Relationship between innervation and forelimb regenerative capacity in the postmetamorphic pond frog *Rana brevipoda porosa*

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ABSTRACT The limb regenerative capacity and the quantity of innervation (the percentage of a cross-sectional area of amputation forelimb stump occupied by nerves) in the pond frog, *Rana brevipoda porosa*, was investigated in postmetamorphic froglets and adults of various sizes by means of amputating forelimbs through the zeugopodium. Nearly all the amputated limbs of newly metamorphosed froglets, 18-19 mm in snout-vent length, showed heteromorphic regeneration. However, the larger the body size, the lower the presence of limb regeneration. Limb regenerative capacity was completely lost in froglets and adults with snout-vents larger than 35 mm. The quantity of innervation of limbs was highest in newly metamorphosed froglets, gradually decreasing with growth. The nerve quantity in adults with a snout-vent length between 60-67 mm was approximately half that of the froglets. When the nerve supply was augmented by deviating ipsilateral sciatic nerve bundles to the forelimb stump, almost all limbs, which were usually non-regenerative with normal innervation, regenerated heteromorphically. These results show that the decline in limb regenerative capacity during postmetamorphic growth is in part attributable to the reduction in innervation levels to below the threshold level required for regeneration.

KEY WORDS: *limb regeneration, nerve, postmetamorphic growth, growth, amphibia*

It is well documented that, except in the case of aneurogenic limbs artificially produced in larval amphibians, nerve is essential for limb regeneration (Wallace, 1981). The absence of regeneration in anuran amphibia adults has been explained by the hypothesis that there is a direct relationship between the quantity of innervation at the amputation surface and the ability of the stump to exhibit regeneration, and that innervation in the limbs of frogs is quantitatively inadequate (Singer, 1954). For example, the regenerating limb of the newt, *Triturus*, has a greater quantity of innervation (expressed as area axoplasm of nerve per area of limb cross-section) than the non-regenerating limb of the ranid frog and the mouse (Rzehak and Singer, 1966; Singer *et al.*, 1967). The *Xenopus'* limb, which generally shows heteromorphic regeneration, has slightly less innervation than the limb of the *Triturus*, which regenerates a morphologically complete limb (Singer *et al.*, 1967). A direct relationship between the quantity of innervation and regenerative capacity was observed in a few other species of amphibia (Scadding, 1982, 1983). Further support came from studies in which regeneration was caused by deviating the sciatic nerve ipsilaterally into the forelimb stump. Such nerve-augmentation resulted in the occurrence of regeneration in non-regenerating limbs

of frogs and lizards. This was because the innervation threshold for limb regeneration was sufficiently supplemented to permit regeneration (Singer, 1954, 1961; Simpson, 1961; Kudokotsev, 1966). By contrast, in several other species, it has been reported that limb regenerative capacity was not related to the nerve density of the limb stump. Even though the forelimbs of adult *Rana ornativentris* usually show heteromorphic regeneration, their forelimbs have a low quantity of innervation, comparable to other non-regenerating limbs of anurans (Kurabuchi, 1990a) when compared to the data of Scadding (1982). Variations in limb regenerative capacity at different amputation levels cannot be explained by variation in the quantity of innervation (Van Stone, 1964; Scadding, 1982, 1983; Kurabuchi, 1990a,b). No correlation between the quantity of innervation and presence of limb regeneration could be established in tadpoles during metamorphosis (Van Stone, 1964; Kurabuchi, 1990b). There was no correlation between innervation and the rate or extent of limb regeneration in various sizes even in the same species (Scadding, 1983).

Thus, the relationship between limb regenerative capacity and the quantity of innervation is not clear. This is because the method used in estimating the amount of nerve tissue occupying a unit area

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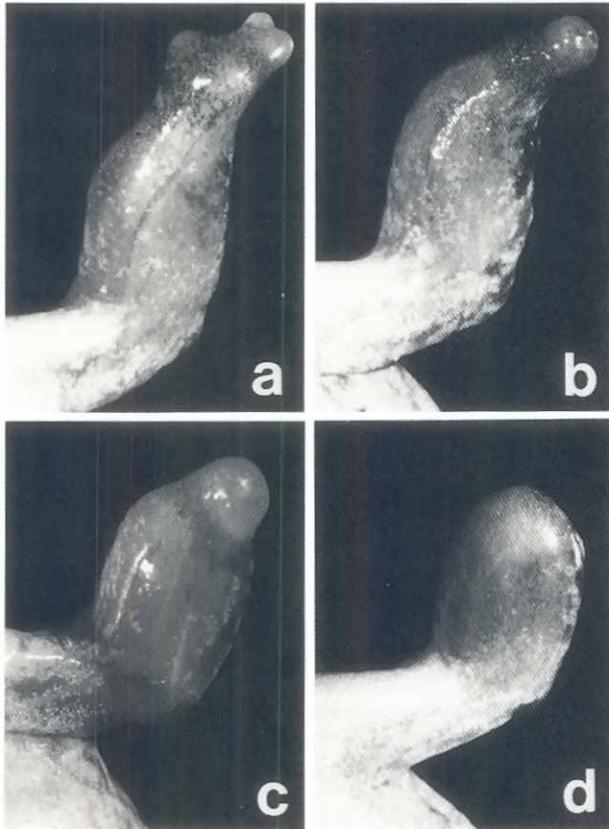
Fig. 1. Ventral view of heteromorphic regenerates obtained in the nerve-augmented forelimb (a) and normally innervated ones (b, c and d), three months after amputation. (a) *Regenerative outgrowth which is slightly flattened, possessing three points at the edge.* (b) *Rod-shaped outgrowth which tapers distally.* (c) *Cone-shaped outgrowth, of which proximo-distal length is shorter than the diameter of the limb stump.* (d) *Non-regenerating limb. x5.*

of limb stump will not always represent the physiological action of the nerve. We currently lack a method for measuring the amount of the neurotrophic factor. As discussed in the author's previous reports (Kurabuchi, 1990a,b), when restricted to the same species, at the same amputation limb level and under the same experimental conditions, the measurement of the quantity of innervation can provide useful information. The current investigation was designed to test whether or not there is a direct relationship between the quantity of innervation and the limb regenerative capacity in froglets and adults of varying sizes/ages in the pond frog, *Rana brevipoda porosa.* Their forelimb regenerative capacity had been previously examined (Kurabuchi and Inoue, 1982a); the youngest froglets regenerated their limbs well, while the rate of occurrence of limb regeneration decreased with postmetamorphic growth, with the limb becoming non-regenerative in adults.

The results of regeneration of normally innervated forelimbs amputated at the zeugopodial level are summarized in Table 1. The forelimbs of young froglets with a snout-vent length of less than 19 mm, which were the ones soon after metamorphosis, yielded a rod-shaped outgrowth in all cases examined. When the body grew larger, the amputated limbs yielded rod- or cone-shaped outgrowths, but some were non-regenerative (Fig. 1). In the froglets having snout-vent lengths between 20-35 mm, the incidence of regeneration decreased from 86% in those with snout-vent lengths between 20-24 mm to 20% in those between 30-34 mm. In froglets and adults with a snout-vent length larger than 35 mm no signs of regeneration

TABLE 1

THE REGENERATIVE CAPACITY OF FORELIMB STUMPS OF POSTMETAMORPHIC FROGLETS AND ADULTS OF VARIOUS SIZES

Snout-vent Length (mm)	No. Limbs Examined	Occurrence of Regeneration No. Limbs(%)	Type of Regenerate
Normally innervated limbs			
18-19	16	16(100)	rod
20-24	14	12(86)	rod & cone
25-29	11	4(36)	rod & cone
30-34	10	2(20)	rod & cone
35-39	10	0(0)	–
40-44[a]	11	0(0)	–
45-49[a]	11	0(0)	–
45-49[a]	5	0(0)	–
50-54[a]	5	0(0)	–
55-59[a]	8	0(0)	–
60-64[a]	7	0(0)	–
65-67[a]	5	0(0)	–
Nerve-augmented limbs			
37-39	3	3(100)	digitoid & rod
40-42[a]	6	6(100)	digitoid, rod & cone
46-49[a]	4	4(100)	rod & cone
52-53[a]	5	5(100)	rod & cone
56[a]	5	5(100)	rod & cone

The number in brackets is the percentage.
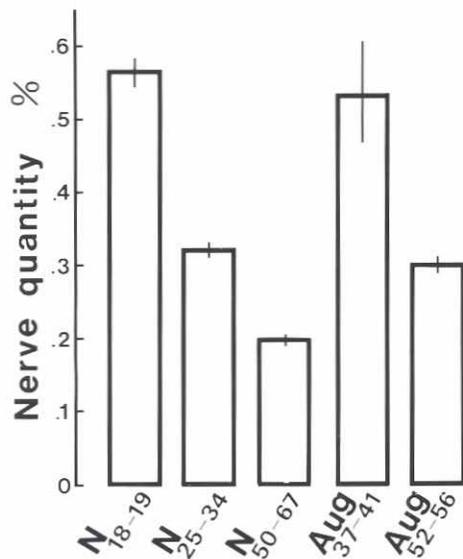[a]Adult body size (Maeda and Matsui, 1989).

Fig. 2. Comparison of the nerve quantity between three groups of the normally innervated limbs (N) and two groups of the nerve-augmented limbs (Aug). *Numbers attached to the groups «N» and «Aug» represent snout-vent length of froglets and adults. Values are expressed as mean± SEM calculated with number of cases given in Table 2.*

were detected in almost all cases examined. Rod-shaped outgrowths appeared frequently in younger froglets and cone-shaped ones in larger froglets. As shown in Table 2, the area of limb cross-section and the area of nerves observed within it gradually increased with body growth. Comparing 60-67 mm long adults and 18-19 mm long froglets, the area of the cross-sectional limb was approximately 20 times larger in the former and the total area of nerves was approximately 5 times larger. The area of limb increased faster than that of nerve. Thus, as shown in Table 2, the relative quantity of innervation of the 18-19 mm long froglets was calculated as having a mean of 0.56±0.02%, with the value falling rapidly until the snout-vent length reached 30 mm, then falling more gradually with further growth. In the case of 60-67 mm long adults, the mean was 0.15±0.02%. The threshold level of innervation required for regeneration seems to range between 25 to 29 mm of snout-vent length, at which the quantity of innervation was approximately 0.35%. In many anurans, the limb regenerative capacity is either lost completely soon after metamorphosis or declines gradually during postmetamorphic growth until adult age/size is reached (Kurabuchi and Inoue, 1982a; Kurabuchi *et al.,* 1985; Kurabuchi, 1990b, c). Also, as with urodeles (Peadon and Singer, 1965; Scadding, 1983), the nerve quantity decreased with increasing age in *Rana brevipoda porosa,* such that this reduction in relative innervation may be a consistent feature of postmetamorphic growth. When in 23 froglets and adults innervation was augmented by the sciatic nerve being ipsilaterally diverted into the forelimb amputation stump, regenerative outgrowths developed in all forelimbs examined (Table 1). These regenerative outgrowths were mainly rod- and cone-shaped, with some furnished with several digitoid protuberances, whose gross-structure resembled the developing forelimb anlage in appearance (Fig. 1). Such morphologically improved outgrowths were occasionally seen in nerve-deviated froglets measuring less than 40 mm in length. This type was not encountered in the regeneration of the normally innervated limbs. In a cross-section of the limb, 1 to 4

bundles of the re-routed sciatic nerve were mostly seen underneath the skin or in the muscle tissue at the dorsal aspect of the forelimb. The nerve quantity of the 23 nerve-augmented limbs was increased

TABLE 2

CROSS-SECTIONAL STUMP AREA, TOTAL NERVE AREA AND NERVE QUANTITY IN THE FORELIMBS OF POSTMETAMORPHIC FROGLETS AND ADULTS OF VARIOUS SIZES

Snout-vent Length(mm)	Cross-sectional area of limb(mm^2)	Total nerve area(10^{-2}mm^2)	Nerve quantity (%)
Normally innervated limbs[b]			
18-19	1.56±0.07	8.70±0.01	0.56±0.02
20-24	1.96±0.22	7.90±0.03	0.42±0.03
25-29	3.44±0.26	11.84±0.07	0.33±0.01
30-34	5.24±0.28	16.42±0.12	0.31±0.02
35-39	7.86±0.35	23.36±0.11	0.29±0.02
40-44[a]	8.46±0.34	22.46±0.15	0.26±0.02
45-49[a]	11.38±1.08	22.54±0.08	0.20±0.02
50-54[a]	16.10±1.48	33.96±0.76	0.20±0.03
55-59[a]	14.96±1.72	29.28±0.36	0.18±0.01
60-67[a]	28.68±5.69	41.78±0.64	0.15±0.02
Nerve-augmented limbs[c]			
37-39	8.41±0.11	37.77±0.71	0.45±0.09
40-42[a]	9.04±0.22	39.73±0.43	0.48±0.06
46-49[a]	12.42±1.71	47.03±0.76	0.38±0.02
52-53[a]	14.21±0.56	40.28±0.41	0.28±0.02
56[a]	20.48±0.81	66.96±0.28	0.33±0.01

[a]Adult body size (Maeda and Matsui, 1989).
[b]Values expressed as mean±SEM calculated with five cases randomly selected.
[c]Values expressed as mean±SEM calculated with number of cases given in Table 1.

(Table 2) and calculated as having a mean of 0.38±0.02%, a value almost equal to the level of innervation of normal limbs of the 25-29 mm long froglets. When the innervation was supplemented by re-routed sciatic nerves, regeneration occurred in normally non-regenerating limbs, a phenomenon also seen in the other frogs (Singer, 1954; Kurabuchi, 1990c) and lizards (Simpson, 1961; Singer, 1961; Kudokotsev, 1962). From the current experiments, it is certain that the decline in regenerative capacity can be in part attributed to the reduction in forelimb innervation to below a threshold level.

Furthermore, divided into groups as shown in Fig. 2, the quantity of innervation of the nerve-augmented limbs of 37-41 mm long froglets and adults, which yielded the highest frequency of morphologically improved outgrowths, was almost the same as that of normally innervated limbs of 19-20 mm long froglets, which usually showed regeneration, being significantly higher ($P<0.001$) than those of normally innervated limbs of 25-34 mm long froglets, which occasionally regenerated and 50-67 mm long adults, which was non-regenerative. However, the quantity of innervation of the nerve-augmented limbs of 50-56 mm long adults, even when regeneration occurred in all cases examined, was almost the same as that of normally innervated limbs of 25-34 mm long froglets, which only occasionally regenerated, being significantly higher ($P<0.05$) than that of normally innervated limbs of 50-67 mm long adults, but, significantly lower ($P<0.001$) than that of normally innervated limbs of 18-19 mm long froglets. Conceivably, the artificial augmentation of nerve supply at the limb stump seems not only to increase the nerve quantity but also to provide the additional function of allowing regeneration. It has been considered that the nerves at the amputation stump sprout branches to convey the nerve influence to all regions of the regenerate (Singer and Egloff, 1949), and that the non-nervous tissues need to possess the competence to respond to the neurotrophic factors produced by nerve (Rzehak and Singer, 1966). On the other hand, it has been found that heteromorphic regeneration can be induced by several means unrelated to nerves; electrical stimulation (Smith, 1967; Borgens et al., 1977), NaCl treatment, and traumatization with needles (Polezhaev, 1945; Rose, 1945; Kurabuchi and Inoue, 1982b). The damage done during the operation of deviating sciatic nerve into the forelimb may promote a physiological action of nerve or the response of non-nervous tissues responding to neurotrophic factors. Unfortunately, the neurotrophic factors thought to be introduced from the nervous system have not yet been identified, as there are currently no methods for measuring either the amount of trophic factor or the competence of tissues responding to nerve.

Experimental Procedures

Tadpoles, froglets and adults of the pond frog, Rana brevipoda porosa were collected in the vicinity of Maebashi City in Japan. Tadpoles were fed boiled spinach and raised to metamorphosis in the laboratory, and then, various sizes of the postmetamorphic froglets and adults 18 mm to 67 mm in snout-vent length were prepared for the current experiments. Maeda and Matsui (1989) have stated in their picture book that the body length of adult frogs is at least 39 mm in the male and 43 mm in the female. After being anesthetized in 0.1% MS 222 (Sandoz), the forelimbs of 112 frogs were amputated unilaterally at the mid-point between the wrist and elbow (zeugopodium). In addition, the nerve supply at the distal forelimb of 23 adults, 45-58 mm in length, was augmented according to the method devised by Singer (1954); the sciatic nerve was deviated into the forelimb.

This procedure was performed about one month prior to the amputation of the limbs. The frogs were classified according to body size at the time of amputation of the limb and kept in separate containers with a small amount of water and maintained at 22-24°C on a 12L/12D photocycle. They were fed crickets twice a week for three months, after which they were examined for signs of limb regeneration.

For measurement of the quantity of innervation, the forelimbs excised as above were fixed in Bouin's solution. After decalcification, a 10 μm thick paraplast cross-section of the forelimb was made as close as possible to the amputation surface, and the sections were stained for nerve fibres using the silver nitrate method of the modified Bodian's method by Otsuka (1962). The resulting cross-sectional area of the limbs and nerves were then measured by video image processor (Olympus, VIP-25); with the image displayed on a TV. The outlines of the skin of the cross-sectional limb and nerves observed in it were traced by digital pen. The quantity of innervation was then expressed as the percentage of cross-sectional area of limb occupied by nerves.

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