Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7194–7197, July 1994
Neurobiology

Hot spots of retinoic acid synthesis in the developing spinal cord

(retinaldehyde dehydrogenase/limb innervation/morphogenesis/pattern formation)

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Communicated by David H. Hubel, April 21, 1994

ABSTRACT **The embryonic spinal cord is known to be rich in retinoic acid, and several indirect lines of evidence point to a dorsoventral concentration difference of this compound. Previous measurements of dorsoventral retinoic acid levels, however, showed only minor differences. By a combination of microdissection and bioassay techniques, we compared retinoic acid levels with retinaldehyde dehydrogenase levels along spinal cords from early embryonic to postnatal mice. Both parameters vary in parallel, indicating that the principal reason for regional retinoic acid differences in the developing spinal cord is different levels of retinoic acid-generating enzyme. Consistent with previous reports, we observed overall quite high synthesis, decreasing with age, and no dorsoventral difference throughout much of the spinal cord length. In two locations, however, ventral synthesis exceeds dorsal synthesis by several orders of magnitude. These hot spots colocalize with the origins of the limb innervations. They are highest during early stages of limb innervation and disappear slowly postnatally. The synthesis hot spots are likely to create local retinoic acid diffusion halos, which may influence the survival of neurons in the limb regions of the spinal cord and which probably promote innervation of the developing limbs.**

Positional information in the spinal cord, as in other organs, is believed to be established by two separate mechanisms acting in the early embryo—one along the anteroposterior and the other along the dorsoventral axis. A component in the establishment of anteroposterior specificity, whose existence is apparent in the innervation patterns from spinal cord segments inverted in this axis (1), is a nested expression mode of sequential homeobox genes (2, 3). Teratogenic retinoic acid doses alter the homeobox expression boundaries, causing an anteroposterior shift in positional identity (4) and pointing to a physiological role of retinoic acid in anteroposterior patterning. Indirect evidence implicates retinoic acid also in dorsoventral patterning, as several observations indicate higher retinoic acid levels in ventral than in dorsal spinal cord (5–9). Measurements, however, revealed only 1.5-fold higher ventral retinoic acid values (10) or even indicated higher dorsal values (11). Moreover, a proposed comprehensive mechanism for dorsoventral specification does not involve retinoic acid (12–14). Here we show that in two restricted locations ventral retinoic acid synthesis exceeds dorsal synthesis by several orders of magnitude. The distribution and timing of these synthesis hot spots are consistent with a role in limb innervation rather than general dorsoventral specification, a process completed considerably earlier (14).

Retinoic acid exerts its actions through activation of nuclear receptors, which regulate transcription of target genes by binding to retinoic acid-responsive elements in their promoter regions (15, 16). The spatial and temporal expression in the developing embryo of different retinoic acid receptors, as well as retinoid binding proteins, points to a widespread and highly specific involvement of this system in morphogenetic patterning (7, 17). Much less is known about synthesis of retinoic acid in the embryo but several studies show regional differences in retinoic acid levels (11, 18–21). How these differences come about is not clear. It is possible that retinoic acid is distributed systemically and that regional differences are created by localized breakdown activities or by localized isomerization processes that convert all-*trans*-retinoic acid into forms with different physiological effectiveness (22, 23). Alternatively, retinoic acid synthesis may take place at restricted locations.

MATERIALS AND METHODS

Retinoic acid measurements shown here were not aimed at absolute values, but they represent internal comparisons of equivalent tissue samples processed in parallel. For retinoic acid detection we used a reporter cell line, teratocarcinoma cells transfected with a sensitive retinoic acid response element driving β-galactosidase expression (10). The dose–response characteristics of these cells are illustrated in Fig. 1. The cells are ≈100-fold more sensitive to all-*trans*-retinoic acid than to the 9- and 13-*cis* isomers. They respond in a linear fashion, by giving double colorimetric readings to a 10-fold retinoic acid increase at higher retinoic acid concentrations and with a more shallow response slope at lower concentrations. Above and below the linear range the response levels out, and at 1 μM retinoic acid and higher it decreases. If retinoic acid levels to be compared vary by several orders of magnitude, as is the case for the measurements described here, they can easily exceed the linear detection range. For this reason, all comparisons shown here were validated by serial dilutions, and the dilution step giving the best dynamic range was taken as the one centered on the linear range of the cell-response capacity.

The trunk regions or spinal cords from a whole litter (younger ages) or part of a litter (older ages) of embryonic and postnatal outbred mice were dissected out in tissue culture medium, lined up in the dish to bring out parallel anatomic landmarks, and cut, as best as possible at equivalent sites, into 8, 12, or 16 consecutive portions. Pooled portions, roughly adjusted for similar volumes, were cultured overnight in 100 μl of L15 medium. The tissue was homogenized and assayed for protein levels with the Micro BCA protein assay reagent kit (Pierce), following the manufacturer's instructions. Retinoic acid amounts released into the culture medium were measured in volumes adjusted for equal tissue protein. The tissue samples were then normalized for protein and processed by a zymography assay to visualize retinoic acid synthesizing enzymes (19). This assay involves separation of native proteins by isoelectric focusing and tests of proteins eluted from gel slices for retinoic acid-generating capacity from 50 nM retinaldehyde with the reporter cells. The enzyme activity mea-

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Abbreviations: DiAsp10, 4-(4-didecylaminostyryl)-*N*-methylpyridinium iodide; E9, embryonic day 9; P1, postnatal day 1.

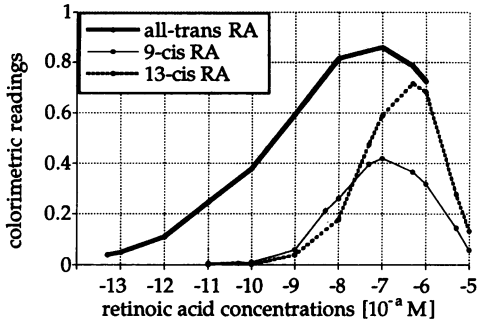

FIG. 1. Response characteristics of the retinoic acid reporter cells to all-*trans*-retinoic acid as described (10) and to 9-*cis*- and 13-*cis*-retinoic acid. The retinoic acid isomers (a gift from Hoffmann–La Roche) were stored in the dark under nitrogen at −80°C, and all handling was carried out under dim yellow light. The illustrated dose–response curves represent a combination of duplicates processed in parallel.

surements were repeated with different fractions of total protein in order to obtain an optimal comparison range.

Pooling of samples, which is likely to smooth out sharp anatomical differences due to the impossibility of cutting at exactly the same places, was necessary for the retinoic acid release assays. The zymography assay, which is at least 10 times more sensitive, can be done on fractions of samples from a single embryo.

For anatomic tracings of the limb innervations, embryos of different ages were fixed in 4% paraformaldehyde, the limbs were cut off, and lipophilic carbocyanine dyes (Molecular Probes), dissolved in dimethyl sulfoxide, were applied to the stumps. Best results were obtained with 4-(4-didecylaminostyryl)-*N*-methylpyridinium iodide (DiAsp10) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). After about a week, the spinal cords were dissected free for inspection and photography.

RESULTS

To characterize the role of retinaldehyde-oxidizing dehydrogenases (19, 24), we undertook a comparison of retinoic acid released from live tissue with dehydrogenase levels in the same samples from a homogeneous tissue, the neural tube from hindbrain to the caudal end of the spinal cord. The trunk regions (neural tubes and surrounding tissues) from a litter of embryonic day 9 (E9) mice, from about the level of the otic vesicle downward, were dissected out and cut into eight consecutive portions, and retinoic acid amounts released into the culture medium were measured (Fig. 2 *Upper*, bar graph). The same tissue samples were then processed by a zymography assay to visualize retinoic acid synthesizing enzyme (19) (Fig. 2 *Upper*, line graph). Similar measurements are illustrated for E10.9 (Fig. 2 *Lower*). These comparisons show good concordance: retinaldehyde dehydrogenase activity levels quantitatively account for released retinoic acid amounts. At both ages no retinoic acid synthesis can be measured in the hindbrain regions and levels are highest in the rostral trunk approximately adjacent to the forelimb bud. In addition, the E10.9 distribution shows a second, caudal maximum. Separate tests indicate that these earliest measurements combine two retinoic acid-generating components, one in the neural tube and the other in the mesoderm (data not shown), as a clean separation by dissection was not practical for the assays presented here. For all later ages, only the isolated spinal cords, dissected free from meninges and dorsal root ganglia, were analyzed.

The development of the two synthesis maxima over the following 22 days was mapped as released retinoic acid amounts in spinal cords dissected into 16 consecutive sam-

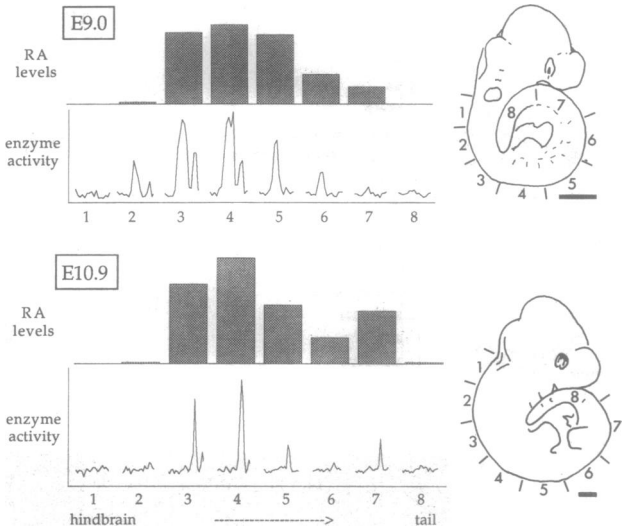

FIG. 2. Comparisons of released retinoic acid amounts and enzyme activities in eight consecutive trunk samples, containing spinal cords and surrounding mesoderm, from litters of E9 (*Upper*) and E10.9 (*Lower*) mice. Approximate locations of the samples in the embryos are indicated. Measurements were done as simultaneous comparisons for each age separately. (Bars = 500 μm.)

ples (Fig. 3*A*). At E13 the rostral maximum exceeds the caudal one, but by E17 and in the newborn (P1; postnatal day 1) the caudal maxima predominate; retinoic acid levels between the maxima are several hundredfold lower. At P13 the retinoic acid level profile is almost even, revealing at best remnants of the caudal maximum. A distribution of retinoic acid synthesis similar to E13 mice was seen in spinal cords of E6 chickens (data not shown). To probe the topography of the maxima, embryos of different ages were fixed, the limbs were cut off, and the stumps were labeled with lipophilic dyes that distribute along neuronal membranes (25). A fluorescent view of an E12.8 spinal cord, labeled with DiAsp10, reveals two bright clusters (Fig. 3*B Left*), the origins of limb innervations. These colocalize with the retinoic acid maxima in samples 6, 7, and 12–14 in the E13 release assay (Fig. 3*A*). The same correlation is evident without tracings in the newborn, where the maxima colocalize with bulges known to represent the origins of limb innervations (Fig. 3*B Right*). While the rostral maximum centers onto the forelimb swelling, the caudal maximum centers to the caudal two-thirds of the hindlimb swelling in the newborn. Comparisons of spinal cords from older pups make it likely that this represents the region of maximal growth at this age. The same growth argument probably accounts for the third maximum at the caudal tip of the spinal cord.

As for the younger ages, released retinoic acid amounts and dehydrogenase activity levels continue to correlate in the isolated spinal cords at least up to P1, the latest age tested. This is shown here for E14.5 (Fig. 4*A*). Apart from the anteroposterior variations in retinoic acid production at any given age, overall synthesis levels decrease substantially with ongoing development. To visualize this temporal decline, the approximate sites of the rostral maxima were dissected from spinal cords of different ages, and the samples, normalized for protein content, were processed for a comparison of retinoic acid-generating enzyme activities: levels decreased by several orders of magnitude (Fig. 4*B*).

As mentioned above, previous comparisons of retinoic acid levels along the dorsoventral axis showed only slightly higher levels in ventral cord; reported values, however, varied substantially between single experiments (10). As we wondered whether this variation could reflect different proximities to the maxima, we tested dorsal and ventral halves of

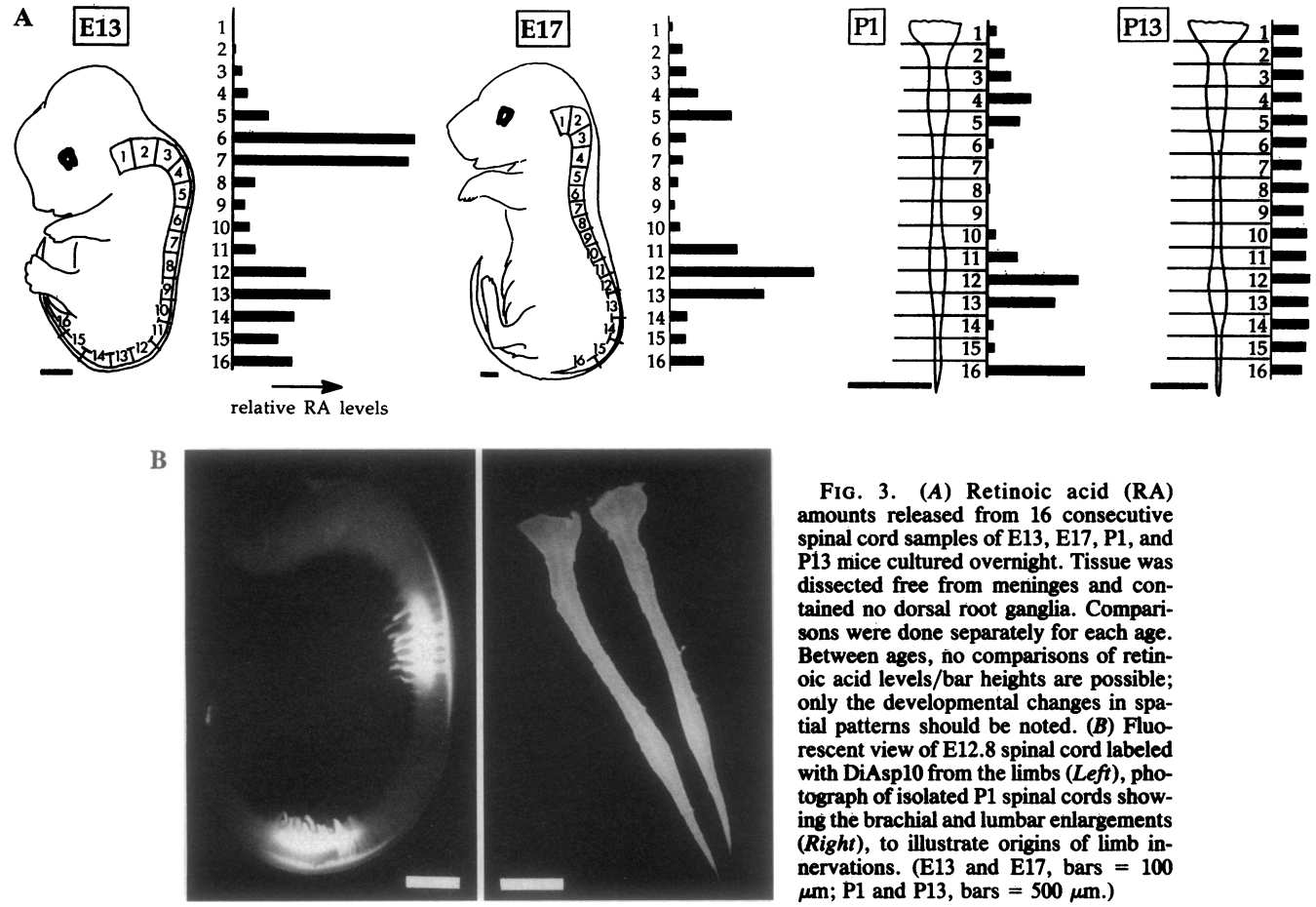

FIG. 3. (*A*) Retinoic acid (RA) amounts released from 16 consecutive spinal cord samples of E13, E17, P1, and P13 mice cultured overnight. Tissue was dissected free from meninges and contained no dorsal root ganglia. Comparisons were done separately for each age. Between ages, no comparisons of retinoic acid levels/bar heights are possible; only the developmental changes in spatial patterns should be noted. (*B*) Fluorescent view of E12.8 spinal cord labeled with DiAsp10 from the limbs (*Left*), photograph of isolated P1 spinal cords showing the brachial and lumbar enlargements (*Right*), to illustrate origins of limb innervations. (E13 and E17, bars = 100 μm; P1 and P13, bars = 500 μm.)

E13 spinal cord pieces dissected from the two maxima and from the intermediate region. The intermediate samples showed identical synthesis levels. Within the maxima, dorsal synthesis was similar to those at intermediate levels but ventral synthesis completely saturated the reporter-cell response. To illustrate this difference, protein amounts were reduced to levels that barely show the dorsal and intermediate enzyme activities (for E16, see Fig. 5); the ventral response still spans almost the entire range of the cell-response capacity, indicating differences that probably exceed 3 orders of magnitude. A similar ventrodorsal difference is apparent for retinoic acid amounts released from cultured live tissue (data not shown). For a better spatial resolution, spinal cord pieces from E13 rostral maxima were dissected into dorsal, lateral, and ventral wall samples, with cuts approximately passing through the anterior and posterior horns, and assayed for dehydrogenase levels (data not shown). Activity in the lateral wall samples was similar to that in the ventral samples, excluding a restriction of high enzyme levels to the floor plate and localizing them to the ventral third of the spinal cord.

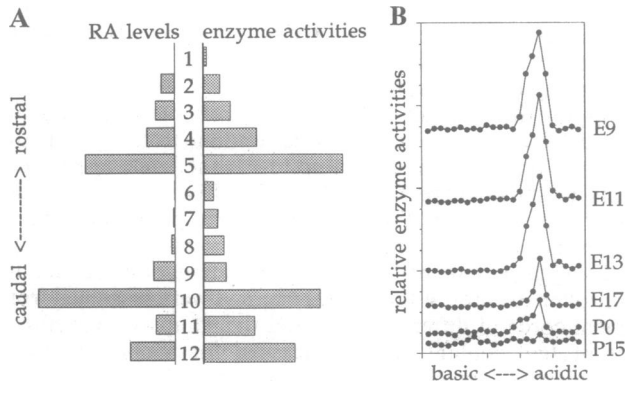

FIG. 4. (*A*) Comparison of released retinoic acid (RA) amounts and dehydrogenase activity levels in 12 consecutive spinal cord samples at E14.5. Bar heights for enzyme activities represent integrated areas under activity peaks from zymographs. (*B*) Age comparison of samples of spinal cord approximately representing the rostral maxima. Samples were normalized for protein and assayed in parallel for enzyme activity levels by zymography.

DISCUSSION

With a combined microdissection/bioassay technique we have shown here for the embryonic hindbrain/spinal cord that locally released retinoic acid amounts and levels of retinoic

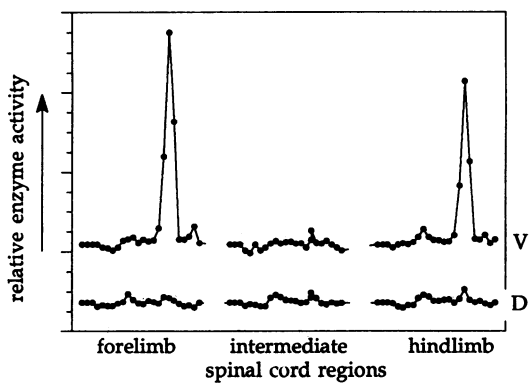

FIG. 5. Zymography bioassays for retinoic acid-generating enzyme activity in dorsal (D) and ventral (V) halves of pieces dissected from the rostral and caudal maxima and the intervening stretch of one E16 spinal cord. Only one-third of the homogenized volumes were tested here in order to bring out the difference between the samples.

acid-generating dehydrogenase correlate well, at least within the limits of the detection system. This is validated by the observation that the same detection system reveals pronounced anomalies at certain other locations—e.g., the adult anterior pituitary lobe contains very high enzyme levels but appears to release very little retinoic acid, a discrepancy that could reflect low precursor levels, high breakdown, or conversion of all-*trans*-retinoic acid into a form to which the reporter cells are less responsive, such as 9-*cis*-retinoic acid (unpublished data). Nevertheless, we found indications for regional differences in retinoic acid breakdown in the embryonic hindbrain/spinal cord, but they have only minor effects on the massive differences due to localized synthesis. For instance, breakdown activity in the early hindbrain is much higher than in the spinal cord (data not shown); as the hindbrain at this age has no detectable dehydrogenase activity, this difference only accentuates the synthesis pattern. Our observations indicate (*i*) the principal reason for local retinoic acid differences in the embryonic spinal cord is different levels of retinaldehyde dehydrogenase; and (*ii*) if part of the retinoic acid in the spinal cord is present in forms other than all-*trans*-retinoic acid, the gross anatomical distribution of isomerization activity cannot be very different from the distribution of retinaldehyde dehydrogenase.

Although the measured dehydrogenase activities account quantitatively for retinoic acid released from the dissected tissue pieces, the described patterns are certain to differ from the retinoic acid distribution *in vivo*, because the lipid retinoic acid diffuses through the tissue relatively unimpeded by cellular boundaries (10). The synthesis hot spots will create retinoic acid halos in the surrounding tissue, which in our experiments were largely washed out during the lengthy dissections. Such diffusion gradients are indicated by the apparently much broader reaction zones in mice transgenic for a retinoic acid response element–*lacZ* construct similar to the one used for the reporter cells (10, 11, 26, 27); these mice show the appearance of a reaction gap at mid-spinal cord levels, but they do not show the ventral retinoic acid origin. On the contrary, ventrodorsal comparisons of *lacZ* reactions in one strain of the transgenic mice, as well as in normal spinal cords cocultured with retinoic acid reporter cells, gave higher intensities for dorsal than for ventral regions (11). To test whether the transgenic *lacZ* pattern might be explained by retinoic acid synthesis in the tissue surrounding the spinal cord, which had been dissected away, pia/meningeal samples from different regions were assayed; retinoic acid synthesis here seems to be rather even (data not shown). When culture supernatants from dorsal and ventral spinal cord pieces from the maxima, with pia left in place, were assayed with the reporter cells, the undiluted medium gave consistently higher readings for dorsal than for ventral samples, and only the dilution series revealed that ventral retinoic acid levels are in fact much higher (data not shown). This indicates that retinoic acid synthesis in ventral spinal cord may reach levels that turn off the reporter construct (see Fig. 1).

The observation of highly localized sources of retinoic acid invokes the importance of specific and varying retinoic acid response thresholds for transcriptional regulation, as described for sequential members of genes in homeobox clusters, which are activated by graded retinoic acid concentrations (28). The hot spots appear with formation of the limbs and persist until limb innervation is about complete. This is clearly too late for a role in general dorsoventral patterning of the spinal cord (14). However, as retinoic acid is known to substantially increase neuron survival and axon outgrowth in spinal cord cultures (29–31), the hot spots are a likely factor in the formation of the limb zones (32), by rescuing relatively more neurons in these regions from morphogenetic cell death and by stimulating neurite outgrowth into the developing limbs.

We thank M. Wagner and T. Jessell for the retinoic acid reporter cells. This work was supported by Grant R01 EY01938 from the National Eye Institute and by a gift from Johnson & Johnson.

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