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Retinoic acid affects the organization of reticulospinal neurons in developing *Xenopus*

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The effects of all-trans retinoic acid (RA) on the differentiation of the reticulospinal system were studied in *Xenopus*. RA was applied in concentrations of 10^{-5} and 10^{-6} M for 30 min at stage 12. When siblings had reached stages 46–48, the spinal cord was transected in anesthetized control and experimental animals and the reticulospinal cells were visualized through retrograde transport of fluorescing dextran amines. The lower concentration of RA led in many animals (22%) to the formation of multiple Mauthner-like cells. Higher concentrations resulted in the formation of two uninterrupted longitudinal columns of rather uniform reticulospinal cells. These data suggest that the normal expression of *Hox* genes pattern – known to be altered by RA – may be necessary for the differential specification of compartments of the reticulospinal system.

The hindbrain of many developing vertebrates forms a series of transverse folds, called rhombomeres, in which branchial and somatic motor neurons specific for a given cranial nerve are generated [10]. The rostral boundaries of rhombomeres coincide often with rostral limitations of *Hox* gene domains [6]. This suggests that *Hox* genes may play a role for the segmental identity of a given rhombomere [7]. Retinoic acid (RA) modulates *Hox*-gene expression in mice [8] and *Xenopus* [16] and causes in *Xenopus* an anterior truncation with an upregulation of *Hox*-gene expression in more anterior areas of the mesoderm and the neuroectoderm [18], resembling to some extent craniofacial malformations in mutants with disrupted *Hox* genes [1, 9].

Besides the motor neurons the hindbrain contains in addition discrete clusters of cells which project either crossed or uncrossed to the spinal cord in all craniate vertebrates: the reticulospinal system [12, 20]. Two of the reticulospinal cells can even be individually identified in most anamniotic vertebrates, the two giant Mauthner cells [3, 4] (Fig. 1). Mauthner cells are characterized in *Xenopus* by a large, contralaterally projecting axon, a position immediately caudal to the VIIIth nerve root, and by three lateral and two medial dendrites [15].

Transplantation of forebrain tissue suggests that development of the medulla and Mauthner cells depends on local regulating factors [13]. Numerical regulation of a single Mauthner cell per hindbrain side is precise and deviates in about 2% of normally developing *Xenopus* [21]. We have therefore used RA in doses previously shown to affect the differentiation of neural tissue [2, 11] and alter the expression of developmental genes [16, 18] to test whether RA treatment affects the development of the reticulospinal system.

A total of 196 *Xenopus laevis* embryos were treated at stage 12 [14] with 10^{-6} or 10^{-5} M RA for 30 min as described elsewhere [11]. When controls were at stages 46–48, the RA-treated experimental and 10 control animals were anesthetized in tricaine methansulfonate and either rhodamine dextran amine (RDA) or fluorescein dextran amine (FDA) was applied to the transected rostral spinal cord [5]. After a transport time of 6 h the animals were anesthetized and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected, mounted flat on a slide and examined and photographed with an epifluorescent microscope using the appropriate filter sets [5].

Incubation with RA causes anterior-dorsal truncation that is more pronounced with higher concentrations [2, 11, 16–19]. The brains are characterized by a progressive fusion and truncation of anterior and dorsal parts. In the

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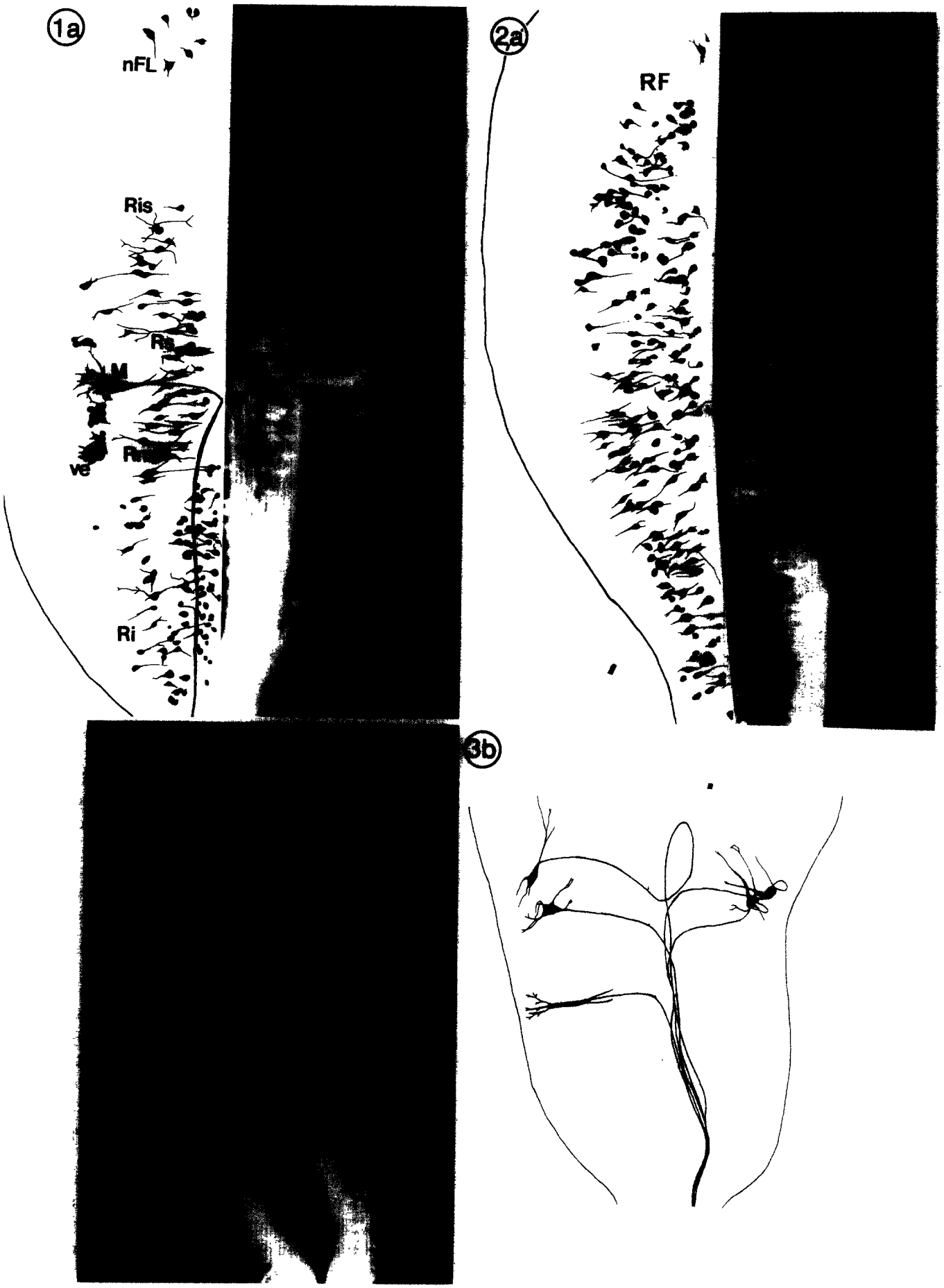
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most extreme cases, the brain consists of a single club-like structure with only an epithelial, choroid plexus-like differentiation of the dorsal parts. This 'brain' is reminiscent of the 'hindbrain' described by Durston et al. [2] but resembles also the neural plate stage of *Xenopus*. Rhombomeres are suppressed at all concentrations of RA as reported elsewhere [16].

Cells which by virtue of their size and lateral position look like Mauthner cells are found in peculiar numbers in RA-treated animals (Figs. 1 and 3). More than one and up to three of these Mauthner-like cells may exist on either side in 22% of the 10^{-6} M RA treated animals (n=104). However, 66% of these animals have only a single Mauthner-like cell labelled or none at all and about 12% display two Mauthner cells per hindbrain. These Mauthner-like cells of RA-treated animals can be identified by their size, but neither their position in the rostrocaudal nor in the medio-lateral direction is constant (Fig. 3). Sometimes these cells look more like anterior and posterior repeats but in other animals they are clustered. We found a noticeable difference between the left and the right side in any given animal, i.e. the transformations were never symmetrical (Fig. 3).

The axons of these cells show in many cases highly unusual pathways. Sometimes they curve first anterior and then loop around before projecting into the spinal cord. Other axons do not cross the midline (Fig. 3). In animals treated with higher concentrations of RA (10^{-5} M) we could not identify in most cases a Mauthner-like cell at all (Fig. 2). Nevertheless, about 6% of all these cases (n=92) had three or more Mauthner-like cells.

Other than these effects on the Mauthner cells there is a consistent reduction of the degree of differentiation of the reticulospinal formation with increased concentrations of RA. Normally, these cells form three rhombencephalic nuclei, one isthmal and one mesencephalic nucleus [20] (Fig. 1). In addition, there is a lateral group of vestibulospinal neurons (ve; Fig. 1). The rhombencephalic and mesencephalic groups are separated by a noticeable gap which harbors the interpeduncular nucleus. In many animals treated with 10^{-5} M RA this gap disappears completely (Fig. 2). Only a uniform population of reticulospinal cells extends uninterruptedly from the spinal cord to the rostral pole of the brain. In contrast to a recent report [16], our data do not indicate any obvious numerical reduction (Figs. 1 and 2). This discrepancy may be due to the different stages (9 compared to 12) or the different RA concentrations used in the two studies. The reduction of the gap between the rhombencephalic and mesencephalic cells is also apparent in some animals treated with 10^{-6} M RA as is the increased cellular uniformity within the reticulospinal formation and the absence of the vestibulospinal cells. It should be emphasized that the effects were not identical for the reticulospinal and vestibulospinal cells: only vestibulospinal neurons disappeared completely in some cases (Fig. 2).

These data show a clear-cut effect of RA on the degree of differentiation of the reticulospinal formation. The variable expression of additional Mauthner-like cells or their complete deletion suggests that their appearance may be critically dependent on information normally specifically expressed only in one rhombomere. This information is apparently even able to transform transplanted forebrain cells into a Mauthner-like cell [13]. A likely source for the distorted information may be the RA-induced alteration of Hox codes at a given rhombomere [6, 8, 16, 18].

Although many 10^{-5} M RA cases have the appearance of an 'all hindbrain' as suggested earlier [2] this appears to come about by a differential suppression of the interpeduncular nucleus. RA may cause respecification of the more caudal cells of the interpeduncular nucleus [8], thus transforming many or all cells of this nucleus to project to the spinal cord. Consistent with this hypothesis is the recent finding of a single rather than two stripes of XKrox-20 in the anterior hindbrain [16]. However, the different effects on reticulospinal and vestibulospinal cells may indicate a more complex disturbance of the specifying factors and not only a simple posteriorization of hindbrain areas.

In summary, the massive effects of RA on the pattern and differentiation of the vestibulo- and reticulospinal system reported here are consistent with the hypothesis that selective expression of developmental genes may be

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Fig. 1a,b. Photomicrograph (b) of one half of a whole mounted brain of a control *Xenopus laevis* (stage 48) tadpole and a camera lucida drawing of the adjacent half (a). Note the prominent labeling of the Mauthner cells (M), the vestibulospinal cells (ve), the inferior (Ri), medial (Rm), superior (Rs) and isthmic (Ris) reticular nuclei and the interstitial nucleus of the longitudinal fascicle (nFL). I, interpeduncular nucleus. Magnification, ×105

Fig. 2a,b. Photomicrograph (b) and camera lucida drawing (a) of a flat mounted brain of a tadpole treated with 10^{-5} M RA for 30 min at stage 12. Notice the uniform appearance of virtually all neurons of the reticular formation (RF) labelled from the spinal cord without formation of any distinct cluster or any Mauthner-like cell. No vestibulospinal cells are recognizable. Magnification, ×149

Fig. 3a,b. This photomicrograph (a) and this camera lucida drawing (b) show the distribution pattern of reticulospinal and vestibulospinal neurons in a tadpole treated at stage 12 for 30 min with 10^{-6} M RA. Notice the presence of three large, Mauthner-like cells on the left side and two large, Mauthner-like cells on the right side. On the left side these cells are closely aggregated and their axons do not cross, on the right side they are

crucial for the patterning of specific areas of the hindbrain [7, 16].

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