation or sectioning of the nerves. However, details on underlying molecular mechanisms remain to be specified in invertebrates. Yet invertebrate research can provide us with important insights concerning the regeneration process and the in vivo involvement of neuronal signalling in a rapid and inexpensive way.

7. ROS: a possible connection between nerves and regeneration?

Reactive oxygen species (ROS) exert essential signalling roles during various physiological processes, including development, ageing, and regeneration. In pathological conditions such as cancer or neurodegeneration, redox imbalances and deregulation often underlie the observed damages (e.g. directly (via mutations) or indirectly (via disturbed signalling) inducing excessive proliferation in cancer development or cell death in neurodegeneration). A correlation between ROS signalling and neuronal development was observed in many research systems, but an interaction between ROS signalling and neuronal signalling in a regenerative context is still unidentified.

It is possible that ROS interact with the nerves to regulate the regeneration process. Many studies, both in vitro and in vivo, demonstrate the importance of ROS signalling in neuroregeneration. Dual oxidase (DUOX)-mediated ROS production is associated with numb-interacting protein 1 (Nip1) expression in neuronal stem cells, regulating neuronal differentiation (Kennedy et al., 2012; Bedard and Krause, 2007; Suzukawa et al., 2000; Kennedy et al., 2010). In cultures of hippocampal cell lines, ROS generation, and more specifically hydrogen peroxide, also induces neurite outgrowth (Min et al., 2006). In C. elegans, a mutation in the pmn-2
gene, which encodes an extracellular peroxidase, results in improved regeneration capacities of mechanosensory axons (Gotenstein et al., 2010).

Since proper neuroregeneration and innervation are crucial for successful regeneration and since neuroregeneration is redox-regulated, it is not surprising that ROS play important signalling roles during the regeneration process (Fig. 4). Rieger and Sagasti were the first to show that increased hydrogen peroxide levels at the wound site are necessary for peripheral sensory axon regeneration following skin injury in zebrafish larvae. Moreover, they noticed impaired fin regeneration with the inhibition of DUOX 1 (Rieger and Sagasti, 2011). Soon thereafter, publications indicating the importance of ROS signalling for successful regeneration of complete body structures followed (Gauron et al., 2013; Love et al., 2013; Niethammer et al., 2009). Both Love and Gauron (Gauron et al., 2013; Love et al., 2013) visualised the ROS burst that takes place during tail regeneration in tadpoles of Xenopus and zebrafish respectively, and showed that inhibition of this ROS production at the wound site via NADPH oxidase (NOX) inhibitors results in impairment of tail and fin regeneration (Gauron et al., 2013; Love et al., 2013). Interestingly, the cellular effects of a diminished ROS production strongly resemble the effects observed after denervation (Fig. 4). Both interventions - inhibition of ROS production and denervation - disturb proliferation.
of the progenitor cells without affecting formation of the wound epithelium. Similarly to denervation, diminished ROS levels disturbed the wnt/β-catenin signalling pathway and FGF signalling, as shown by Love and colleagues (Love et al., 2013). Gauron and colleagues investigated the effects of ROS production inhibition on JNK MAPK signalling cascade and apoptosis following amputation, thereby affecting cell proliferation and the expression of various signalling factors, which were also deregulated following denervation (fgf20, sdf1, wnt5b, wnt10, and igf2b) (Gauron et al., 2013). The necessity of a balanced ROS production for proper stem cell differentiation and patterning, as well as the establishment of correct polarity, was recently demonstrated in planarians (Pirotte et al., 2015). In this study, diminished ROS levels also resulted in the formation of reduced cephalic ganglia so it is possible that the observed morphological effects of impaired regeneration, which are similar to those observed following denervation, are the result of a diminished brain and thus diminished neuronal signalling.

We can state that there is a clear connection between ROS signalling and regeneration (and neuronal formation) on one hand, and neuronal signalling and regeneration on the other hand. However, the direct link between ROS and neuronal signalling has not yet been shown in a regenerative context. Thorough knowledge of the exact interaction between both processes is essential in order to understand all aspects of the regeneration process. Interestingly, the indirect link between the redox balance, neuronal signalling and regeneration capacities is also present in the physiological progress of ageing. During the process of ageing, the inability of elderly neurons to re-grow or produce the vital nerve-derived factor(s) probably underlies the loss of the regeneration capacity which organisms possess in early life stages (Seifert and Voss, 2013; Stevens et al., 2015). Different studies have shown that the ability of mammals to regenerate their peripheral nerves decreases with age, which is also the case in most other vertebrates (Tanaka and Ferretti, 2009; Verdu et al., 2000). In Xenopus laevis, axons are able to regrow across a spinal cord lesion, but this ability is lost following metamorphosis (Gibbs et al., 2011). Spinal cord extracts from regenerating limbs of young axolotls induce a stronger mitogenic effect in cultures of blastema cells in comparison to the extracts of old individuals (Boilly and Albert, 1988). Thus, it seems clear that ageing and metamorphosis reduce the regenerative capacity of the nervous system in various organisms, and this will likely contribute to the reduced capacity of tissue and appendage regeneration observed in elderly individuals (Seifert and Voss, 2013; Seifert et al., 2012). Interestingly, the redox-balance shifts with age as well. Already in the 1950s, Denham Harman speculated the “free-radical theory of ageing”, in which he suggests that endogenous oxygen radicals were responsible for cumulative damage in the cells (Harman, 1956, 1992, 2006). Since then, numerous studies confirmed the association between the cellular response to oxidants and the mechanisms that regulate cellular longevity. Alterations in intracellular ROS levels have been linked with an age-related decline of cellular functioning (Finkel and Holbrook, 2000; Finkel, 2003). So, it is possible that the loss of redox control is responsible for improper neuronal cell functioning, thus affecting cell renewal and regeneration capacities. Studying the interaction between ROS and neuronal signalling in regeneration can provide important information to address age-related pathologies.

8. Conclusion

A lot of interesting research has led to crucial insights and important improvements on neural signalling during both vertebrate and invertebrate regeneration. Innervation is necessary for successful regeneration. The nerves are involved in the production of trophic factors that promote and regulate different processes during initial and later phases of regeneration, such as cell proliferation, differentiation, and patterning. However, investigation on the complex network of signalling factors and the affected processes during regeneration following denervation is still a work in progress. A better understanding on this topic is essential, especially towards medical applications. To achieve that goal, it is necessary to characterize involved pathways in both regeneration and development across different species.

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