0014-4754/96/090865-06$1.50 + 0.20 © Birkhäuser Verlag Basel, 1996

Effect of a dipeptide inhibiting ubiquitin-mediated protein degradation on nerve-dependent limb regeneration in the newt

C. H. Taban, H. Hondermarck[a], R. A. Bradshaw[b] and B. Boilly[a,*]

[a]*Centre de Biologie Cellulaire, Unité 'Dynamique des Cellules Embryonnaires et Cancéreuses', SN3, Université de Lille 1, F-59655 Villeneuve d'Ascq Cedex (France), Fax +33 20 43 40 38, e-mail: Benoni.Boilly@univ-lille1.fr*
[b]*Department of Biological Chemistry, College of Medicine, University of California, Irvine (California 92717, USA)*
Received 18 March 1996; received after revision 7 May 1996; accepted 9 May 1996

Abstract. The dipeptide Leu-Ala, which inhibits ubiquitin-mediated protein degradation, has been shown to act in vitro as an inhibitor of neurite outgrowth of PC12 cells (Hondermarck et al. [1992] Biochem. Biophys. Res. Commun. **189:** 280). Using agarose beads as vehicles, we tested, in vivo, the effect of this dipeptide (and the inactive inverse, Ala-Leu, as a control) on limb regeneration in the newt (*Triturus cristatus*), a nerve-dependent developmental process. Leu-Ala inhibited the growth of mid-bud blastemas without altering blastema differentiation, while Ala-Leu had no effect. Cytological observations of dipeptide-treated blastemas using Bodian staining or neurofilament antibodies showed that all the blastema tissues were unmodified except with regard to innervation. Leu-Ala-treated blastemas were devoid of nerve fibers in the epidermal cap, while the mesenchyme distal to the dipeptide impregnated bead exhibited fewer nerve fibers than did Ala-Leu-treated blastemas, which were similar to the control nontreated blastemas. Thus, Leu-Ala, in reducing blastema innervation, inhibits its growth in the same manner as surgical denervation.
Key words. Newt; limb regeneration; nerve regeneration; ubiquitin-mediated proteolysis; ubiquitin ligase (E3).

Limb regeneration of urodeles is nerve-dependent; thus, denervation prevents regeneration. Moreover, the regeneration of an amputated limb is stopped if denervation occurs before the regenerate begins to differentiate (reviewed in [1, 2]). The identification (sensory, motor, sympathetic) of nerve fibers acting in limb regeneration is still open. It is known that several types of nerve fibers are able to sustain regeneration and that the total section surface of the nerve fibers related to the limb amputation surface may be germane to whether or not regeneration occurs. Nevertheless, Singer [3, 4] has shown that sensory fibers are the most active in this process, since only 40% of the animals regenerate their limbs when only motor fibers are kept after section of the sensory roots. Although the nature of the nerve factor(s) (the so-called neurotrophic factor) that promotes limb regeneration is still unknown, the effects of nerves on the metabolism of blastema cells are well documented; they are related to mitosis, protein and nucleic acid synthesis (reviewed in [1]), extracellular matrix deposition [5–7], and second messenger changes [8–11].

The action of nerves on limb regeneration depends on regeneration of the amputated peripheral nerves. We previously showed that nerves produce more growth factors when regrowing after amputation [12]. Moreover, we observed that extracts of axolotl spinal cord 14 days after forelimb amputation are more mitogenic than are extracts 7 days after forelimb amputation or extracts from axolotls without amputation [13], suggesting that the mitogenicity of nerves depends on their regenerating status. Thus, the control of nerve growth appears to be key in limb regeneration.

Recently, it has been shown that the ubiquitin-mediated path of proteolysis is implicated in the process of neurite outgrowth. A major step of the ubiquitin-mediated degradation pathway is recognition between a substrate protein and the enzyme ubiquitin ligase (E3) (reviewed in [14, 15]). Hondermarck et al. [16] have shown that dipeptide inhibitors of this molecular recognition also block growth factor-induced neurite outgrowth of pheochromocytoma (PC12) cells, indicating that the E3 pathway is necessary for this process. These results have also been extended to neuronal precursor cells from amphibian embryos [17].

To determine whether the E3-related ubiquitin pathway plays a role in nerve fiber regrowth during limb regeneration, we implanted beads containing inhibiting or non-inhibiting dipeptides into blastemas. The results show that the inhibiting dipeptide Leu-Ala, when administered to the blastema of *Triturus cristatus*, reduces the number of histologically detectable nerve fibers and concomitantly slows the regeneration process.

* Corresponding author.

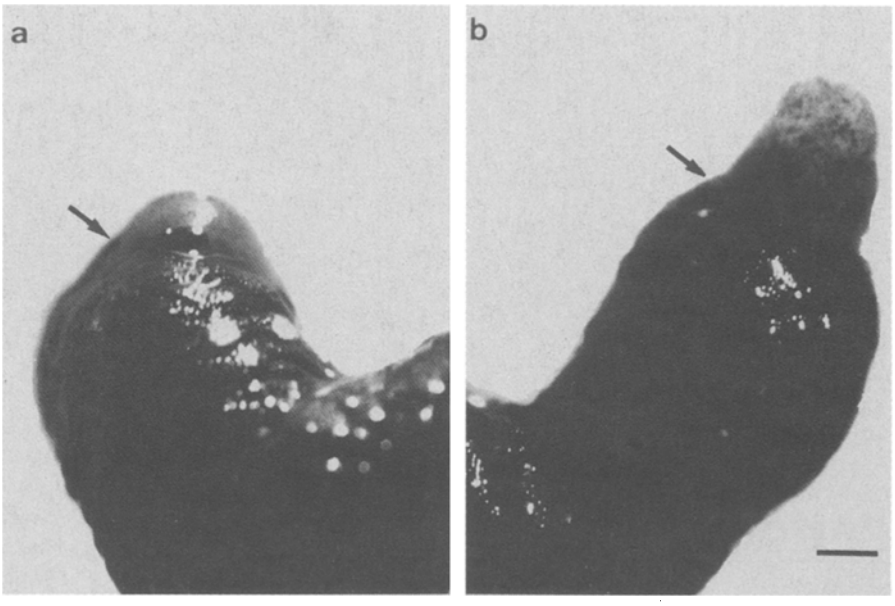

Figure 1. Forelimb regeneration of *T cristatus*: 26 days postamputation, 15 days after implantation of dipeptide-soaked beads. (*a*) Leu-Ala treatment. (*b*) Ala-Leu treatment. A clear difference appears between Leu-Ala-treated blastema (which reaches only the early/mid-bud stage), and Ala-Leu-treated blastema (which exhibits a palette stage). No morphological difference was observed between Ala-Leu-treated and sham-operated blastemas. Arrows indicate the level of amputation (same animal). Bar: 1 mm.

Material and methods

Animals and experimental procedures. One-year-old *T. cristatus* were collected from a private pond in Cologny, close to Geneva. They were maintained in large aquariums at room temperature (19 ± 1 °C) and fed twice a week with beef or calf liver. All animals were anesthetized with MS 222 (Sandoz, Basel, Switzerland) before experimental procedures. Both forearms were amputated at either mid-humerus level or between wrist and elbow. Dipeptides were introduced into blastemas at the time of wound healing, or at early bud stage using Affigel blue beads (Bio-Rad) soaked in 0.5 M aqueous solution of dipeptide for 24 to 48 h. The beads were implanted as follows: A stretched tip Pasteur pipette containing the beads was inserted through a thin incision in the skin at the distal part of the stump, and one bead was pulled under the wound epithelium or epidermal cap. In an initial control series, we showed that implantation of such beads, which are known to passively absorb and release hydrophilic compounds, does not alter the limb regeneration process. The left stump of each animal was treated with the active dipeptide and the right side with the control dipeptide, Leu-Ala and Ala-Leu, respectively. Additional controls were performed by inserting Ala-Leu on one side and a PBS (phosphate-buffered saline) bead on the other. This operation was repeated five times at 2- to 3-day intervals, and the length of the regenerating blastemas measured under the dissecting microscope. Twenty-eight bilateral amputations were performed, and 14 treated regenerates were sampled at the end of the experiment for histological studies. In three additional animals, 4 weeks after the first implantation we reversed the side of each dipeptide implantation. Measurement of the blastemas 6 months after the end of the experiment was performed to evaluate the possible impact of the transient action of dipeptides during the regenerating process

Chemicals. Dipeptides (Leu-Ala and Ala-Leu, all L form) were obtained from Research Organics, USA; ubiquitin and anti-ubiquitin (polyclonal, rabbit) were from Sigma (St Louis, MO). Anti-neurofilament AD1 and 345 antisera were kindly provided by Dr A. Delacourte, Lille and Sanofi, Montpellier. AD1 is a monoclonal antibody raised in mice treated with PHF (paired helicoidal filaments) from human brain cells with Alzheimer lesions [18]; it very probably recognizes the site KSPV of high- (200 kDa) and medium-sized (150 kDa) subunits of neurofilament proteins (NF-H and NF-M respectively) but only when it is phosphorylated. Antibody 345 is a polyclonal raised in rabbits against bovine brain neurofilaments and recognizes three proteins of the neurofilament triplet, mainly the 200 (NF-H) and 140–160 (NF-H) neurofilament protein in man and several mammals.

Cytological procedures. At the end of the experiment the animals were sacrificed, and the regenerated limbs were fixed in PBS-buffered 4% formaldehyde (Merck, Darmstadt, Germany) solution (pH 7.4), washed in PBS 5% sucrose solution and frozen with dry ice. Samples were then processed immediately or frozen at

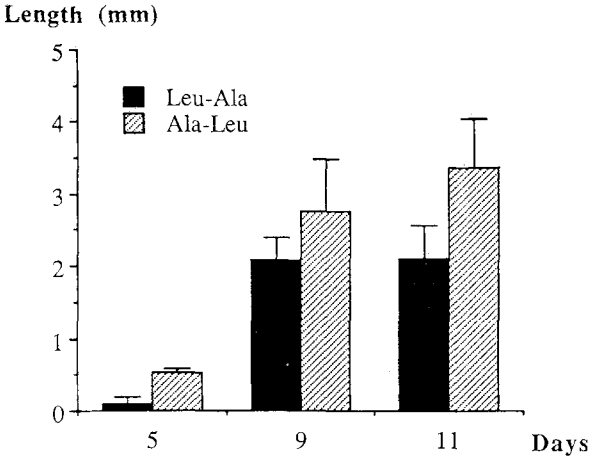

Figure 2. Growth of limb blastema of *T. cristatus* treated with either Leu-Ala or Ala-Leu. The blastema was treated for the first time 15 days after amputation (early bud stage). The length (mean ±SD in mm) of the blastema was measured 5, 9 and 11 days after the first treament. Significant differences (t-test) were found between the length of Leu-Ala- and Ala-Leu-treated blastemas for each of the three measurements (seven animals); $p < 0.05$ (9 days); $p < 0.01$ (5 and 11 days).

−20 °C until use. Twelve-µm sagittal sections were cut with the cryostat and placed on poly-L-lysine or gelatin-coated slides. The total number of sections was recorded in order to estimate the regenerate thickness. Staining was performed with Goldner trichrome and Bodian. Antibodies recognizing the corresponding epitopes in the tissues were revealed according to Coon's method followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) treatment.

The number of nerve fibers was estimated on Bodian-stained serial sections of four animals. Eight sections chosen in the median part of each regenerate were scanned across the whole section with a grid composed of 25 squares. Scans passing proximally or distally to the implanted beads were analysed separately in Leu-Ala- and Ala-Leu-treated limbs. When entire or partially sectioned nerve fibers were present in a grid square, this square was counted as positive; when no nerve fiber was observed, the square was counted as negative. The total number of squares observed (with the magnification given by a 10× ocular and 100× objective) was noted, and the proportion of positive and negative squares was computed. Results were obtained from 37 grids for each level (proximal vs distal) and for each treatment (Leu-Ala vs Ala-Leu).

Statistical analysis was performed using the Student's t-test.

Results

Leu-Ala-treated regenerates grew less well than the Ala-Leu-treated ones (fig. 1). The length difference between Leu-Ala- and Ala-Leu-treated regenerates was significant 5, 9 and 11 days after the first treatment, as shown in figure 2. Moreover, the number of 12-µm sections

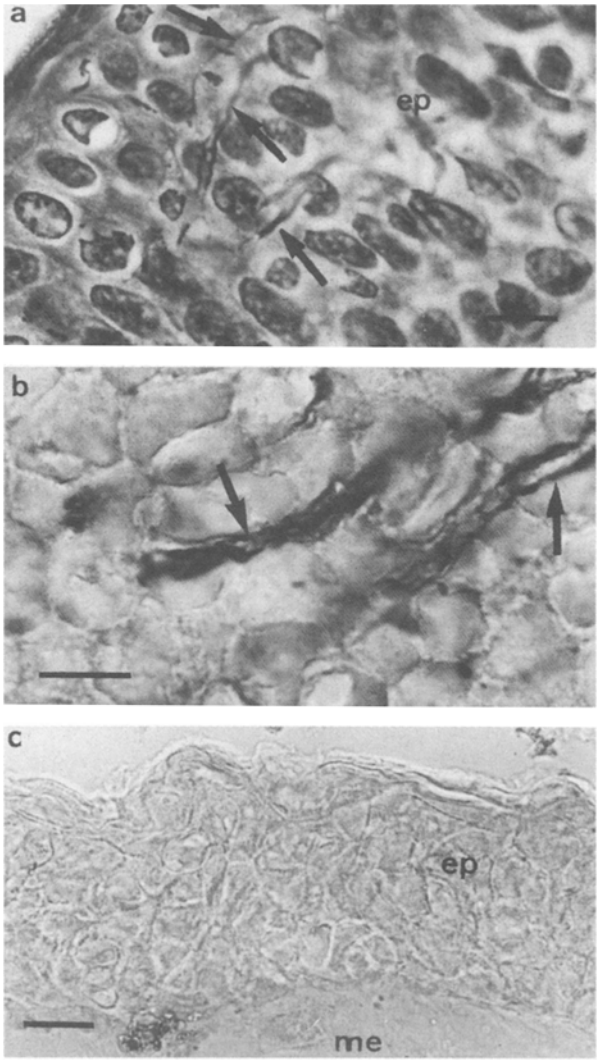

Figure 3. Epidermal cap (ep) of dipeptide-treated limb blastema (12-µm-thick mid-sagittal cryostat sections). (*a*) Ala-Leu-treated blastema, Bodian staining. Arrows: silver-stained nerve fibers. Bar: 20 µm. (*b*) Ala-Leu-treated blastema, AD1 antibody. Note the black intraepidermal nerve fibers revealed by DAB (arrows). Bar: 20 µm. (*c*) Leu-Ala-treated blastema, AD1 antibody. This picture shows the entire epidermal cap at a low magnification; it exhibits no intraepidermal nerve fibers after DAB application. *me*: underlying mesenchyme. Bar: 60 µm.

obtained from Leu-Ala-treated blastemas was less than that for Ala-Leu, which also indicated a clear difference in regenerate thickness (not shown). No significant difference either in length or width was detected between sham-operated stumps and Ala-Leu-treated ones. The effect of the dipeptide inhibition is reversible, since left and right limb regenerates of treated animals observed 5 months after the completion of the experiment were of normal appearance and of similar length (9 ± 0.5 mm). Moreover, in the three animals for which a reversal of the type of implanted dipeptide was carried out 4 weeks after the first series of implantations, an inversion of the relative growth was detected in accordance with the type of dipeptide used.

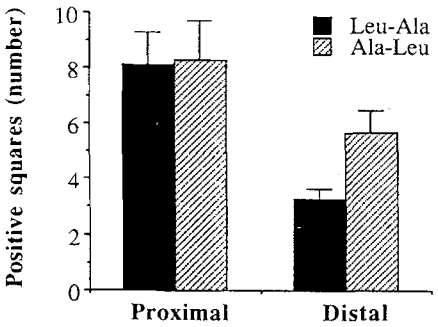

Figure 4. Number of grid squares (400 μm^2 per square) of blastema histological sections (12-µm-thick mid-sagittal sections) containing Bodian-stained nerve fibers, proximal and distal to the Leu-Ala- or Ala-Leu-implanted bead. For each of the four animals studied, 925 squares (corresponding to 37 grids composed each of 25 squares) were observed.

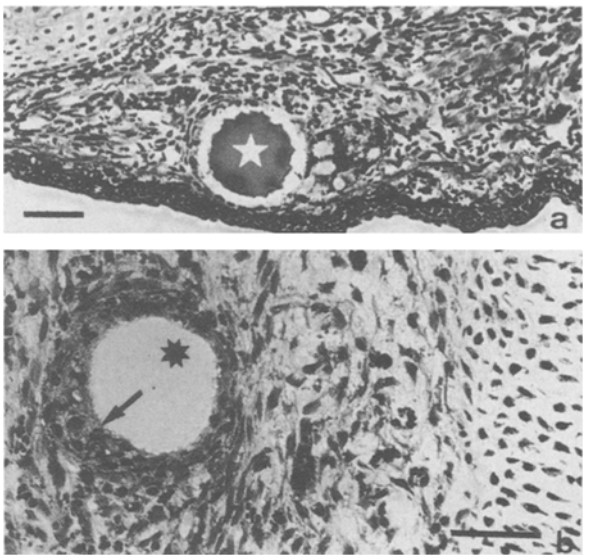

Figure 5. General histological view of dipeptide-treated blastema (Goldner staining, 12-µm-thick cryostat sections) 1 month after amputation. All tissues are of normal histological appearance. There is, however, a restricted inflammatory reaction (arrow) around the dipeptide-soaked bead (star). (*a*) Leu-Ala bead; (*b*) Ala-Leu bead. Bar: 100 µm.

Microscopic observations of blastemas implanted with beads showed no histological differences regardless of the type of dipeptide used (Leu-Ala, Ala-Leu) except with regard to innervation. Differentiation of the precartilage and cartilage appeared to be undisturbed by the presence of either type of dipeptide, and no histological difference could be noted in the regenerate, either proximal or distal to the implant, thus indicating that the dipeptides did not interfere with the differentiation process. Both Bodian staining and immunolocalization of neurofilaments with the AD1 antibody showed that Leu-Ala-treated blastemas were almost entirely devoid of present intraepidermal stained nerve fibers, whereas they were present in Ala-Leu-treated blastemas (fig. 3) or untreated regenerating animals. In the connective tissue proximal to the bead, nerve fibers were in an equivalent number in blastemas treated with Leu-Ala or with Ala-Leu (fig. 4). In contrast, the connective tissue distal to the inserted Leu-Ala-soaked bead showed significantly fewer grid squares positive for nerve fibers than did the connective tissue distal to an Ala-Leu-soaked bead (fig. 4). Leu-Ala- and Ala-Leu-soaked beads produced a moderate local inflammatory reaction and were surrounded after 2 weeks by one or two sheaths of polynuclear and macrophage cells; most of the time the bead kept its compact ball-shaped appearance and was present inside the tissue of the blastema (fig. 5).

The two different antibodies used, AD1 and 345 (both of which detect neurofilaments), showed some differences in the number of filaments revealed in sections of the same bud. In contrast to the AD1 monoclonal antibody, the polyclonal 345 antibody which recognizes NF-H, NF-M and NF-L revealed fewer filaments, but their distribution was similar. Finally, strong ubiquitin-like immunoreactivity was found in almost all tissues of mid-bud stage blastemas, mainly in growing cartilages and the epidermal cap (not shown). The specificity of the antibody was shown by the fact that sections treated with ubiquitin preabsorbed with anti-ubiquitin (5 mg/ml) gave no specific marking.

Discussion

In regenerating Urodele limbs, fibers from the severed brachial nerves grow rapidly toward the tip of the blastema. In particular, sensory nerve fibers invade the wound epithelium, invasion being easily observed during the cone stages [19, 20]. The early innervation of the blastema is a prerequisite for regeneration. Previous studies have generally used surgical denervation [21], but more recently chemical destruction of neurotubules with colchicine was shown to inhibit limb regeneration in a similar manner [22]. Colchicine is also known as a potent inhibitor of mitosis and does not allow one to differentiate neurotoxic effects from direct antiproliferative ones. In this study, we showed that a competitor of the ubiquitin-mediated protein degradation pathway, namely the dipeptide Leu-Ala, specifically inhibits nerve fiber regeneration into the blastema. This dipeptide has been shown to competitively inhibit the in vivo recognition by E3 of substrate proteins and consequently their degradation [23]; other peptides (peptide aldehydes) have also been shown to inhibit the degradation of ubiquitinated proteins and, as a consequence, to block the generation of peptides presented on MHC class I molecules [24]. The dipeptide Leu-Ala has no known direct effect on cell proliferation but inhibits neurite outgrowth in FGF- or NGF-treated PC12 cells [16] or

NGF-treated neural plate cells of pleurodele embryos [17]. The present result is of special interest since it is the first time that this dipeptide has been shown to control nerve fiber regeneration in an in vivo system. The observation that ubiquitination of proteins seems to be necessary for nerve regrowth extends our previous results to peripheral nerve regeneration. The result is confirmed by the fact that the inverse dipeptide (Ala-Leu) has no effect, and also by the demonstration of ubiquitin in newt limb regenerates by immunocytochemistry (not shown). A surprising result was the observation that the inhibiting effect of the Leu-Ala dipeptide occurred only distal to the source of dipeptide, suggesting that there is a proximo-distal flow, within the blastema, that carries away small molecules like dipeptides.

We consider that dipeptide inhibition of nerve fiber regeneration in the blastema correlates with the fact that blastema growth stops following treatment with these inhibitors, since the number of observed mitotic figures decreased (not shown). This decrease appears not to be the result of cytotoxicity or of a direct effect on mitosis, but is likely to be associated with a primary effect on the regenerating nerve's release of stimulating factors for regenerating tissues [25]. This interpretation is confirmed by the fact that cartilage was not histologically modified in the presence of Leu-Ala implants, and that nerve fibers are missing in the epidermal cap. Their number also decreased in the connective tissue distal to the implant of Leu-Ala-treated blastemas. The absence of an effect of dipeptides on blastema histodifferentiation is probably related to the fact that the dipeptides were used, at least for the last treaments, after the onset of the differentiative phase; it is known that denervation after this phase fails to inhibit differentiation and morphogenesis of the blastema into a normal and functional limb, although the regenerated limb is of a smaller size [26, 27].

The absence of nerve fibers in the epidermal cap can explain the inhibition of regeneration after dipeptide treatment, since sensory nerve fibers, which are known to invade the wound epithelium rapidly after amputation [19, 20], are effective for triggering limb regeneration [3, 4]. The importance of sensory fibers in limb regeneration was supported by several in vivo [28–31] and in vitro [32–34] assays which demonstrated the high mitogenic effect of sensory neurons. More recently, the efficiency of spinal ganglia vs other parts of the nerve tissue was shown using the formation of inositol phosphates in blastemal mesoderm tissue of *Notophthalmus viridescens* [11]. Spinal ganglia extracts stimulated the formation of inositol phosphates at a very low level (10^{-12} g/ml) compared with brain and spinal cord extracts (10^{-10} and 10^{-8} g/ml, respectively). Although these last in vitro results might be explained by a higher content of neurons and/or mitogenic factors in spinal ganglia (substance P for Globus and Alles [35]), in vivo observations suggest two special features of sensory fibers which might account for their function during limb regeneration. The first is the rapidity of their regrowth into blastema tissues [19, 20], a behavior which might be related to their mitogenicity [12]; the second concerns their targets, i.e. wound epithelium and epidermal cap, which are known to perform an important function during limb regeneration (reviewed in [36]) and to contain a high level of growth factors [37], such as acidic FGF [38], which is known to trigger blastema cell proliferation [39].

Acknowledgements. This work was supported in part by the region of Nord-Pas de Calais and the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (EA 1033).

1 Tassava R. A. and Olsen C. L. (1985) Neurotrophic influences on cellular proliferation in urodele limb regeneration: in vivo experiments. In: Regulation of Vertebrate Limb Regeneration, pp. 81–92, Sicard R. E. (ed.), Oxford University Press, New York
2 Carlone R. L. and Mescher A. L. (1985) Trophic factors from nerves. In: Regulation of Vertebrate Limb Regeneration, pp. 93–105, Sicard R. E. (ed.), Oxford University Press, New York
3 Singer M. (1946) The nervous system and regeneration of the forelimb of adult *Triturus*. IV. The stimulating action of a regenerated motor supply. J. Exp. Zool. **101:** 221–240
4 Sidman R. L. and Singer M. (1960) Limb regeneration without innervation of the apical epidermis in the adult newt, Triturus. J. Exp. Zool. **144:** 105–110
5 Mescher A. L. and Munaim S. I. (1986) Changes in the extracellular matrix and glycosaminoglycan synthesis during the initiation of regeneration in adult newt forelimbs. Anat. Rec. **214:** 424–431
6 Vanrapenbusch S. and Lassalle B. (1988) Effects of denervation on the extracellular collagen matrix of limb regenerate of the newt, *Pleurodeles waltlii*. In: Recent Trends in Regeneration Research, pp. 217–227, Kiortsis V., Koussoulakos C. and Wallace H. (eds), Plenum Press, New York
7 Boilly B., Oudghir M., Deudon E., Boilly-Marer Y. and Hondermarck H. (1995) Nerve dependent sulphated glycosaminoglycan synthesis in limb regeneration of the newt *Pleurodeles waltl* Roux. Arch. Devl Biol. **204:** 509–512
8 Cathieni M. and Taban C. H. (1992) Cyclic nucleotide fluctuations during newt limb regeneration depends on injury and nerve action. In: Keys for Regeneration, pp. 85–92, Taban C. H. and Boilly B. (eds), Karger, Basel
9 Oudkhir M., Martelly I., Castagna M., Moraczewski J. and Boilly B. (1989) Protein kinase C activity during limb regeneration of amphibians. In: Recent Trends in Regeneration Research, pp. 69–79, Kiortsis V., Koussoulakos S. and Wallace H. (eds), Plenum Press, New York
10 Boilly B., Oudkhir M., Moraczewski J. and Martelly, I. (1992) Signal transduction pathway of nerve-derivated mitogen in regenerating limb of newt. In: Keys for Regeneration, pp. 93–99, Taban C. H. and Boilly B. (eds.), Karger, Basel
11 Smith M. J., Globus M. and Vethamany-Globus S. (1995) Nerve extracts and substance P activate the phosphatidylinositol signaling pathway and mitogenesis in newt forelimb regenerate. Devl Biol. **167:** 239–251
12 Boilly B. and Bauduin B. (1988) Production in vitro by spinal cord of growth factor(s) acting on newt limb regeneration: influence of regeneration of the nerve fibers. Devl Brain Res. **38:** 155–160
13 Boilly B. and Albert P. (1988) Blastema cell proliferation in vitro: effects of limb amputation on the mitogenic activity of spinal cord extracts. Biol. Cell **62:** 183–187

14 Varsharsky A. (1992) The N-end rule. Cell **69:** 725–735
15 Bradshaw R. A., Sy J., Stewart A. E., Kendall R. L., Hondermarck H. and Arfin S. N. (1994) Co-translational modification, stability and turnover of eukaryotic proteins. In: Biological Membranes: Structure, Biogenesis and Dynamics, pp. 155–164, Op den Kamp J. A. F. (ed.), Springer Verlag, Berlin
16 Hondermarck H., Bradshaw R. A., Sy J. and Arfin S. N. (1992) Dipeptide inhibitors of ubiquitin-mediated protein turnover prevent growth factor-induced neurite outgrowth in rat pheochromocytoma PC12 cells. Biochem. Biophys. Res. Commun. **189:** 280–288
17 Maufroid J. P., Bradshaw R. A., Boilly B. and Hondermarck H. (1996) Nerve growth factor-induced neurite outgrowth from amphibian neuroepithelial precursor cells is prevented by dipeptides inhibiting ubiquitin-mediated proteolysis. Int. J. Devl Biol. (in press)
18 Condamines O., Buée-Scherrer V., Boissier L., Wattez A., Delacourte A., Bau B. and Mourton-Gilles C. (1995) New immunoassay for the mapping of neurofibrillary degeneration in Alzheimer's disease using two monoclonal antibodies against human PHF-tau proteins. Neurosci. Lett. **192:** 81–84
19 Singer M. (1949) The invasion of the epidermis of the regenerating forelimb of the urodele, *Triturus*, by nerve fibers. J. Exp. Zool. **111:** 189–210
20 Taban C. H. (1949) Les fibres nerveuses et l'épithélium dans l'édification des régénérats de pattes (in situ ou induites) chez le triton. Archs Sci. **2:** 553–561
21 Locatelli P.(1924) L'influenza del systema nervoso sui processi di regenerazione. Archs Sci. Biol. **5:** 362–378
22 Scadding S. R. (1988) Treatment of brachial nerves with colchicine inhibits limb regeneration in the newt *Notophthalmus viridescens*. J. Exp. Zool. **247:** 56–61
23 Baker R. T. and Varshavsky A. (1991) Inhibition of the N-end rule pathway in living cells. Proc. Natl Acad. Sci. USA **88:** 1090–1094
24 Rock K. L., Gramm C., Rothstein L., Clark K., Stein R., Dick L., Hwang D. and Goldberg A. L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78:** 761–771
25 Boilly B., Lheureux E., Boilly-Marer Y. and Bart A. (1990) Cell interactions and regeneration control. Int. J. Devl Biol. **34:** 219–231
26 Schotté O. E. (1926) Regeneration of asensory limbs of *Ambystoma* larvae. Rev. Suisse. Zool. **33:** 371–373
27 Singer M. and Craven L. (1948) The growth and morphogenesis of the regenerating forelimb of adult *Triturus* following denervation at various stages of development. J. Exp. Zool. **108:** 279–308
28 Kamrin A. A. and Singer M. (1959) The growth influence of spinal ganglia implanted into the denervated forelimb regenerate of the newt, *Triturus*. J. Morph. **104:** 415–439
29 Tomlinson B. L. and Tassava R. A. (1987) Dorsal root ganglia grafts stimulate regeneration of denervated urodele forelimbs: timing of graft implantation with respect to denervation. Development **99:** 173–186
30 Goldhamer D. J., Tomlinson B. L. and Tassava R. A. (1992) Ganglia implantation as a means of supplying neurotrophic stimulation to the newt regeneration blastema: cell-cycle effects in innervated and denervated limbs. J. Exp. Zool. **262:** 71–80
31 Globus M. and Liversage R. A. (1975) In vitro studies of limb regeneration in adult *Diemictylus viridescens*: neural dependence of blastema cells for growth and differentiation. J. Embryol. Exp. Morph. **33:** 813–829
32 Globus M. and Vethamany-Globus S. (1977) Transfilter mitogenic effect of dorsal root ganglia on cultured regeneration blastemata in the newt *Notophthalmus viridescens*. Devl Biol. **56:** 316–328
33 Tomlinson B. L., Globus M. and Vethamany-Globus S. (1981) Promotion of mitosis in cultured newt limb regenerates by a diffusible nerve factor. In Vitro **17:** 167–172
34 Lassalle B., Oudkhir M., Vanrapenbusch S. and Boilly B. (1985) Rôle du système nerveux dans la prolifération cellulaire des constituants épidermique et mesenchymateux des blastèmes de régénération de membres d'un triton Pleurodèle (*Pleurodeles waltlii* Michah) en culture. Biol. Cell **53:** 37–40
35 Globus M. and Alles P. J. (1990) A search for immunoreactive substance P and other neural peptides in the limb regenerate of the newt *Notophtalmus viridescens*. Exp. Zool. **254:** 165–176
36 Stocum D. L. (1985) Role of the skin in urodele limb regeneration. In: Regulation of Vertebrate Limb Regeneration, pp. 32–53, Sicard R. E. (ed.), Oxford University Press, New York
37 Boilly B. and Albert P. (1990) In vitro control of blastema cell proliferation by extracts from epidermal cap and mesenchyme of regenerating limbs of Axolotl. Roux Arch. Dev. Biol. **198:** 443–447
38 Boilly B., Cavanaugh K. P., Thomas D., Hondermarck H., Bryant S. V. and Bradshaw R. A. (1991) Acidic fibroblast growth factor is present in regenerating limb blastemas of Axolotl and binds specifically to blastema tissues. Devl Biol. **145:** 302–310
39 Albert P., Boilly B., Courty J. and Barritault D. (1987) Stimulation in cell culture of mesenchymous cells of newt limb blastema by EDGF I or II (basic or acidic FGF). Cell. Diff. **21:** 63–68