

Molecular Basis for the Nerve Dependence of Limb Regeneration in an Adult Vertebrate

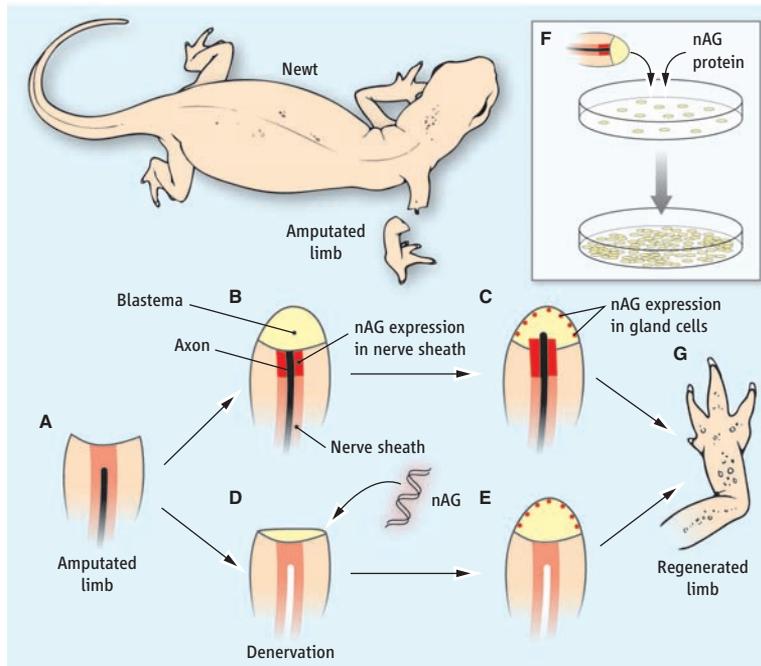
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AUTHORS' SUMMARY

Unlike human, salamanders can regrow an amputated limb. This regeneration occurs only if there is simultaneous regeneration of the severed nerves. Although we usually think of the nervous system as carrying information between nerve cells and their sensory and motor targets, nerves are also essential for tissue regeneration. When a salamander limb is amputated at any position, from the shoulder to the fingertips, the stump forms a blastema, a mound of stem cells from which regeneration begins. The nerve supply cut by the amputation also regenerates, and this regrowth is required for the proliferation of the blastemal cells. If the nerves are cut at the base of the limb, deeper than the regenerative tissue, the limb stump is permanently denervated, the axons cannot regenerate, and limb regeneration fails.

The nerve dependence of limb regeneration was discovered in 1823 by Tweedy John Todd, an English physician, and analyzed in the 1940s and 1950s by Marcus Singer. Nerves are required for many different sorts of regeneration in both vertebrates and invertebrates (1), perhaps to ensure that the regenerated tissue receives adequate innervation. Singer showed that either sensory or motor nerves could support regeneration and that neither conduction of the nerve impulse nor neurotransmitter release was required. The molecular basis for communication between nerves and regenerating tissue has been unclear, although various growth factors have been proposed as mediators. Our paper now identifies a protein that can rescue the denervated blastema and induce regeneration of the limb.

The limb blastema gives rise to structures precisely distal to its site of origin (that is, a blastema at the wrist gives rise to a hand, not an upper arm). The stem cells of the blastema can thus be specified according to what structures are being formed, not just what cell types are needed. Analysis of this property led to the previous identification of Prod 1, a small protein anchored to the surface of blastemal cells and expressed in a gradient along the limb (more Prod 1 is found proximally and less at distal points). Prod 1, a member of a protein superfamily called Ly6, determines the proper identity of the regenerating tissues. To search for possible ligands for Prod 1, we conducted a screen in yeast cells and identified the n(evt)AG protein. This candidate ligand, nAG,



Regeneration of salamander limbs requires concomitant regeneration of the severed nerves. A single protein, nAG, can substitute for the regenerating nerve cells.

showing that expression in both locations depends on axons. In isolated cells cultured from the blastema, nAG promotes proliferation (F). nAG can be artificially expressed in the denervated stump by injecting plasmid DNA that encodes nAG and passing an appropriate current across the limb, a procedure called electroporation that encourages cells to take up external DNA (D). In denervated blastema so treated, nAG is expressed widely and is secreted, and the otherwise missing glands in the wound epidermis are re-established (E). These results suggest that nAG that is normally released from the Schwann cells during regeneration induces the glands (B, C). Most striking, the artificial expression of nAG rescues the denervated blastema so that the limb regenerates through the entire missing proximal-distal axis and forms digits (G). Therefore, the requirement for the presence of nerve cells to achieve normal, regenerated limb shape and structure can be met by expression of a single protein—nAG.

A geneticist might prefer to see the function of nAG tested by leaving the nerve intact and knocking out the expression of the nAG gene in the relevant cells after amputation. This manipulation is not possible on the salamander at present, but the rescue experiment we report may hold promise for future efforts to promote limb regeneration in mammals (2).

Summary References

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is a member of a large conserved family, the anterior gradient proteins, each of which contains a fold named after the protein thioredoxin and is secreted from cells.

With the help of antibodies reactive to two regions of nAG, we identified where in the regenerating newt limb the protein is expressed. After amputation, the severed axons retract within the stump and then grow back along the nerve sheath (see the figure, A and B). The Schwann cells that make up the nerve sheath in this region express nAG (B and C) as the first blastemal cells divide (B). Later, nAG appears within gland cells in the specialized wound epidermis at the end of the limb (C). This epidermis is critical for sustaining later cell proliferation in the blastema. The appearance of nAG in both the Schwann cells and the wound epidermis is abrogated by denervation (D),

FULL-LENGTH ARTICLE

The limb blastemal cells of an adult salamander regenerate the structures distal to the level of amputation, and the surface protein Prod 1 is a critical determinant of their proximodistal identity. The anterior gradient protein family member nAG is a secreted ligand for Prod 1 and a growth factor for cultured newt blastemal cells. nAG is sequentially expressed after amputation in the regenerating nerve and the wound epidermis—the key tissues of the stem cell niche—and its expression in both locations is abrogated by denervation. The local expression of nAG after electroporation is sufficient to rescue a denervated blastema and regenerate the distal structures. Our analysis brings together the positional identity of the blastema and the classical nerve dependence of limb regeneration.

Limb regeneration occurs in various species of salamander and offers important insights into the possibilities for regenerating a complex structure in adult vertebrates (1). Regeneration proceeds from the limb blastema, a mound of mesenchymal stem cells that arises at the end of the stump. A blastema always regenerates structures distal to its level of origin; a wrist blastema gives rise to the hand, whereas a shoulder blastema gives rise to the arm (2). Distal blastemal cells are converted to more proximal cells by exposure to retinoic acid or other retinoids over a relatively narrow range of concentration (3, 4). This finding led to the identification of Prod 1, a determinant of proximodistal (PD) identity that is expressed at the cell surface as a glycosylphosphatidylinositol (GPI)-anchored protein of the Ly6 superfamily (5). Its expression is graded ($P > D$) in both normal and regenerating limbs (6), and distal cells of the larval axolotl blastema are converted to more proximal cells following focal electroporation of a plasmid expressing Prod 1 (7). We have suggested that a ligand for Prod 1 could be an important player in PD identity (5).

The stem cell niche for limb regeneration has been studied intensively, and the key tissues are the regenerating peripheral nerves and the wound epidermis (8). The severed axons retract after amputation and then regenerate back along the nerve sheath and into the blastema. Axonal regeneration can be prevented or arrested by transecting the spinal nerves at the base of the limb, distant from the amputation level (9). The generation of the initial cohort of blastemal cells occurs in a denervated limb, but the growth and division of these cells depends on the concomitant regeneration of peripheral axons (10). Both motor and sensory axons have this activity, and it is independent of impulse traffic or transmitter release (11, 12). If a peripheral nerve is cut and deviated into a skin wound, it can even evoke the formation of an ectopic appendage (13, 14). Limb regeneration is abrogated if the blastema

is denervated during the initial phase of cellular accumulation, but denervation after the mid-bud stage allows the formation of a regenerate (15). The wound epidermis is not required to support proliferation during the first week of regeneration in an adult newt, but it is critical for subsequent division (16).

It remains unclear which molecules are responsible for the activity of the nerve and wound epidermis (8). The candidates considered to date include neuregulin (17, 18), fibroblast growth factor (19), transferrin (20) and substance P (21). In no case has it been demonstrated that a rigorously denervated blastema can be rescued such that it regenerates to form digits. We have identified a secreted protein that is a ligand for Prod 1 and a growth factor for limb blastemal cells. It is induced after amputation as axons regenerate along the nerve sheath, and then appears in the wound epidermis. The expression in both locations is abrogated by denervation. Most notably, the expression of this protein can rescue the denervated limb blastema and support regeneration to the digit stage.

Results

Identification of nAG protein as a ligand of Prod 1. We performed a yeast two-hybrid screen with the 69 amino acid newt Prod 1 protein (without N or C terminal signal/anchoring sequences) as bait and with prey libraries derived from both normal newt limb and limb blastema. In a search for potential extracellular ligands, two secreted proteins were identified as positives from the screen and subsequent control experiments (Fig. 1A). One was a newt member of the family of anterior gradient proteins, originally defined by the XAG2 protein, which is expressed in the cement gland of the *Xenopus* tadpole (Fig. 1B) (22). These proteins have a single thioredoxin fold with a secretory signal sequence (23). They are expressed in secretory epithelia and have been identified as elevated in various examples of rodent and human cancer (24, 25).

Epitope-tagged versions of bacterially expressed nAG and Prod 1 were found to complex together in a standard pull-down assay (Fig. 1C). When myc-tagged nAG was expressed after transfection of mammalian Cos 7 cells, it was secreted and then detected in immunoblots using two different antibodies directed at non-

overlapping sequences (Fig. 1D). The conditioned medium was reacted with live mouse PS cells transfected so as to express the anchored newt Prod 1 on the surface. The binding of nAG to the surface was detected by phosphatase-labeled secondary antibodies (Fig. 1E) and was absent in untransfected PS cells or after reaction with control medium from mock transfected Cos 7 cells.

Nerve-dependent expression in regeneration.

The expression of nAG protein was analyzed by reacting sections of the newt limb with the two antibodies, and these gave comparable results (fig. S1). In the normal limb, there was weak staining of a subset of glands in the dermis (fig. S2), but after amputation the distal end of the nerve sheath reacted strongly, as illustrated by a longitudinal section at 5 days after amputation [post-amputation (pa)], corresponding to the early dedifferentiation stage (26) (Fig. 2A and fig. S3). We analyzed cross sections of the sheath by staining for both nAG and the Schwann cell marker HNK1, and the nAG was expressed in the Schwann cells but not in axons (Fig. 2B). The wound epidermis was initially negative during regeneration, but after day 10 pa it reacted in glandular structures, as shown at day 12 pa, corresponding to the early bud stage (26) (Fig. 2C and fig. S4).

Newts were denervated by cutting the spinal nerves at the brachial plexus of the right limb and then amputated on both sides. The nerve sheath in the innervated limb showed strong expression at day 8 pa, whereas the sheath on the denervated side showed no reactivity (Fig. 3, A and B). Interestingly the expression in the wound epidermis was also dependent on the nerve. Figure 3C shows a low power image of the wound epidermis on the innervated side with nAG positive glands clearly visible, whereas the contralateral limb showed no reactivity and no glandular structures (Fig. 3D). We conclude that the nAG protein is expressed in the key niche tissues early in regeneration and that expression in both locations is abrogated by denervation.

Activities of nAG on the denervated blastema.

To deliver the protein to the adult newt limb, we electroporated plasmid DNA into the distal stump at day 5 pa. In trial experiments, red fluorescent protein (RFP) was strongly expressed in about 30 to 50% of the mesenchymal cells in this region (Fig. 4A) and persisted for up to 3 weeks. We expressed nAG from a plasmid with the N terminal signal sequence, and the protein was readily detectable both in the electroporated cells (Fig. 4B) and after secretion in the extracellular space of the early regenerate. Because this procedure appeared to deliver the protein effectively, we denervated animals on the right side, amputated both limbs, and then electroporated the nAG plasmid or empty vector on the denervated side. At day 8 after electroporation, we sectioned the distal limbs on both sides and stained with the nAG antibodies. None of the animals electroporated with the control vector

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showed the appearance of nAG positive glands in the wound epidermis, but five out of six animals electroporated with the nAG plasmid showed the induction of nAG positive glands (Fig. 4C). Therefore, the delivery of this protein to a denervated blastema can induce these elements in the wound epidermis.

To determine whether nAG can rescue the nerve dependence of limb regeneration, groups

of animals were denervated on the right side, and then amputated bilaterally (Fig. 4D). At day 5 pa, the right limb was electroporated either with nAG plasmid or with empty vector. The animals were allowed to regenerate, and the progress of limb regeneration was monitored up to day 40 pa. Two representative newts are shown at day 40 in Fig. 4E. The position of the initial denervation is marked with a yellow star.

The newt on the left has regenerated its control left limb, whereas the right denervated limb has failed to regenerate. In some animals, the axons may subsequently regenerate from the level of the star to the amputation plane, but denervated adult newt blastemas undergo fibrosis and other tissue changes that stop them from making a delayed regenerative response (27). All animals electroporated with the vector resembled the left newt in Fig. 4E. The right animal has also regenerated on the control left side, but the expression of nAG has rescued the denervated blastema and regeneration has proceeded to the digit stage. We analyzed the animals of different batches at day 30 to 40 pa, and half of the nAG-electroporated animals showed digit-stage regeneration (Fig. 4E). Some animals regenerated more slowly and were not included as

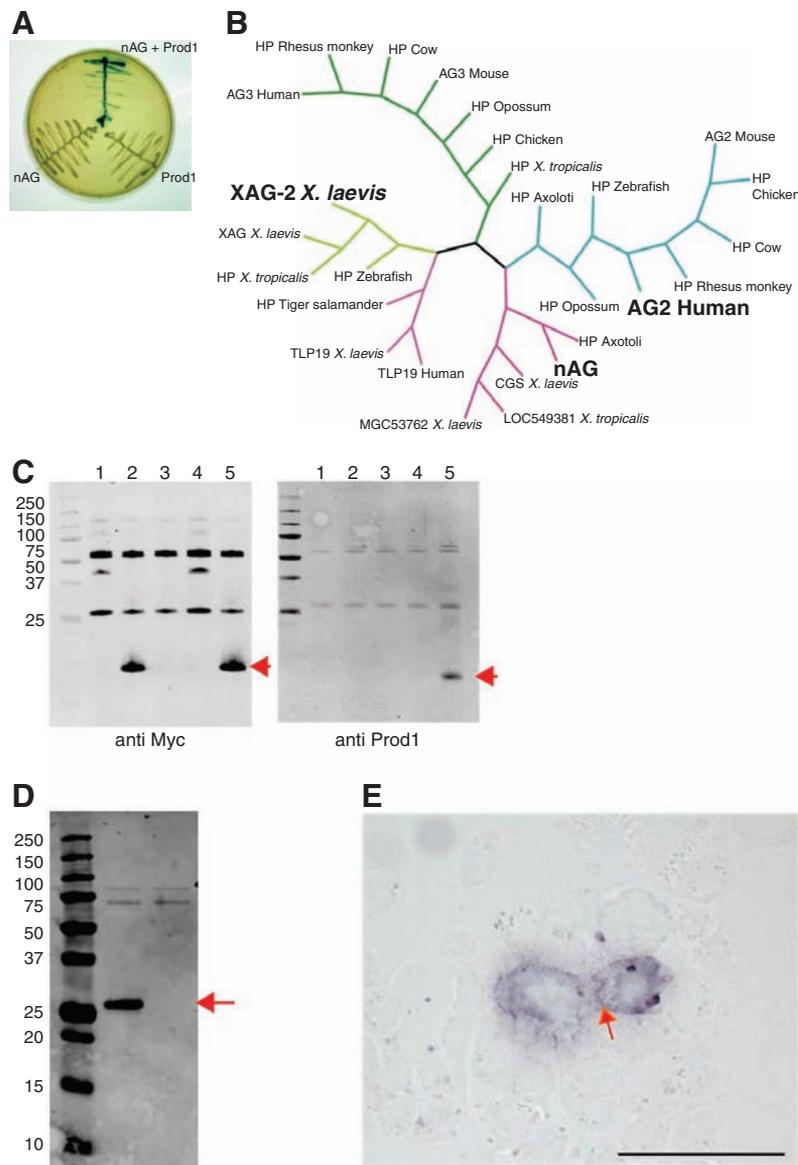


Fig. 1. Identification of nAG protein as a ligand for Prod 1. **(A)** Yeast two-hybrid assay illustrating the interaction between nAG and Prod 1. **(B)** Consensus Bayesian phylogenetic tree of representative members of the AG family of secreted proteins, highlighting nAG, the founder member XAG2, and the human AG2, which is up-regulated in several examples of cancer. **(C)** Pull-down assay with epitope-tagged forms of nAG and Prod 1 purified after bacterial expression. Lane 1, connective tissue growth factor (CTGF) beads; 2, nAG beads; 3, control beads + Prod 1; 4, CTGF beads + Prod 1; 5, nAG beads + Prod 1. Note that Prod 1 is only pulled down in lane 5. **(D)** Secretion of nAG after transfection of Cos 7 cells. Cos 7 cells were transfected with a plasmid expressing the myc-tagged nAG, or RFP, as control. The medium was analyzed by Western blotting with antibody to myc. The central lane is the nAG-transfected sample, the right is the RFP, and the left is the molecular weight markers. **(E)** Reaction of myc-tagged nAG at the surface of Prod 1 transfected mouse PS cells. nAG-conditioned medium derived as in (D) was reacted at 4°C with live PS cells transfected to express Prod 1. Note the purple reaction product at the membrane junction between the two cells (arrow). Scale bar, 50 μm.

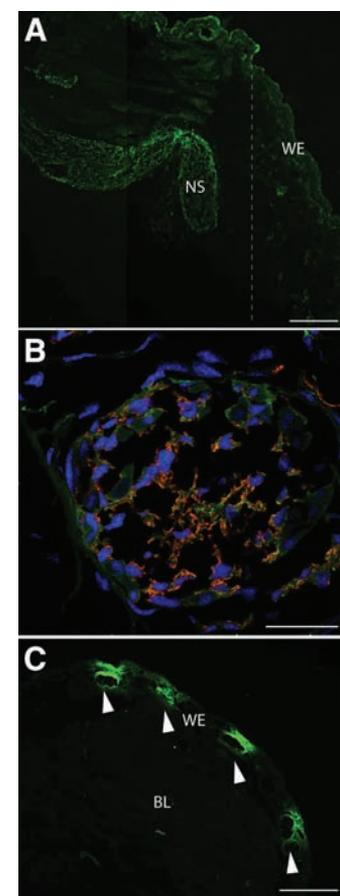


Fig. 2. Expression of nAG after amputation of the adult newt. **(A)** Longitudinal section of a blastema at day 5 pa, stained with antibodies to nAG (green). Note the strong reaction of the nerve sheath and lack of reaction in the WE. The dotted line indicates the position of the amputation plane. **(B)** Cross section of a nerve sheath in a blastema at day 10 pa, stained for nAG (green), the Schwann cell marker HNK1 (red), and nuclei (blue). **(C)** Longitudinal section of a blastema at day 12 pa, stained with antibodies to nAG and showing nAG positive glands (arrow). NS, nerve sheath; BL, blastema; WE, wound epidermis. Scale bars: (A) 200 μm; (B) 50 μm; (C) 250 μm.

reaching digit stage, whereas others did not regenerate, possibly because of the variability in the nAG expression level observed after electroporation of plasmids into adult limbs.

Limbs rescued by nAG expression were sectioned and stained with antibodies, along with

their contralateral control limbs. After staining with antibody to acetylated tubulin, which stains peripheral nerves, the rescued limb showed few labeled profiles, whereas the control limb was densely innervated (Fig. 5, A and B). This result also indicates that nAG

did not rescue the denervated blastema by enhancing the rate of nerve regeneration. These limbs were usually atrophic compared with the contralateral controls (Fig. 4E), and Fig. 5, C and D, shows sections stained with antibody to myosin. The experimental limbs had less muscle than the innervated controls, and it appears that the dependence of skeletal muscle on its innervation (28) was not satisfied by substituting nAG. It is clear, however, that the nerve requirement for completion of the PD axis was met in these animals.

nAG acts as a growth factor for cultured blastemal cells. It is difficult to understand the events underlying cell division in limb mesenchyme because of the complexity of epithelial-mesenchymal interactions in development and regeneration (29, 30). To determine whether nAG acts directly on limb blastemal cells to stimulate their proliferation, the wound epidermis was removed from limb blastemas, and the cells were dissociated and allowed to attach to micro-wells in serum-free medium before maintenance in medium containing 1% serum (Fig. 6A). These cultures were reacted under live conditions with antibody to Prod 1, and ~70% of the cells were specifically stained on their cell surface. The cells were incubated with medium from Cos 7 cells transfected with a nAG plasmid or with a control plasmid. The nAG protein was detected in the medium after immunoblotting under both reducing and nonreducing conditions as a band at 18 kD (fig. S5). The mean stimulation index for S-phase entry, as determined by bromodeoxyuridine (BrdU) pulse labeling, was 8.3 ± 3.3 fold (SD as determined in eight independent experiments) (table S1). All cell preparations were responsive to nAG; an example is shown in Fig. 6, B and C. This evidence supports the view that nAG can rescue the denervated blastema by acting directly on blastemal cells to stimulate their proliferation and, therefore, that it mediates the nerve-dependent growth of the early regenerate.

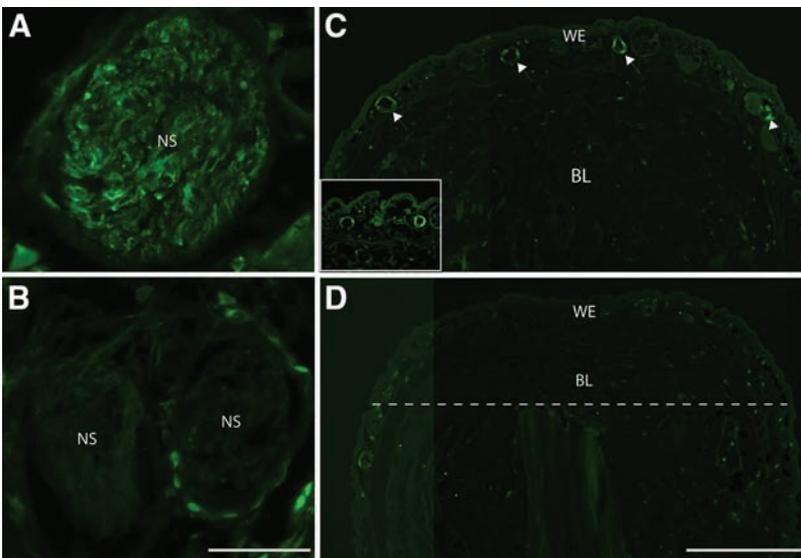
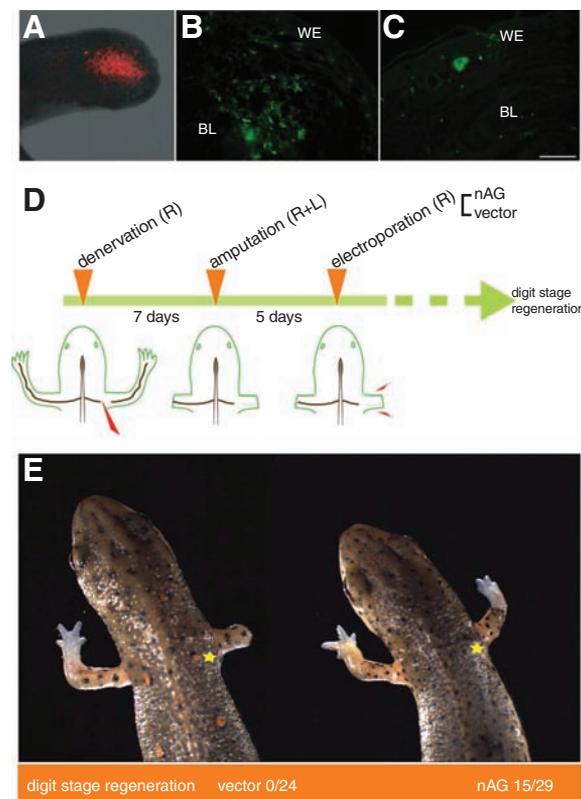


Fig. 3. Expression of nAG in the early blastema depends on innervation. (A) Cross section of a nerve sheath on the innervated side at day 8 pa, and (B) cross section of a sheath on the contralateral denervated side, both stained in parallel with antibodies to nAG. (C) Longitudinal section of a blastema on the innervated side at day 13 pa, showing nAG-positive glands (arrow). The inset shows two glands at higher magnification. (D) Section of the contralateral denervated epidermis, with the amputation plane (dotted line) and the blastema. Scale bars: (A) and (B), 100 μ m; (C) and (D) 500 μ m.

Fig. 4. Delivery of nAG protein to regenerating new limbs. (A) RFP expression at the end of the limb stump at day 10 pa after electroporation at day 5 pa. (B) Expression of nAG in cells of the limb after electroporation of nAG plasmid at day 7 pa. The section was stained with antibodies to nAG (green). (C) Section of a nAG positive gland in the WE after electroporation of nAG plasmid into a denervated limb blastema at day 5 pa and analysis at day 17 pa. (D) Experimental design for assaying activity of nAG on the denervated blastema. Newts were denervated and amputated before electroporation on the denervated side with either vector or nAG plasmid DNA. (E) Representative animals at day 40 pa from the two groups of an experiment outlined in (D). The yellow star indicates the position of the initial denervation. Scale bars: (B) and (C), 250 μ m.



Discussion

Our identification of nAG as a ligand for the PD determinant Prod 1 has underlined that patterning and cell division are linked at the molecular level. We envisage that PD identity is manifested by the quantitative gradation of Prod 1 (6) and that nAG has no role in specifying that identity but rather acts through Prod 1 to promote cell division. Blastemal growth is stimulated in experimental confrontations of cells differing in positional identity—for example in PD intercalation, in which a wrist level blastema is grafted onto a shoulder stump (31, 32)—and this is always dependent on the presence of the nerve. In a recent study of supernumerary limb formation in the axolotl, the deflection of the brachial nerve into a skin wound provided a growth stimulus to form an ectopic blastema or “bump”; such bumps only progress to form limbs if a piece of skin is grafted from the

contralateral skin to the wound site so as to provide dermal fibroblasts of disparate identity (14). It is interesting that dermal fibroblasts express Prod 1 and that this expression is up-regulated by retinoic acid (6).

Two previous studies on AG proteins are relevant to the present results. First, the human AG2 protein was used as bait in a yeast two-hybrid assay and found to complex with a GPI-anchored protein called C4.4, which is associated with metastasis (24, 33). This protein has two Ly6-type domains that are related in sequence to urokinase-type plasminogen activator receptor (34). The degree of relatedness between the three-dimensional structures of Prod 1 and C4.4 domains is not yet resolved, but taken together these results suggest that functional interactions between AG proteins and this class of small Cys-rich protein domains may be conserved.

In the second study, it was found that over-expression of the XAG2 protein in early cleavage stage *Xenopus* embryos could induce formation of an ectopic cement gland that expressed XAG2 (22). We find that expression of nAG induces formation of nAG-positive glands in the denervated newt wound epidermis. After amputation, nAG appears first in the Schwann cells of the distal nerve sheath and then in glands in the wound epidermis. If axonal regeneration is prevented by denervation, neither the Schwann cell nor the glandular expression is detected. Our results suggest that nAG is released by the distal sheath and induces the formation of glands in the wound epidermis. It appears that the secreted nAG acts directly on the limb blastemal cells. It is unclear how the regenerating axons act on the sheath cells, although the membrane form of neuregulin is a candidate, in view of its importance for such interactions (35). The nerve

dependence of regeneration offers a distinction between limb development and regeneration, because the outgrowth of the limb bud is not dependent on its innervation (36). Nonetheless, the ingrowth of the nerve is critical for establishing the nerve dependence, as shown in elegant transplantation experiments on axolotl larvae (37). The identification of nAG offers a new opportunity to study the mechanisms underlying this switch.

Nerve dependence of regeneration is conserved in phylogeny. It has been studied in regeneration of the fish fin, the taste barbel in catfish, the arms of crinoid and asteroid species in echinoderms, and the body axis in annelids (28, 38). In most vertebrate appendages, the density of innervation is lower than in salamanders, and Singer suggested that this is a primary determinant for the loss of regenerative ability, for example in mammals (9, 39). This hypothesis now seems unlikely because there are other variables apparently curtailing regeneration (1). It is notable that the expression of a single protein can rescue limb regeneration in an adult animal (Fig. 4E), and this finding underlines that the blastema is an autonomous unit of organization for which there is no obvious mammalian counterpart. We have suggested that one approach for regenerative medicine would be to understand the specification of the blastema at a level of detail that would allow it to be engineered in mammals (1). The local delivery of permissive regulators such as nAG could then evoke formation of the appropriate structures without the need for subsequent intervention.

Fig. 5. Nerve and skeletal muscle are deficient in nAG-rescued limbs. Rescued limbs were analyzed along with the contralateral innervated limbs, generally at mid-radius/ulna level. Sections of innervated (A) and rescued (B) regenerate limbs were stained with antibody to acetylated tubulin. Sections of innervated (C) and rescued (D) limbs were stained with antibody to skeletal myosin to label muscle. M, muscle. Scale bars, 200 μ m.

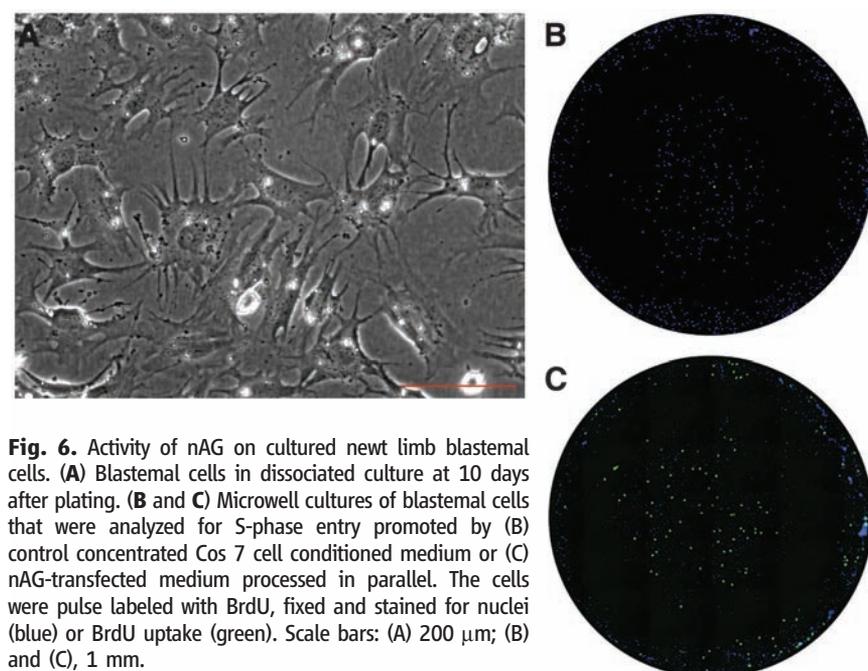
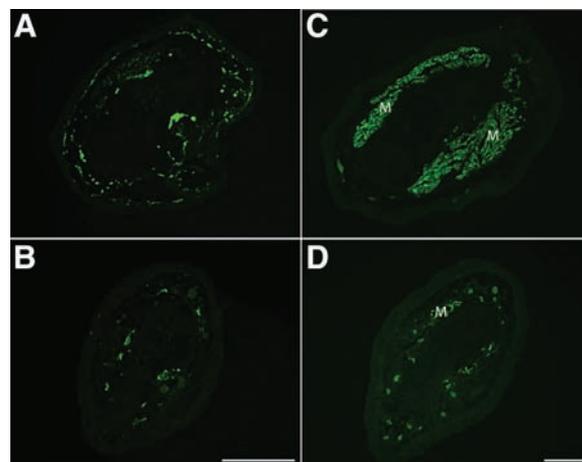


Fig. 6. Activity of nAG on cultured newt limb blastemal cells. (A) Blastemal cells in dissociated culture at 10 days after plating. (B and C) Microwell cultures of blastemal cells that were analyzed for S-phase entry promoted by (B) control concentrated Cos 7 cell conditioned medium or (C) nAG-transfected medium processed in parallel. The cells were pulse labeled with BrdU, fixed and stained for nuclei (blue) or BrdU uptake (green). Scale bars: (A) 200 μ m; (B) and (C), 1 mm.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/318/5851/772/DC1
 Materials and Methods
 Figs. S1 to S5
 Table S1
 References

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REPORTS

A Bright Millisecond Radio Burst of Extragalactic Origin

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Pulsar surveys offer a rare opportunity to monitor the radio sky for impulsive burst-like events with millisecond durations. We analyzed archival survey data and found a 30-jansky dispersed burst, less than 5 milliseconds in duration, located 3° from the Small Magellanic Cloud. The burst properties argue against a physical association with our Galaxy or the Small Magellanic Cloud. Current models for the free electron content in the universe imply that the burst is less than 1 gigaparsec distant. No further bursts were seen in 90 hours of additional observations, which implies that it was a singular event such as a supernova or coalescence of relativistic objects. Hundreds of similar events could occur every day and, if detected, could serve as cosmological probes.

Transient radio sources are difficult to detect, but they can potentially provide insights into a wide variety of astrophysical phenomena (1). Of particular interest is the detection of short radio bursts, no more than a few milliseconds in duration, that may be produced by exotic events at cosmological distances, such as merging neutron stars (2) or evaporating black holes (3). Pulsar surveys are currently among the few records of the sky with good sensitivity to radio bursts, and they have the necessary temporal and spectral resolution required to unambiguously discriminate between short-duration astrophysical bursts and terrestrial interference. Indeed, they have recently been successfully mined to detect a new galactic population of transients associated with rotating neutron stars (4). The burst we report here, however, has a substantially higher inferred energy output than this class and has not been observed to repeat. This burst therefore represents an entirely new phenomenon.

The burst was discovered during a search of archival data from a 1.4-GHz survey of the Magellanic Clouds (5) using the multibeam receiver on the 64-m Parkes Radio Telescope (6) in Australia. The survey consisted of 209 telescope pointings, each lasting 2.3 hours. During each pointing, the multibeam receiver collected independent signals from 13 different positions (beams) on the sky. The data from each beam were one-bit sampled every millisecond over 96 frequency channels spanning a band 288 MHz wide.

Radio signals from all celestial sources propagate through a cold ionized plasma of free electrons before reaching the telescope. The plasma, which exists within our Galaxy and in extragalactic space, has a refractive index that depends on frequency. As a result, any radio signal of astrophysical origin should exhibit a quadratic shift in its arrival time as a function of frequency, with the only unknown being the integrated column density of free electrons along the line of sight, known as the dispersion measure (DM). Full details of the data reduction procedure to account for this effect, and to search for individual dispersed bursts, are given in the supporting online material. In brief, for each beam, the effects of interstellar dispersion were minimized for 183 trial DMs in the range 0 to 500 cm⁻³ pc. The data were then searched for individual pulses with signal-to-noise (S/N)

ratios greater than 4 with the use of a matched filtering technique (7) optimized for pulse widths in the range 1 to 1000 ms. The burst was detected in data taken on 24 August 2001 with DM = 375 cm⁻³ pc contemporaneously in three neighboring beams (Fig. 1) and was located ~3° south of the center of the Small Magellanic Cloud (SMC).

The pulse exhibited the characteristic quadratic delay as a function of radio frequency (Fig. 2) expected from dispersion by a cold ionized plasma along the line of sight (8). Also evident was a significant evolution of pulse width across the observing frequency band. The behavior we observed, where the pulse width W scales with frequency f as $W \propto f^{-4.8 \pm 0.4}$, is consistent with pulse-width evolution due to interstellar scattering with a Kolmogorov power law [$W \propto f^{-4}$ (9)]. The filter-bank system has finite frequency and time resolution, which effectively sets an upper limit to the intrinsic pulse width $W_{\text{int}} = 5$ ms. We represent this below by the parameter $W_5 = W_{\text{int}}/5$ ms. Note that it is entirely possible that the intrinsic width could be much smaller than observed (i.e., $W_5 \ll 1$) and that the width we observe in Fig. 2 results from the combination of intergalactic scattering and our instrumentation.

We can estimate the flux density of the radio burst in two ways. For the strongest detection, which saturated the single-bit digitizer in the observing system, we make use of the fact that the integrating circuit that sets the mean levels and thresholds is analog. When exposed to a source of strength comparable to the system equivalent flux density, an absorption feature in the profile is induced that can be used to estimate the integrated burst energy. For a 5-ms burst, we estimated the peak flux to be 40 Jy (1 Jy $\equiv 10^{-26}$ W m⁻² Hz⁻¹). Using the detections from the neighboring beam positions, and the measured response of the multibeam system as a function of off-axis position (6), we determined the peak flux density to be at least 20 Jy. We therefore adopt a burst flux of 30 ± 10 Jy, which is consistent with our measurements, for the remaining discussion. Although we have only limited information on the flux density spectrum, as seen in Fig. 2,

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**Molecular Basis for the Nerve Dependence of Limb
Regeneration in an Adult Vertebrate**

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