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Nerves innervate the ectopic limbs

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In this communication, we report the innervation of the ectopic hindlimbs formed as a result of homeotic transformation mediated by vitamin A in the Indian jumping frog, *Polypedates maculatus* (Anura: Rhacophoridae). Histology and nerve staining revealed that the ectopic limbs are innervated.

IT is well known that vitamin A causes severe embryonic malformations in several animals ever since their discovery in 1909 (refs 1–3). However, its effect is more pronounced in amphibians causing proximodistal, anteroposterior and dorsoventral duplication of parts of the limbs during limb regeneration in several species of amphibians[4–14]. But the most remarkable of all the effects of vitamin A is the homeotic transformation of tails to limbs in *Uperodon systoma*[15]. Since then several workers have confirmed similar phenomenon in other species of amphibians[16–19]. As many as 8–9 ectopic limbs arise from the tail tissue. The limbs arise either singly or in pairs. Most of the ectopic limbs have the normal hindlimb structure but none of them is functional. The tadpoles dragged the extra limbs and died as a result of overgrowth. As the ectopic limbs were functionless, it was possible that they were devoid of nerves. The present study was therefore undertaken to ascertain whether the ectopic limbs of *P. maculatus* were supplied with nerves.

The egg mass of *P. maculatus* was collected from Utkal University campus in July, 1995 and reared in the laboratory up to the hindlimb bud stage following the standardized procedure of Mohanty-Hejmadi[20]. Prior to amputation in the middle of the tail, the tadpoles were anaesthetized in MS 222 (tricaine methanesulphonate) and exposed to vitamin A 10 IU/ml for 72 h. Following the above treatment they were transferred to aerated, conditioned water and allowed to grow till the emergence of ectopic limbs. Throughout the experiment they were fed with boiled egg and *Amaranthus ad libitum*. Once the ectopic limbs had developed and the forelimbs emerged, the tadpoles were fixed in 10% buffered formalin.

For histological studies of the tail region of tadpoles with ectopic limbs, the tails of those tadpoles who possessed ectopic limbs (Figure 1 *a*) were amputated and fixed in aqueous Bouin's fluid, embedded in paraffin (m.p. 58°C–60°C), sectioned longitudinally at 10 µm thickness and stained with Mallory's triple stain for examination under the light microscope.

Interestingly, histological studies revealed that multiple sections of the nerve cord were found in the extreme distal and ventral regions of the tail (Figure 1 *b*). As the limbs always arise from the ventral side, it is quite likely that the nerve cord which is located on the dorsal side, takes several turns in the distal region of the tail to reach the ventral side, perhaps to supply the ectopic limbs. As a result, multiple sections of the nerve cord were visible on the ventral side in the distal region of the tail.

To be more sure that the ectopic limbs were innervated, Sihler's differential nerve staining technique[21] was used on the tadpoles with ectopic limbs. In both the tadpoles (Figure 1 *c*, *d*) a thin, faint nerve could be discerned in the ectopic limbs. The continuity of the nerves could not be tracked due to the shrinking of the specimens during fixation. On the other hand, the nerves innervating the normal hindlimbs were thick and prominent (Figure 1 *c*) and were therefore easily distinguishable.

The present study therefore confirms that, because of sparse and weak innervation the ectopic limbs are not fully functional, although morphologically they are well-developed hindlimbs. They are richly vascularized too as revealed from histology (unpublished data). Singer[22] transplanted the upper arm segments of the adult *Notophthalmus viridescens*, in the flank region of the same animal and found that a few of the grafts regenerated after some delay. The arms which had regenerated contained an average less than one-third of the normal density of nerve fibres. A similar fraction of the total nerve supply can induce the formation of supernumerary limbs when diverted to a surface wound. Thornton and Tassava[23] also recorded regeneration in orthotypically transplanted arms of *Ambystoma mexicanum* larvae which were kept sparsely innervated by repeated denervation. On the other hand, if the hind brain and trunk nerve cord are excised when they first form a neural tube, a defective embryo develops which survives and develops normally but is incapable of normal movements or feeding. The arms develop quite well with either very few or no detectable nerve fibres and can reach the four digit stage in this species[22]. This might be the

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Figure 1. *a*, Supernumerary ectopic limbs in the tail amputated tadpoles of *P. maculatus* following exposure to vitamin A 10 IU/ml for 72 h. The ectopic limbs were fully developed after 35–40 days of the initial amputation (× 10). *b*, Longitudinal section of vitamin A treated tail showing multiple sections of the nerve cord (C) on the ventral side (V) of the distal region of the tail. The nerve cord, supposed to be on the dorsal side is seen here taking a turn from the dorsal side to the ventral side (× 100). *c*, Sihler's differential nerve preparation shows a thin nerve (marked by arrowhead) in the single ectopic limb of *P. maculatus*. The normal hind limb shows a thick and distinct nerve (marked by arrow) (× 11). *d*, Sihler's differential nerve preparation shows a thin nerve (marked by arrowhead) in the paired ectopic limbs of *P. maculatus* (× 11).

reason why the ectopic limbs formed in *P. maculatus* in the present study though seemed normal, may not be fully functional because they are sparsely and scantily innervated.

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