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Short Contribution

Protein synthesis in the brain of newts undergoing limb regeneration

PANAGIOTIS A. TSONIS*, CHARLES H. WASHABAUGH and KATIA DEL RIO-TSONIS

Laboratory of Molecular Biology, Department of Biology, University of Dayton, Dayton, Ohio, USA

ABSTRACT The nervous system plays an important role during the process of amphibian limb regeneration. However, the molecules that are involved in such a control of regeneration are largely unknown. We have attempted to map protein synthesis in the brains of intact newts and from newts undergoing limb or tail regeneration. Our results show unique protein synthesis in the brain of newts undergoing limb regeneration. Such an analysis can lead to the identification and characterization of these proteins.

KEY WORDS: *nervous system, newt, limb regeneration*

Among amphibians the urodeles possess the unique capacity to regenerate, during adulthood, appendages that have been amputated. The first event manifested during regeneration is the covering of the wound by a specialized epithelium. The tissues at the amputation plane undergo dedifferentiation, forming a population of undifferentiated mesenchymal cells called blastema cells. The dedifferentiation aspects of limb regeneration have been shown by histological criteria (Hay, 1958; Hay and Fischman, 1961). Support has also come from molecular biology studies dealing with metaplasia (Casimir *et al.*, 1988). However, none of the experiments so far have ruled out the possibility of the existence of stem cells, nor has the existence of stem cells been proven in limb regeneration (for discussion on this issue see Ferretti and Brockes, 1991). The blastema cells proliferate under the influence of the nervous system for about two to three weeks, and subsequently redifferentiate to the various tissues that will reconstitute the lost part. Most of the limb structures will be replaced in about two months (Wallace, 1981; Tsonis, 1990, 1991).

The role of nerves in limb regeneration was first suggested by Todd in 1823. It was, however, the classical experiment by Singer (1952) which provided definite proof of the role of the nerve supply during blastema formation and subsequent regeneration. When stump denervation is performed, regeneration does not advance until new nerve fibers regrow. This suggested the existence of the so-called neurotrophic factor(s) essential for regeneration. Lebowitz and Singer (1970) subsequently showed that protein synthesis in the blastema is inhibited due to denervation and that it could be restored by infusion of nerve homogenates. Similarly, the inhibition of protein synthesis can be reversed by infusion of brain extracts, especially basic proteins, even though such an activity of brain extracts should not be regarded as neurotrophic (Singer *et al.*, 1976; Choo *et al.*, 1978). Inhibition of regeneration occurs upon hypophysectomy, suggesting that the pituitary gland might be involved in this process (Liversage and Globus, 1977; Liversage *et al.*, 1985).

Certain studies have been performed in order to elucidate the identity of the important protein(s) involved in nerve-dependent cell proliferation and regeneration (Tassava and Olsen, 1985). Related to this it has been noted that fibroblast growth factor infused in denervated blastemata normalizes mitotic activity (Mescher and Gospodarowitz, 1979). The role of FGF might be to promote dedifferentiation upon release from injured nerves, or it could be mitogenic (Mescher, 1983). Albert *et al.* (1987) and Boilly *et al.* (1991) have shown that FGF stimulates the proliferation of blastema cells *in vitro* and *in vivo*. Brockes (1984) and Brockes and Kintner (1986) have presented evidence that glial growth factor (GGF), which acts on Schwann cells, is present in the newt blastema and absent after denervation. In fact it was shown that when nerve-dependent blastema cells from denervated limbs are cultured in the presence of GGF, their mitotic index increases seven-fold. In other studies the presence of several neuropeptides has been investigated. Using radioimmunoassay and immunofluorescence staining techniques, it was shown that substance P is localized in the epidermis of the regenerating limb, especially in the outer layers. Substance P was also found to be mitogenic to the blastema cells. Of other peptides only neurotensin (a non-tachykinin peptide) showed weak staining in the basal layer. Bombesin, metenkephalin and the tachykinin family peptides eledoisin, kassinin, substance K, and neuromedin K were not detected (Globus and Alles, 1990). Mescher

Abbreviations used in this paper: 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; FGF, fibroblast growth factor; GGF, glial growth factor.

***Address for reprints**: Laboratory of Molecular Biology, Department of Biology, University of Dayton, Dayton, OH 45469-2320, USA. FAX: 1-513-229.4000.

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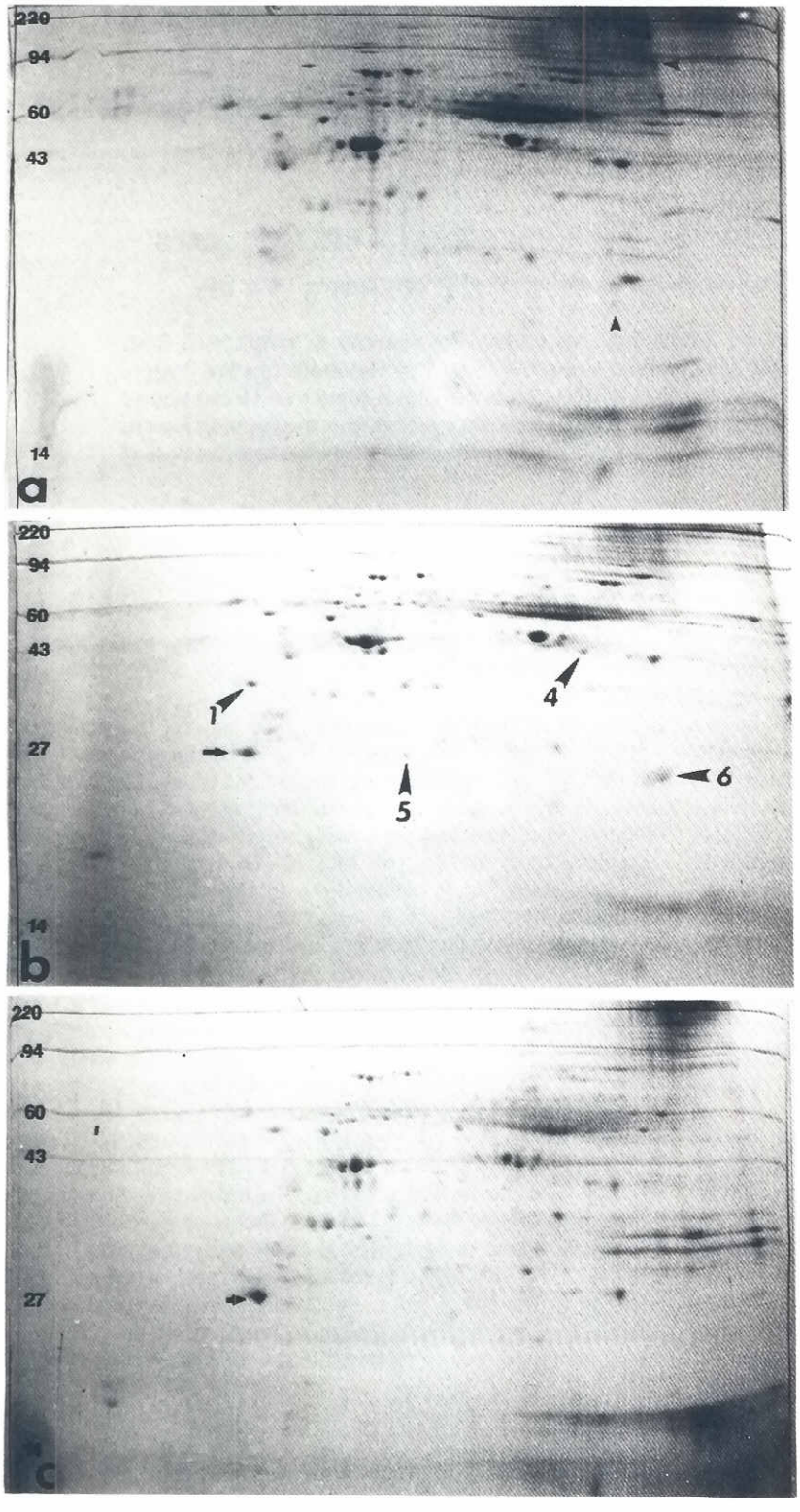

Fig. 1. 2-D PAGE of brain proteins (a) *from intact newts;* **(b)** *from newts undergoing limb regeneration;* **(c)** *from newts undergoing tail regeneration. The markers are: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000) and lysozyme (14,000). These standards appear as horizontal lines on the Coomassie Brilliant Blue R-250 stained gels. The 27kDa, pI 5.2 standard protein is shown by an arrow. In (a) the small arrowheads indicate two proteins found only in this case, brain from intact animals. In (b) the big arrowheads indicate the proteins 1, 4, 5 and 6 that were quantified (see Table 1) and show specific expression only in the brain of newts undergoing limb regeneration.*

and Munaim (1984) have demonstrated that one of the neurotrophic factors of peripheral nerves is transferrin, the iron transport protein. Addition of transferrin increases DNA labeling and mitotic index twice in cultured blastema. In other studies it has been shown by 2-D PAGE that a group of basic proteins ranging from 15-31KDa are expressed in ganglia. Protein synthesis increased 4-fold in the ganglia from newts with amputated limbs as compared with newts with unamputated limbs. New proteins were not detected in that

TABLE 1

QUANTITATION OF PROTEINS SHOWN IN FIG. 1

Spot#	Gel a	Gel b	Gel c
1	nd	7,151	nd
4	4,691	11,511	nd
5	nd	1,685	nd
6	31,830	18,485	18,159

Integrated density values of polypeptide spots resolved by 2-D electrophoresis (gel a, brain from intact newt; gel b and c, brain from newt undergoing limb and tail regeneration respectively. nd: not detected.

study, but it was suggested that amputation affects protein synthesis in the nerves quantitatively (Bao *et al.*, 1986). Increased protein synthesis in the whole spinal cord has been shown by Boilly and Scaps (1988).

Given the role of brain extracts in reversing the effects of denervation and the apparent lack of detailed studies on protein synthesis, we undertook the task of examining protein synthesis in the brain of newts undergoing limb or tail regeneration. It is important to know whether or not new proteins are synthesized in the brain due to the amputation and subsequent regeneration. Our results suggest that in contrast with protein synthesis in the ganglia, qualitative differences in protein synthesis can be observed in the brain of the newts undergoing limb regeneration versus brain from newts undergoing tail regeneration or with intact limbs.

The results of the 2-D electrophoresis are shown in Fig. 1. Fig. 1a presents protein synthesis in the brain of newts with intact limbs. Fig. 1b and 1c present protein synthesis in the brain of newts undergoing limb and tail regeneration respectively. Proteins showing some differences among the different gels were used for quantification (Table 1). From Fig. 1 and Table 1 several qualitative and quantitative differences can be observed. Two proteins indicated by large arrowheads are found only in brains from animals with regenerating limbs (Fig. 1b proteins numbered 1,5), while proteins pointed to by small arrowheads in Fig. 1a are present only in the brain of intact animals. Protein 4 shows a marked increase only in brain from animals with regenerating limbs, while protein 6 shows a decrease as a result of the repair in general (Table 1). This pattern of expression suggests that proteins 1 and 5 could be specifically synthesized due to the process of limb regeneration. Some other proteins that seem unique in Fig. 1a-c but are not marked in Fig. 1 indicate that these differences were not reproducible when different experiments were performed. In Table 1 we present the quantification data of the proteins under discussion (1, 4, 5 which show good reproducibility). Proteins with poor reproducibility could be unique to the individual animals that we used. Related to this, the qualitative and quantitative differences observed in the present study and marked in Fig. 1a-c are unlikely to be attributed to differences between individual newts because these differences were also observed when proteins were isolated from mixed population (pooled brain samples from a number of newts) or from individual newts of the same sex (females). Similarly, the differences are not likely to reflect variations in size since the selected newts were of approximately the same size. Each entire experiment always involved newts from the same population.

The qualitative differences are in clear contrast with previous studies on protein synthesis in the ganglia from newts with or without amputated limbs. The specific protein synthesis observed in our experiments is not restricted to basic proteins of low molecular weight, as in the case of the protein synthesis in the ganglia (Bao *et al.*, 1986). Rather, acidic and basic proteins of different spectrum of molecular weights were detected. Nevertheless, it should be noted that differential protein synthesis in the brain does not necessarily correlate to the blastema cell proliferation but it could be attributed to sensory deafferentation, which results from the amputation of the branchial nerves. For example, protein kinase C activity increases in the spinal cord but does not change in the brain after amputation of the newt limb (Oudkhir *et al.*, 1988; see also on deafferentation Pons *et al.*, 1991).

Our results demonstrate for the first time that specific protein synthesis might happen in the brain of newts due to regeneration. Given the role of brain extracts in stimulating the process of limb regeneration, our results could imply the presence or absence of these proteins in limb regeneration. Furthermore, this analysis could allow characterization of these proteins by microsequencing, making possible the isolation and study of these proteins during limb regeneration.

Experimental Procedures

Animals

Adult newts *Notophthalmus viridescens* used in this study were purchased from Amphibia of North America (C. Sullivan).

Isolation of brain proteins

Brains were isolated from intact newts (no amputation or trauma) and from newts with amputated limbs or tails two weeks after amputation. The newt brain is divided into four parts: 1) telencephalon, which contains the olfactory bulb, the cerebral hemispheres, the amygdala and the optic chiasma; 2) diencephalon, containing the epithalamus, thalamus and the hypothalamus; 3) mesencephalon, containing the tectum mesencephalis, the pituitary and the isthmus; 4) rhombencephalon, containing the cerebellum and the medulla oblongata which connects to the spinal cord. These anatomical features are very similar to the ones described for other species including the tiger salamander *Ambystoma tigrinum* (Herrick, 1948; Kokoros and Northcutt, 1977), *Ambystoma mexicanum* (Opdam and Nieuwenhuys, 1976), *Salamandra salamandra* (Francis, 1934) and *Necturus* (Chiasson, 1965). The tissues were placed in Osmotic Lysis Buffer (10mM Tris, pH 7.4 and 0.3% SDS).

The brain samples were freeze-thawed twice before 1/10 of 10X nuclease solution was added (50mM $MgCl_2$, 100mM Tris pH 7.0, 500μg/ml RNase from Sigma R5125 and 1000μg/ml DNase from Sigma D4527). The nucleases were allowed to react for 15 min. Next an equal amount of SDS boiling buffer (5% SDS, 5% beta-mercaptoethanol, 10% glycerol and 60 mM Tris pH 6.8) was added and the samples were boiled in a water bath for 15 min. The samples were then cooled, the undissolved material removed and stored in -70°C until use. All solutions were purchased from Kendrick Laboratories, Madison, WI. Protein determination was performed by taking an aliquot before the addition of the nuclease solution using the BCA total protein assay from Pierce Chemicals.

2-D electrophoresis

This was performed according to the method of O'Farrell (1975) as follows. Glass tubes of 2.0mm inside diameter were prefocused using 2.0% pH 4-8 ampholines (BDH Chemical, LTD Poole, England) for one hour at 300 volts and two hours at 600 volts with 30 μl of urea buffer. An equal amount of samples (150μg) was loaded and isoelectric focusing was carried out for 11.6 h at 700 volts. One μg of an IEF internal standard, vitamin D-dependent calcium binding protein, MW 27,000 and pI 5.2 was added to one of the

samples. The final tube gel pH gradient usually extended from pH 4.1 to pH 8.1 as measured by a surface pH electrode (BioRad) and colored acetylated cytochrome pI markers (Calbiochem-Behring, La Jolla, CA) run in an adjacent tube. After equilibration for 10 min in SDS sample (10% glycerol, 50mM dithiothreitol, 2.3% SDS and 62.5mM Tris pH 6.8) the tube gel was sealed to the top of a two-day aged and prerun 12.5% acrylamide slab gel (0.75 mm thick). To the agarose that sealed the tube gel to the slab gel MW standards (Sigma) were added. The slab gel had been prerun for about 2 h at 12.5 mA/gel with 0.07% thioglycolic acid in the upper chamber. After addition of the tube gels the slab gel electrophoresis was carried out for about four h at 12.5 mA/gel with 0.07% thioglycolic acid. The electrophoresis was stopped when the bromophenol blue dye front had reached the bottom of the slab gels. The gels were next placed in 0.1% Coomassie Brilliant Blue R250, 50% methanol for 10 min, then destained in two rinses of 50% methanol for 10 min and twice in distilled water for 5 min each, and they were air dried.

Quantification of proteins

The three Coomassie blue-stained gels (Fig. 1) were scanned over the optical density range of 0-0.25 OD units using an LGS 50 laser densitometer (Digital Instruments, Kemblesville, PA). The digitized images were transferred to an IBM system 60 computer where integrated density above background for each spot of interest was determined using QGEL software (Kendrick Laboratories, Madison, WI) as previously described (Burgess-Cassler *et al.*, 1989).

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