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Segment-specific pattern of sympathetic preganglionic projections in the chicken embryo spinal cord is altered by retinoids

(pattern formation/segmentation/RA)

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ABSTRACT **Sympathetic preganglionic neurons exhibit segment-specific projections. Preganglionic neurons located in rostral spinal segments project rostrally within the sympathetic chain, those located in caudal spinal segments project caudally, and those in midthoracic segments project either rostrally or caudally in segmentally graded proportions. Moreover, rostrally and caudally projecting preganglionic neurons are skewed toward the rostral and caudal regions, respectively, of each midthoracic segment. The mechanisms that establish these segment-specific projections are unknown. Here we show that experimental manipulation of retinoid signaling in the chicken embryo alters the segment-specific pattern of sympathetic preganglionic projections and that this effect is mediated by the somitic mesoderm. Application of exogenous retinoic acid to a single rostral thoracic somite decreases the number of rostrally projecting preganglionic neurons at that level. Conversely, disrupting endogenous synthesis of retinoic acid in a single caudal thoracic somite increases the number of rostrally projecting preganglionic neurons at that level. The number of caudally projecting neurons does not change in either case, indicating that the effect is specific for rostrally projecting preganglionic neurons. These results indicate that the sizes of the rostrally and caudally projecting populations may be independently regulated by different factors. Opposing gradients of such factors along the longitudinal axis of the thoracic region of the embryo could be sufficient, in combination, to determine the segment-specific identity of preganglionic projections.**

The role of retinoids in neural development has become the focus of intense investigation since the discovery that application of exogenous retinoic acid (RA) to vertebrate embryos can alter the longitudinal patterning of the central nervous system (1–3). In the spinal cord of avians and mammals, such patterning is most evident in the thoracic region. Here, the preganglionic sympathetic neurons exhibit segmental and intrasegmental patterns of differentiation with respect to their projection pathways in the sympathetic chain. In each segment, there is a segregation of the preganglionic neurons that project rostrally versus caudally in the chain, and the proportions and distributions of the two populations of neurons vary systematically from segment to segment (4). We now show that manipulation of retinoid signaling in the thoracic region alters selectively the number of rostrally projecting neurons, suggesting a mechanism by which retinoids can influence the segment-specific pattern of sympathetic projections.

We have targeted our manipulations to the somites, the segmental blocks of mesodermal tissue that lie along side the spinal neural tube, because several lines of evidence indicate that inductive interactions between the somites and the neural tube are involved in establishing segmental patterns of differentiation in the spinal cord. For example, zebrafish bearing a mesoderm-autonomous mutation that prevents the normal segmentation of the somitic mesoderm fail to develop the normal segmental patterns of neuronal differentiation in the spinal cord (5). In the chicken embryo, shifting the segmental relationship between the thoracic neural tube and the somites by heterotopic transplantation causes preganglionic neurons to project aberrantly (6). To determine whether retinoid signaling may mediate interactions between somites and the spinal neural tube, we have applied RA or inhibitors of RA synthesis to individual developing somites.

MATERIALS AND METHODS

Exogenous Application of Retinoic Acid or Inhibitors of its Synthesis. White Leghorn chicken eggs (Oliver Merrill and Sons, Londonderry, NH) were incubated at 37°C for 55–75 hours. Embryos were exposed by cutting a small window into the shell, and RA or inhibitors of its synthesis were applied to individual somites by using a slow-release bead delivery system (7) (Fig. 1*a*). A single anion exchange resin bead soaked in 20 μg/ml RA, 10^{-4}M citral, or 10^{-6}M disulfiram in dimethyl sulfoxide (DMSO) was placed at the second or sixth thoracic level (T2 or T6) *in ovo* shortly after somite formation at that level. Citral and disulfiram each inhibit RA synthesis; citral is a competitive inhibitor of the aldehyde dehydrogenase mediated oxidation of either retinol or retinal to RA (8, 9) whereas disulfiram interferes with the activity of the aldehyde dehydrogenase by forming mixed disulfide bonds with essential sulfhydryl groups in the enzyme (10). Two other compounds were used to substantiate that the effects of citral and disulfiram were related to their ability to inhibit RA synthesis. For citral (3, 7 dimethyl 2, 6 octadienal), we used as a control the closely related nitrile (3, 7 dimethyl 2, 6 octadienitrile, 10^{-4}M), which does not act as a competitive inhibitor for retinal. For disulfiram, we used as a control diethyldithiocarbamate (10^{-6}M), a metabolite of disulfiram that has little effect on aldehyde dehydrogenases *in vitro* (11). Control beads for all experiments were soaked in the vehicle, DMSO. For application at T2, beads were placed on somites 20 or 21 in 20–23 somite embryos. For application at T6, beads were placed on somites 25 or 26 in 25–28 somite embryos. Beads (AG1-X2, 200–400 mesh, Bio-Rad) were 50–100 μm in diameter and thus smaller than an individual somite. Beads releasing RA

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Abbreviations: BrdU, bromodeoxyuridine; DMSO, dimethyl sulfoxide; E10, embryonic day 10; T2, thoracic segment 2; T6, thoracic segment 6; RA, retinoic acid; RAR, retinoic acid receptor.

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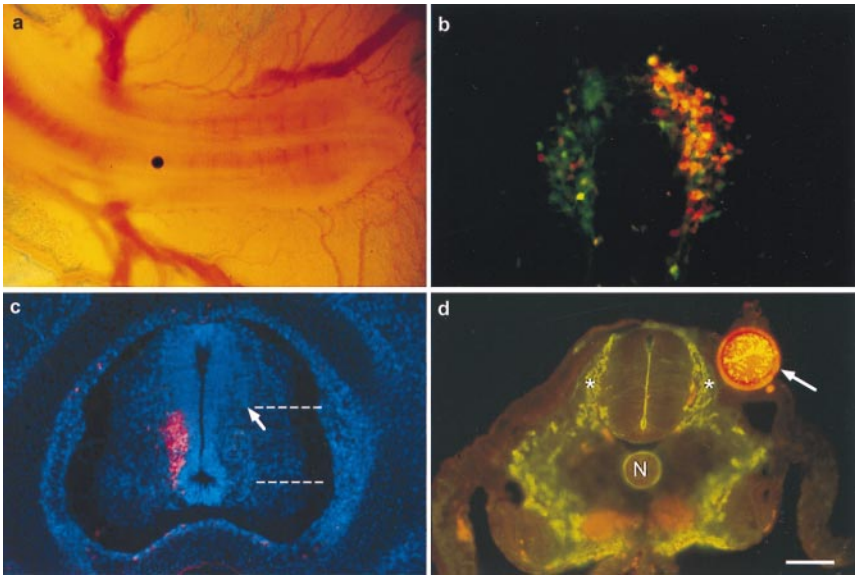

FIG. 1. Experimental paradigm. (*a*) This *in ovo* photograph shows a bead in place on a somite 24 hours after bead placement (caudal is to the right). (*b*) Retrograde labeling of preganglionic neurons in T6 at E10. Preganglionic neurons that project rostrally along the sympathetic chain are labeled red, and those that project caudally are labeled green. This 30-μm section is taken from an experimental animal in which the left side is the control and the right side was treated with disulfiram. (*c*) Location of T6 sympathetic preganglionic neurons at E7. Sympathetic preganglionic neurons on the left side were labeled retrogradely (red) from the T6 sympathetic chain; sections were stained with Hoechst dye to label all of the nuclei. Counts of BrdU-labeled cells were made from a restricted region of the spinal cord equivalent to the area containing preganglionic neurons. The dorsoventral extent of the counted region extends from the bottom of the central canal to the point at which the ventricular neuroepithelium fans out laterally (marked by dotted lines). The mediolateral extent of the counted region extended from the edge of the ventricular epithelium to the most lateral extent of the ventricular epithelium (arrow). To determine the percentage of nuclei in this area that represent preganglionic neurons, nuclei and retrogradely labeled preganglionic neurons were counted in this restricted region in four sections such as these from five different preparations. (*d*) Development of the somites and migration of neural crest and formation of sympathetic ganglia were apparently normal after all treatments. Here, migrating neural crest (green fluorescence) is shown at somite 26 20 hours after treatment with a citral-containing bead (arrow). Coalescing dorsal root ganglia are marked by asterisks. *n* = notochord. (Calibration: *a* and *b*, 200 μm; *c*, 100 μm; *d*, 50 μm.)

were placed at T2 whereas beads releasing either citral or disulfiram were placed at T6. In either case, the beads were placed on the somites shortly after their formation so that the relative stage of differentiation of the treated somites was similar. Bead placement occurred before the start of cell proliferation within the preganglionic cell column (12).

Retrograde Labeling of Sympathetic Preganglionic Neurons. The effect of the manipulations on the segmental patterning of the sympathetic preganglionic neurons was assessed by mapping the numbers and distributions of rostrally and caudally projecting neurons in the same segments on embryonic day 10 (E10), after the projections had developed (Fig. 1*b*). E10 embryos were killed, and the sympathetic preganglionic projections were labeled retrogradely *in vitro* with fluorescently tagged dextran amines as described (4). Because of occasional inaccuracy in somite counting in the older embryos, some of the caudally placed beads were found at T5 or T7, instead of T6. In these cases, the retrograde labeling was performed at T5 or T7, respectively. No skeletal abnormalities were apparent in any of the DMSO, RA, citral, nitrile, or diethyldithiocarbamate-treated embryos; two of eight disulfiram-treated embryos exhibited a fork in the distal aspect of the vertebral rib at the treated segment.

Immunohistochemical Detection of Migrating Neural Crest. To assess whether exogenous addition of RA or inhibitors of its synthesis disrupted neural crest migration and/or sympathetic ganglion formation, tissue sections were stained with an antibody to the neural crest marker, HNK1 (Beckton Dickinson; 1:100 in PBS containing 10% normal goat serum and 0.3% Triton X-100) (13). A fluoresceinated secondary antibody was used to visualize the primary antibody (Chemicon goat anti-mouse IgM; 1:50 in the same diluent).

Detection of Cell Proliferation with Bromodeoxyuridine. To determine whether retinoid manipulations altered the proliferation of preganglionic neuron precursors, cell proliferation was assayed by immunohistochemical detection of exogenously applied bromodeoxyuridine (BrdU; Sigma) (14). BrdU is incorporated into cells during the S-phase of the cell cycle. Citral-treated anion exchange beads were applied unilaterally to T6 as described above. DMSO-treated anion exchange beads were placed on the corresponding contralateral somites to control for possible local concentration of the BrdU by the anion exchange bead. Subsequently (8–16 hours later), 20 μl of BrdU (5.5 μg/ml) was pipetted onto the embryos at T6. The number of labeled cells was assayed at E7, after preganglionic cell division was over (12) but before the period of programmed cell death in the preganglionic cell column (15). Embryos in which both beads were found at T6 were processed for BrdU detection in paraffin sections. Transverse sections (6–11 μm) were permeated with 6 M HCl before treatment with a mAb to BrdU (Beckton Dickinson; 1:50 in PBS containing 4% BSA, 5% nonfat powdered milk, and 10% normal goat serum) followed by visualization by using a biotinylated secondary antibody (Vector Laboratories; anti-mouse IgG, 1:50 in the same diluent) and Vectastain ABC kit (Vector Laboratories). BrdU-containing cell profiles were counted on every fifth section in the region of the presumptive Column of Terni (see Fig. 1*c*). This delimited region encompasses the extent of retrogradely labeled preganglionic neurons after tracer application to the sympathetic chain at E7 (Fig. 1*c*) (16). Counts of retrogradely labeled T6 preganglionic neurons and of all cell nuclei (stained with Hoechst 33342, Molecular Probes) indicate that at least 58% of cells within this region at E7 are preganglionic neurons (57.7 $\pm$ 2.2%; 20 sections from each of 5 different embryos; Fig. 1*c*).

Detection of Cell Death. To determine whether retinoid manipulations alter the pattern of cell death in the spinal cord, the number of cells exhibiting DNA fragmentation was determined in T6 after citral treatment at Stage 15 (17). Citral-treated anion exchange beads were applied unilaterally to T6

as described above, and embryos were returned to the incubator until they reached Stage 18. In embryos in which the bead was recovered at T6, cells exhibiting DNA fragmentation were visualized in 8-μm serial sections by using Oncor's Apoptag kit according to manufacturer's instructions. Cells were counted in the ventral pyknotic zone as described by Homma *et al.* (18).

Data Analysis. The numbers of retrogradely labeled neurons, BrdU-labeled cell profiles, or cell profiles exhibiting DNA fragmentation in the treated hemisegment were compared with those in the contralateral control hemisegment. Because only relative counts were made, no correction for counting split cells was performed. Because of relatively small sample sizes in some cases, statistical significance was assessed by nonparametric analysis (Mann–Whitney Rank Sum test for grouped data and Wilcoxon Signed Rank Sum test for paired data) by using SIGMASTAT software (Jandel, San Rafael, CA). Significance was defined as $P \leq 0.05$.

RESULTS

Somitic treatment with RA or inhibitors of its synthesis significantly alters pattern formation in the preganglionic cell column of the adjacent neural tube. Normally, T2 is populated predominantly by rostrally projecting preganglionic neurons (>95%) whereas T6 contains only a minority of such neurons (<20%) (4). This difference reflects a progressive segmental shift in the proportion of rostrally and caudally projecting preganglionic neurons observed as one moves from rostral to caudal thoracic segments (4). Manipulations of retinoid signaling selectively altered the number of rostrally projecting preganglionic neurons compared with control (untreated or vehicle-treated). Treatment of the T2 somite with RA decreased significantly the number of rostrally projecting neurons without changing the number of caudally projecting neurons (Table 1, Fig. 2*a* and *b*). This observation suggested that the progressive rostral to caudal segmental decrease in the number of rostrally projecting preganglionic neurons in the normal embryo might be related inversely to segmental differences in the availability of RA. According to this premise, low endogenous levels of RA in rostral thoracic segments would be correlated with a large number of rostrally projecting preganglionic neurons whereas progressively higher levels in more caudal segments would be correlated with progressively fewer rostrally projecting neurons. We therefore tested whether inhibition of RA synthesis in T6, which normally contains relatively few rostrally projecting neurons, would increase their number. Indeed, treatment of the T6 somite with citral increased significantly the number of rostrally projecting neurons without changing the number of caudally projecting neurons (Table 1; Fig. 2 *c* and *d*). Treatment with disulfiram gave results similar to those seen with citral (Fig. 1*b*; Table 1). Treatment with nitrile, diethyldithiocarbamate, or DMSO had no significant effect on preganglionic neurons (Table 1).

In these experiments, there are several potential sources of variability. Those associated with manipulation of retinoid signaling per se include the amount of RA sequestered by the bead, the metabolic stability of RA, the rate and time course of release, and the response to the changed retinoid levels. In addition, there are sources of variability not associated with experimental manipulation, including the efficiency of the retrograde labeling, small differences in staging at bead application and retrograde labeling, and interanimal and intersegmental variability in the size of preganglionic populations. Not surprisingly, the data in Table 1 exhibit large variability. In Fig. 2, we have attempted to eliminate (at least partially) the variability not associated with the manipulation of retinoid signaling by normalizing to the average number of labeled neurons (both rostrally and caudally projecting) on the control side. The data presented in Fig. 2 also are restricted to those cases in which the bead was placed on the penultimate somite at T2 (RA) or T6 (citral). Thus the *n*, average, SEM, and *P* values are different than the values in Table 1, which include a more varied population of experiments. The normalized data are presented in matched pairs, thus illustrating the range and magnitude of the experimental effects.

To determine whether the large increase in rostrally projecting neurons in caudal segments treated with inhibitors of RA was caused by increased proliferation of preganglionic precursors, mitotic activity was assessed by assaying the relative incorporation of the thymidine analog BrdU. BrdU was applied to the embryos 8 or 16 hours (approximately one or two cell cycles; ref. 19) after application of citral to somite 25 in 26 somite embryos. Both BrdU applications were within the period of preganglionic neuron generation, which begins about the time of citral application and peaks at about the time of the second BrdU application (12). There was a small but statistically significant (Wilcoxin signed rank test) increase in the number of BrdU-labeled cell profiles on the citral treated side relative to the vehicle-treated side at T6 (assayed at E7) with BrdU application at either time point. The average increase was 10% in the 8-hour group and 8% in the 16-hour group.

An early phase of cell death in the brachial and lumbar spinal cord occurs between Stages 16 and 22, during the period of preganglionic cell birth, with a peak at Stage 18 (18). To determine whether a similar period of cell death occurs in the thoracic cord and whether the number of dying cells decreases after citral treatment, cell profiles exhibiting DNA fragmentation were counted in T6 at Stage 18 after citral treatment at Stage 15. There was no significant change in the number of cells with fragmented DNA at Stage 18 in citral treated animals ($n = 11$; average $\pm$ SEM $= 8.54 \pm 1.4$ for the treated side and 8.27 ± 1.6 for control side). The number of dying cells observed bilaterally in T6 at stage 18 was similar to the number in the ventral pyknotic zone of the brachial or lumbar cord reported by Homma *et al.* (18).

DISCUSSION

The experiments described above show that manipulating retinoid signaling in the paraxial mesoderm of chicken em-

Table 1. The effect of different treatments on the number of rostrally or caudally projecting preganglionic neurons in rostal (T2) or caudal (T5, 6, or 7) segments

	Rostrally projecting preganglionic neurons			Caudally projecting preganglionic neurons		
Treatment (*n*)	Control side	Treated side	*P* value	Control side	Treated side	*P* Value
RA at T2 (16)	429 ± 39	249 ± 48	0.0004	21 ± 5	26 ± 7	n.s.
DMSO at T2 (5)	399 ± 103	457 ± 109	n.s.	27 ± 7	15 ± 7	n.s.
Citral at T5 or T6 (10)	127 ± 44	303 ± 65	0.0078	450 ± 31	443 ± 31	n.s.
Disulfiram at T6 or T7 (8)	73 ± 16	155 ± 23	0.0281	423 ± 63	438 ± 63	n.s.
Carbamate at T5 or T6 (5)	160 ± 35	146 ± 43	n.s.	473 ± 104	425 ± 85	n.s.
Nitrile at T6 or T7 (7)	124 ± 21	112 ± 25	n.s.	419 ± 48	455 ± 75	n.s.
DMSO at T6 or T7 (8)	144 ± 38	153 ± 24	n.s.	359 ± 54	406 ± 46	n.s.

All values are mean ± SEM. *P* values for statistically significant results determined by the Wilcoxon Signed Rank Sum test. *n*, number of treated embryos; n.s., not significant.

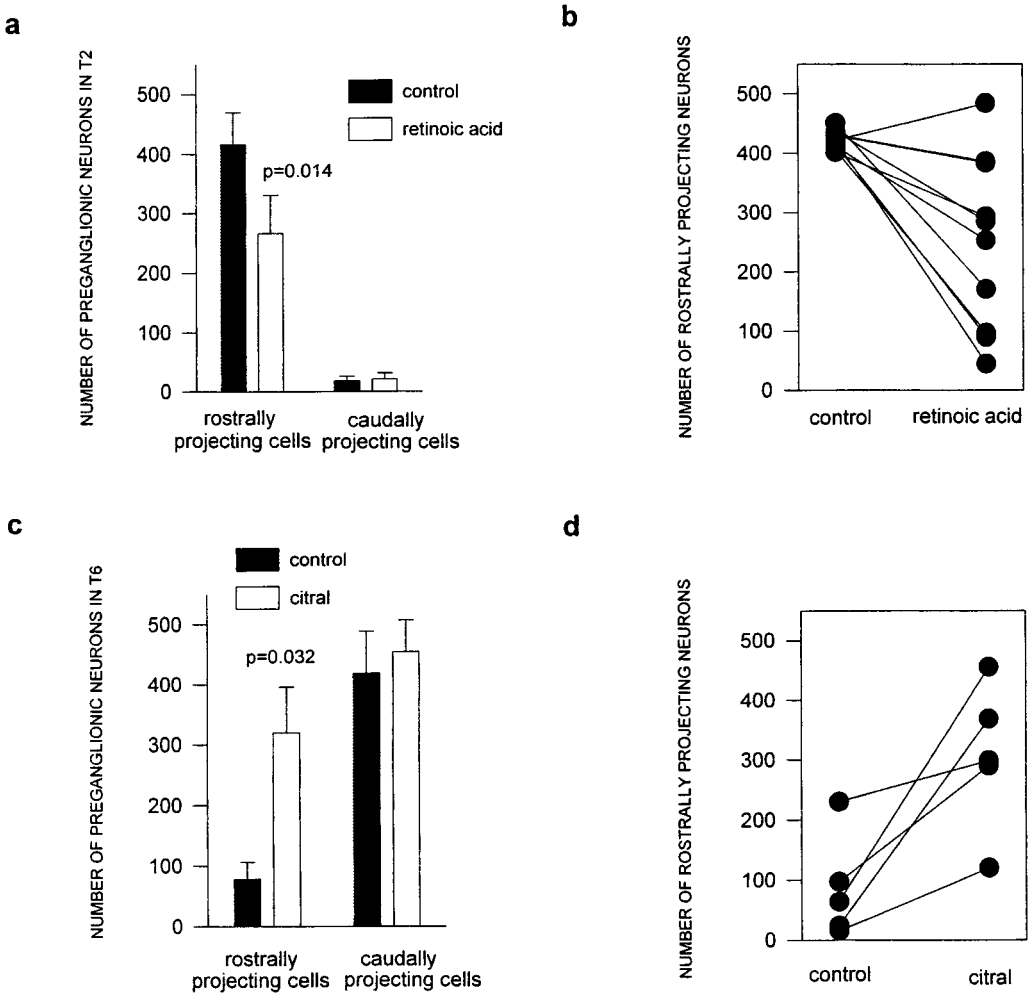

FIG. 2. Effect of altering retinoid signaling on sympathetic preganglionic projections. In contrast to Table 1, the data plotted in this figure are restricted to those cases in which the bead was placed on the penultimate somite and was recovered at T2 or T6 only. (*a*) Treatment of T2 somites with RA selectively decreases the number of preganglionic neurons that project rostrally. Values are mean ± SEM; significance was assessed with Wilcoxon Signed Rank test, $n = 10$. In 9 of 10 cases, the experimental side contained fewer rostrally projecting neurons than the control side. (*b*) Same data as in *a*, following a normalization procedure in which we normalized all data points to the average number of labeled neurons (both rostrally and caudally projecting) for the control side. The normalized data are presented in matched pairs, thus illustrating the range and magnitude of the experimental effects. (*c*) Treatment of T6 somites with citral selectively increases the number of preganglionic neurons that project rostrally. Data presented as in *a* ($n = 5$). In all five cases, the experimental side contained more rostrally projecting neurons than the control side. (*d*) Same data as in *c*, following the normalization procedure described in *b*.

bryos alters numerically a specific population of central nervous system neurons. The results are striking in their specificity; perturbing retinoid signaling selectively changes the number of sympathetic preganglionic neurons that project rostrally in the sympathetic chain without affecting the number of those that project caudally. In preliminary experiments, RA-soaked beads placed directly on the spinal cord did not alter the pattern of sympathetic preganglionic projections (C.J.F., unpublished results), suggesting that the retinoid influence is mediated indirectly via the somite.

Retinoids have been shown to modify neuronal proliferation (20), differentiation (21, 22), and survival (23–27) and to stimulate neurite outgrowth (28, 29). Each of these effects could influence the size of the rostrally projecting population of preganglionic sympathetic neurons. In many cell types, the influence of RA on proliferation is inhibitory (30), an effect that could explain our results if specific for the rostrally projecting population of preganglionic neurons. We did observe a modest increase in proliferation at T6 after inhibition of RA synthesis with citral. The increase, however, is not of sufficient magnitude to explain the ultimate increase in cell number observed; the number of T6 cells in S phase 8 or 16 hours after citral treatment increased by 10% and 8%, respectively, whereas the total number of T6 rostrally projecting preganglionic neurons more than tripled. Thus, it seems unlikely that retinoid signaling selectively regulates the number of rostrally projecting preganglionic neurons solely through an effect on proliferation.

It also seems unlikely that retinoid signaling selectively regulates cell number in this system through control of cell death. The retinoid manipulations were performed almost 1 week before the period of target-dependent cell death (between E8 and E10 in the preganglionic neuron population; ref. 15). Both the segmental and intrasegmental patterns of preganglionic projections already are established by E7 (the earliest time we can selectively label rostral and caudal projections). Additionally, we have shown that disruption in retinoid signaling does not affect an earlier period of cell death in the spinal cord that has been proposed as a potential phenotype selection mechanism (18).

Two other potential mechanisms by which retinoids might control the number of rostrally projecting preganglionic neurons remain to be explored. One possibility is that retinoids selectively suppress the outgrowth of rostrally projecting ax-

ons. This possibility might easily explain the decrease of rostrally projecting neurons in T2 after exogenous RA application. However, to use this line of reasoning to explain the large increase in the number of rostrally projecting neurons in T6 after citral treatment would require that a substantial population of committed, rostrally projecting preganglionic neurons in T6 normally are inhibited from projecting into the chain until at least E10 (when we assay the projections) but are disinhibited during the presumed fall in retinoid levels after experimental inhibition of retinoid synthesis. Because growth of rostrally projecting axons into the sympathetic chain in the appropriate segmental pattern already has occurred by E7 at T6 (16), such a latent population clearly does not establish the segment-specific pattern of preganglionic projections. Moreover, in normal E10 preparations in which preganglionic neurons have been labeled from the paravertebral chain, there is no indication that there is a large population of unlabeled preganglionic neurons as visualized by cresyl violet counterstain of biotinylated dextran labeled preparations.

A second, and to us more attractive, possibility is that retinoids bias the differentiation of noncommitted cells away from the rostrally projecting preganglionic neuron fate, such that, in the normal situation, fewer differentiate in caudal than rostral segments. This scenario is easier to reconcile temporally both with the manipulations we have performed and with the events that specify the differentiation of somatic motoneurons into pools with different axonal trajectories (31). Moreover, it is reminiscent of the differential effects of RA on rod and cone photoreceptor differentiation in the zebrafish (22).

How can RA effect such changes? RA can bind either to cytoplasmic binding proteins or to nuclear receptor proteins. The binding proteins are thought to act as buffers that regulate the intracellular concentration and trafficking of RA (32, 33). The receptors bind as dimers to response elements in DNA, thereby regulating gene transcription (34). The receptors comprise two classes with different ligand affinities: the retinoic acid receptors (RARs), which bind the all-trans and 9-cis isomers of RA (9-cis RA) about equally well, and the retinoid X receptors, which are predominantly selective for 9-cis RA. In the chicken embryo, three RARs (α, β, and γ) and two retinoid X receptors (α and γ) have been identified (35–37). These can form different heterodimer configurations that are targeted to different response element nucleotide sequences (34). Genes known to be involved in the patterning of the central nervous system, most notably *Hox* genes, are sensitive to RA, and several contain RA response elements (38–42). There is thus a tangible molecular link between RA and neural patterning, including the regulation of axon outgrowth (43), but the cellular responses involved are clearly complex and potentially pleiotropic. Kessel (44) has shown explicitly that retinoid modulation of *Hox* codes in mouse embryos alters neuronal cell fate in the brainstem; phenotypic expression of the alteration in fate includes altered axonal pathfinding. However, as noted by Kessel (44), whether the altered axonal pathfinding of these brainstem neurons represents an alteration in intrinsic specification of the neurons, an influence on the surrounding neuroepithelium or attraction by other cells cannot be determined from the phenotypic observation of axon growth. Our data for preganglionic projections suggest a retinoid-induced alteration in intrinsic identity of spinal segments as indicated by the quantitative distribution of cells of a particular type within the segment and suggest that this effect is mediated via the somite. In addition to its alteration of *Hox* codes in the neural tube, manipulation of mesodermal retinoid signaling in the chicken embryo recently has been shown to alter the expression of the leucine zipper transcription factor *MafB/Kr* in the brainstem (45). In this study, exogenous application of RA to a rostral somite mimicked the effect of transplantation of a more posterior somite in place of the rostral somite, suggesting a possible role for a somitic gradient of endogenous retinoids in specifying neural tube identity (45).

A full understanding of the effects of RA on the patterning of preganglionic sympathetic projections will require a comprehensive regional characterization not only of RA synthesis and availability but also of RA binding proteins and receptors. Substantial progress on this line of inquiry has been made in the mouse (46, 47), but in the chicken embryo the description is still fragmentary.

At the stages exposed to RA here, some RA binding proteins and receptors exhibit regional patterns of expression in the chicken embryo. Those that have been detected in the spinal neural tube or emigrating neural crest or both, either as transcripts or translated protein, include the cellular RA binding protein CRABP (48), RAR β (49, 50) (J.C.G. and P. Chambon, unpublished results), retinoid receptor α (51), and retinoid receptor γ (ref. 35; F. Hoover, A. Kielland, and J.C.G., unpublished results). RAR β has been detected by immunohistochemistry and *in situ* hybridization of message in the somites (ref. 50; J.C.G. and P. Chambon, unpublished results). RARα and RARγ have not been investigated yet in the chicken embryo; in the mouse, transcripts of the RARα gene have been detected in spinal neural tube, neural crest, and somites (52, 53).

Chronological profiles of RA content during specific periods of embryonic development have been reported in several species. During primary axis formation in both *Xenopus laevis* and chicken embryos, a concentration gradient of endogenous RA exists along the longitudinal axis with the highest concentration located posteriorly (54, 55). After neurulation, hot spots of RA synthesis and release have been demonstrated in the mouse embryo in the neural tube adjacent to limb innervating regions, creating a bipolar gradient of endogenous retinoids in the thoracic region (56). This bipolar gradient evidently is sculpted from an initially uniform or longitudinally graded distribution of endogenous RA (46, 57). Unfortunately, chronological profiles of RA content have not been obtained yet at the appropriate stages for the chicken embryo, and those reported for the mouse embryo do not have single segment resolution, precluding a segment-by-segment comparison of RA levels and the number of rostrally projecting preganglionic neurons in either species.

At the stages at which we have performed our experiments, RA is known to be synthesized by the floor plate of the neural tube in chicken embryos (58). In the mouse embryo, McCaffery and Dräger (56) note that, at corresponding stages (E9–11), RA also is generated by the mesoderm. Similarly, Horton and Maden (57) have shown that RA and retinol are present in both the spinal cord and somites of E10.5 mouse embryos. These studies indicate that RA may be synthesized and released by the somites as well as the neural tube. As both tissues express RA receptors, retinoid signaling between them is likely to be bidirectional.

We have observed in separate studies that transplantation of caudal somites to more rostral segmental levels within the thoracic region increases selectively the number of caudally projecting preganglionic neurons at those levels (E.B.E. and C.J.F., unpublished results). Thus, the sizes of the rostrally and caudally projecting populations may be independently regulated by different factors, each potentially derived from the somites. Opposing gradients of such factors along the thoracic region could be sufficient, in combination, to determine the segment-specific identity of preganglionic projections. Based on our present results, the factor specific for the rostrally projecting neurons appears to be a retinoid or some other substance whose availability is under the control of retinoids.

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