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Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects

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SUMMARY

Both retinoid receptor null mutants and classic nutritional deficiency studies have demonstrated that retinoids are essential for the normal development of diverse embryonic structures (e.g. eye, heart, nervous system, urogenital tract). Detailed analysis of retinoid-modulated events is hampered by several limitations of these models, including that deficiency or null mutation is present throughout gestation, making it difficult to isolate primary effects, and preventing analysis beyond embryolethality. We developed a mammalian model in which retinoid-dependent events are documented during distinct targeted windows of embryogenesis. This was accomplished through the production of vitamin A-depleted (VAD) female rats maintained on sufficient oral retinoic acid (RA) for growth and fertility. After mating to normal males, these RA-sufficient/VAD females were given oral RA doses which allowed for gestation in an RA-sufficient state; embryogenesis proceeded normally until retinoids were withdrawn dietarily to produce a sudden, acute retinoid deficiency during a selected gestational window. In this trial, final RA doses were administered on E11.5, vehicle at E12.5, and embryos analyzed on E13.5; during this 48 hour window, the last RA dose was metabolized and embryos progressed in a retinoid-deficient state. RA-sufficient embryos were normal. Retinoid-depleted embryos exhibited specific malformations of the face, neural crest, eyes, heart, and nervous system. Some defects were phenocopies of those seen in null mutant mice for RXRα$^{-/-}$, RXRα$^{-/-}$/RARα$^{-/-}$, and RARα$^{-/-}$/RARγ$^{-/-}$, confirming that RA transactivation of its nuclear receptors is essential for normal embryogenesis. Other defects were unique to this deficiency model, showing that complete ligand 'knock-out' is required to see those retinoid-dependent events previously concealed by receptor functional redundancy, and reinforcing that retinoid receptors have separate yet overlapping contributions in the embryo. This model allows for precise targeting of retinoid form and deficiency to specific developmental windows, and will facilitate studies of distinct temporal events.

Key words: retinoid receptors, vitamin A deficiency, eye, neural crest, cranial nerves, nervous system, craniofacial development, ontogenesis, rat embryo

INTRODUCTION

That retinoids are essential for normal embryogenesis has been known for over three quarters of a century. Animal feeding trials identified vitamin A (the prohormone retinol) as essential for normal development. Vitamin A deficiency (VAD) resulted in offspring born with limb truncations, anophthalmia, cleft palate, foreshortened skulls, and malformed kidney, heart, eyes, diaphragm, lungs and urogenital tract (e.g. Wilson et al., 1953; Morriss-Kay and Sokolova, 1996 and references therein). More recently, the VAD in ovo quail has demonstrated retinoid requirements during early events including hindbrain patterning, neural crest development and cardiogenesis (e.g. Dersch and Zile, 1993; Maden et al. 1996). That vertebrate embryos contain RA and spatially restrict its synthesis during organogenesis also is consistent with its importance (reviewed by Hofmann and Eichele, 1994).

A second line of evidence for a developmental retinoid requirement comes from analysis of murine embryos having null mutations for retinoid receptors, which are ligand-activated transcriptional regulators (reviewed in Lohnes et al., 1995). Multiple RA receptor isoforms (RARα, β, γ) are expressed during development in specific spatiotemporal patterns. Loss of a single retinoid receptor generally results in modest or no physical anomalies (reviewed in Lohnes et al., 1995), a notable exception being the cardiac and ocular defects in RXRα mutation (Sucov et al., 1994; Kastner et al., 1994; Dyson et al., 1995). Compound RAR and/or RXR null mutations recapitulate several VAD phenotypes, affecting patterning and/or differentiation within the nervous system, face, heart, neural crest, urogenital tract, and limb (Kastner et al., 1994; Mendelsohn et al. 1994). The findings reinforce that interactions between RA and its receptors are crucial for normal embryogenesis.

While classic deficiency models and receptor null mutants have proven importance in identifying retinoid-dependent embryonic events, these approaches are not entirely satisfactory. In null mutant models the RA ligand is still present and capable of trans-activating the remaining receptors, presenting

the potential for functional redundancy with the remaining receptors; indeed, no receptor null mutant yet recapitulates the entire spectrum of changes exhibited in vitamin A deficiency. A second complication is that the deleted gene or retinoid ligand is absent during the entire gestational period, such that a particular developmental window cannot be targeted in isolation; this can make it difficult to distinguish primary and secondary effects. Embryo lethality prohibits analysis of the downstream developmental progression. While approaches using conditional mutation can spatially and temporally target gene mutations (e.g. Gu et al., 1994), developing such models is laborious and not yet available for retinoid receptors.

To circumvent these problems, we present a model for studying the RA hormone requirement during specific temporal events of mammalian development. The method relies upon a vitamin A-deficient rat model system in which the investigator controls the retinoid status of the dam and embryos during defined gestational windows. Retinoid-depleted dams are supplemented daily with sufficient RA during early gestation to permit normal development, and then discontinued at a specified gestational time such that subsequent embryogenesis continues in a retinoid-deficient state. Retinoid deprivation during a period of organogenesis (E11.5-E13.5) causes specific perturbations in ocular, craniofacial, cardiac, and nervous system development. That these results recapitulate several of the RAR/RXR null mutant phenotypes confirms the requirement for RA/receptor transactivation. Several other phenotypes are novel or significantly exaggerated as compared with those seen in traditional deficiency or null mutants, suggesting some retinoid actions were masked in previous studies. The model provides experimental versatility not matched by current genetic techniques. It can be used to create a 'complete' retinoid null phenotype targeted to specific developmental windows, permits supplementation with diverse retinoid metabolites in a whole animal bioassay to test ligand efficacy, and allows for targeted ligand rescue to follow subsequent consequences to the fetus, neonate and adult.

MATERIALS AND METHODS

Production of vitamin A-deficient rats

The protocol produces rapid, synchronous retinoid deficiency (Anzano et al., 1979) while minimizing lactational retinoid transfer (Davila et al., 1985; Smith et al., 1990) and relies upon all-trans-RA's ability to support fertility and gestation prior to E15 (Thompson et al., 1964; Wellik and DeLuca, 1995). Timed pregnant Sprague-Dawley rats (gestation day 14) were fed a vitamin A-deficient (VAD) powder diet based on AIN-93G (Reeves et al., 1993, Table 1); animals were maintained in a pathogen-free environment. Female pups were weaned at day 19, housed in wire drop cages to reduce coprophagy, and weighed three times weekly to monitor growth and health.

At growth plateau #1 (defined as 1-2 g weight gain/day over 5 days; generally at 7 weeks old), these female offspring were supplemented with 20 μg RA/day, administered orally with a positive displacement pipette. All-*trans*-RA (Sigma) was stored as 5 mg/ml and diluted in cottonseed oil before use. Serum retinoids were measured at growth plateau #1 to assess retinoid status. When growth again slowed (plateau #2; generally at 10 weeks old), the supplement was increased to 50-100 μg RA/day for the duration, depending on the individual; these rats were considered deficient.

When weight gain on the higher dose was consistent for 7 consecutive days, VAD RA-sufficient (RAS) females were mated overnight; presence of a copulation plug or sperm in the vaginal smear the following morning confirmed the mating and was designated embryonic day 0.5 (E0.5). During gestation, all dams were given their daily 50-70 μg RA dose on E0.5 to E5.5, and 70-100 μg RA every 12 hours from E5.5 to E11.5. Thereafter, the RAS group continued to receive RA every 12 hours, while RADs received cottonseed oil only. Embryos were collected on E13.5, and maternal serum and liver samples analyzed for retinoid content. A third group of dams maintained on commercial rat chow (Teklad) assessed the efficacy of the RAS control.

Immunohistochemistry

Alternating 6 μm embryonic sections were prepared with a 36 μm repeat. Immunochemistry followed standard protocols (Dickman and Smith, 1995), using antibodies directed against α-, β-, γ-crystallin (D. Carpen), type 1α-collagen I (D. Greenspan), HNK-1 (Becton-Dickinson), NCAM (C.-M. Chuong), and α-smooth muscle actin (Sigma), and detected with peroxidase-conjugated goat anti-mouse-IgM for all except NCAM, which was detected with β-galactosidase-conjugated goat anti-rabbit IgG. Apoptotic cells were identified using the ApopTag In Situ apoptosis detection kit-peroxidase (Oncor). Peroxidase was detected using diaminobenzidine and counterstained with methyl green; β-galactosidase was detected using X-gal.

Retinoid analysis

Serum retinoids were analyzed according to the method of Barua et al. (1995) with slight modifications. Retinyl acetate (Acros) served as an internal standard. Retinoid separation was achieved using a C18 column (Rainin 3 μm Microsorb-MW 4.6 mm × 10 cm) with acetonitrile:dichloromethane: methanol:water (89:14:1:4) + 0.05% ammonium acetate as the isocratic phase, on a Waters 440 HPLC. Absorbance was monitored at 325 nm via a Waters 996 photodiode array detector. Peak identity was confirmed by coelution with known standards. Peak area was determined by integration using the Millenium 2010 program and compared against a retinol standard curve. All manipulations were perfomed under yellow light to minimize retinoid isomerization.

Liver retinoids of dams were quantified according to Amedee-Manesme et al. (1984) and Furr et al. (1986). Briefly, 1 g of scissor-minced liver was ground with 3 g of anhydrous sodium sulfate. Retinyl acetate (50 μg, Acros) was added to the liver paste which was then extracted 3× with 5 ml dichloromethane, and the extract brought to 50 ml final volume with dichloromethane. Aliquots of this extract were concentrated under argon, redissolved in 2-propanol: dichloromethane (4:1), and separated on a Waters 501 HPLC using a C18 column (Rainin 5 μm Microsorb 4.6 mm × 15 cm) with acetonitrile:dichloromethane (80:20) as the mobile phase. Absorbance was monitored at 325 nm using a Waters photodiode array detector; peaks corresponding to retinyl esters were integrated and compared to a retinyl palmitate standard curve.

Embryonic retinoid content was measured at various times after the last administration of 100 μg RA at E11.5. Tissue was homogenized by sonication in 300 μl stabilizing buffer (Thaller and Eichele, 1987) after addition of [^{3}H]RA as internal standard. The samples were extracted twice with 1 ml of ethylacetate:methylacetate (8:1). After concentration under nitrogen the extracts were fractionated on a C18 HPLC column (Rainin 5 μm, Microsorb 4.6 mm × 25 cm) with methanol: acetonitrile: 0.1 M ammonium acetate pH 6.8 (64:20:16) as the isocratic phase. Absorbance was monitored at 350 nm and at 280 nm using Shimadzu SPD 6A detectors. One minute fractions were collected and counted to determine the recovery of the internal standard. Peaks corresponding to RA or retinol were integrated and compared to standards. Using the wet weight, molar concentrations of RA and retinol were calculated.

RESULTS

Depletion of endogenous vitamin A stores

We developed a protocol to study targeted gestational windows

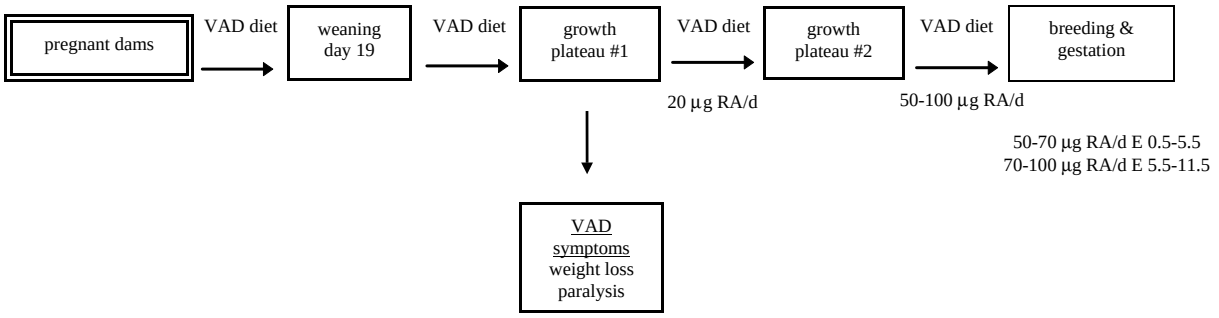

Fig. 1. Schematic of protocol used to generate retinoid-deficient embryos, as detailed in Methods and Results.

under conditions of severe retinoid deficiency while allowing prior development under retinoid-sufficient conditions (Fig. 1). Pregnant rats consumed a retinoid-deficient diet (Table 1) during gestation and lactation to minimize retinoid transfer and stores in newborn pups (Smith et al., 1990; Davila et al,. 1985). Their daughters were weaned at day 19 and fed the VAD diet thereafter, and became the pregnant dams who would carry the retinoid-deficient embryos.

Female pups gained weight steadily until the 7th week (approximately 4 weeks post-weaning), when their growth plateaued (Fig. 2); several rats exhibited characteristic retinoid deficiency symptoms including forelimb paralysis, appetite loss, ocular porphyrin deposits, and weight loss (Rogers and Bieri, 1969). Rats were supplemented with 20 µg RA daily when they exhibited any of the above symptoms, or if their weight gain was <1 g/day over 5 days; supplementation rapidly reversed these symptoms. This RA dose allowed for continued body growth but also forced these future dams into depleting their endogenous retinoid stores (Anzano et al., 1979). RA substitutes for retinol in most functions except for the visual cycle and spermatogenesis and is not appreciably stored. By 10 weeks old, female rats again stopped growing (Fig. 2) and some again exhibited VAD symptoms, indicating 20 µg/day RA was no longer sufficient. Daily supplementation with 50-70 µg RA reversed deficiency symptoms and restored body growth; the precise dose varied somewhat with individual requirements.

Table 1. Composition of vitamin A-deficient diet

Substance	g/kg diet
Casein, 'vitamin-free' test	190
l-Cystine	3
Corn starch	523
Sucrose	150
Cottonseed oil	50
Cellulose	40
Mineral mix, AIN-93G-MX†	35
Vitamin mix‡	5
Choline dihydrogen citrate	3.5
dl-α-tocopheryl acetate (1000 U/g)	0.121
Vitamin D_2, in corn oil (400,000 U/g)	0.0055

†Reeves et al., 1993.

‡TD 83171 (Teklad), without choline and vitamins A, D, E. Supplies (mg/kg diet): biotin (0.4), calcium pantothenate (66.1), folic acid (2.0), inositol (110.1), menadione (49.6), niacin (99.1), p-aminobenzoic acid (110.1), pyridoxine-HCl (22), riboflavin (22), thiamin-HCl (22), vitamin B12 (29.7), ascorbic acid (1016.6).

Retinoid status was assessed by serum retinol levels at growth plateau #1 (7 weeks old) and by serum and liver retinoid levels at embryo collection. Serum retinol levels of VAD females averaged <2.5 µg retinol/dl serum at 7 weeks old and 0.36 µg/dl at the time of embryo collection. Serum RA was undetectable at 7 weeks and averaged 0.10 µg/dl with RA supplementation (100 µg twice daily); this latter value was in the range for that of chow-fed controls (0.06 µg/dl, Table 2). Maternal liver retinoid analysis at embryo collection confirmed the depletion, as liver retinoid stores were below the detection limit (<1 µg retinyl palmitate/g liver wet weight); other retinyl esters (stearate, oleate) were undetectable. In contrast, control dams fed commercial chow had considerable retinyl palmitate stores (148±11 µg/g liver wet weight) and adequate serum retinol levels (26.3±4 µg/dl). We conclude that hepatic retinoid stores of VAD breeder rats were essentially depleted, and thus their predominant retinoid source was exogenous supplementation.

Dose dependence of embryogenesis

Three RA supplementation levels were tested for their ability to support normal embryogenesis through E13.5. Oral supplementation with 20 µg RA/day did not maintain body weight, fertility and estrus cycling, and gestation; matings at this dose produced frequent implantations but only empty deciduas were recovered, consistent with the inability of this dose to prevent deficiency symptoms. In contrast, 50-100 µg RA every 12 hours was sufficient for both implantation and normal embryo development through day 13.5 (Fig. 3). The precise dose was individually determined and tended to increase as depletion progressed. We recommend use of 12-hour dosing during gestation to reduce the potential for deficiency.

Gross anomalies in retinoid deficiency

Using the above protocol, we produced embryos which were RA-sufficient from E0 to E11.5 and RA-deficient until collected at E13.5; RAS controls continued to receive RA. Embryos collected from RAS dams were indistinguishable from those obtained from chow-fed dams (not shown). Direct visual inspection of RAD embryos revealed severe malformations including an underdeveloped hindbrain, absent cranial flexure, microphthalmia, narrow limb buds, and a straight tail which failed to curl (Fig. 3A; Table 3). Craniofacial defects included foreshortening of the snout and branchial arches, and an enlarged oral cavity distended rostrally. The severity of phenotype was somewhat heterogenous within a given litter. RAD embryos had a significantly smaller crown-to-rump length than did RAS controls (5.5 mm±0.1 vs. 6.4 mm±0.3;

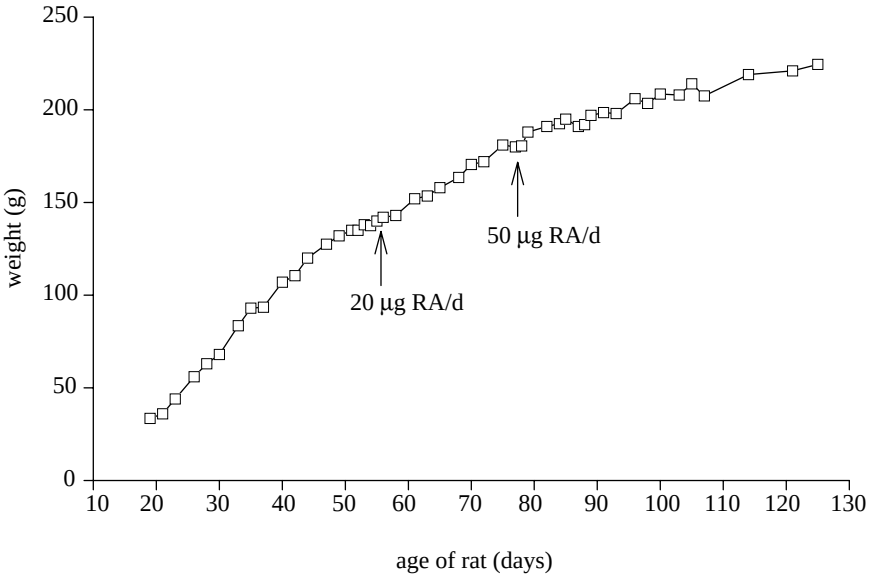

Fig. 2. Growth curve of representative breeder rat maintained on VAD diet; note the initiation of daily oral RA supplements at times of growth plateau (open arrows).

$P<0.05$), suggesting impaired growth. However, direct comparison of the E13.5 RAD embryo with normal E13.5 and E12.5 embryos suggests the observed dysmorphology is not due to simple growth arrest, as the RAD phenotype is quite distinct from the normal E13.5 and E12.5 morphology (Fig. 3B).

Embryonic retinoid content was measured at various times after the last administration of RA at E11.5 and is summarized in Table 2. Twelve hours after the last RA dose, embryonic RA content from VAD-RAD dams is 63% (55 nM) of the RA concentration found in the same stage embryos from ad libitum chow-fed dams (88 nM), and falls to 25% (21 nM) after 27 hours. After 73 hours only 2% of the RA found in untreated control embryos can be detected. Retinol was not found in any of the VAD-RAD embryos but was readily detected in chow control embryos.

Stained histological sections and immunohistochemical analysis of cranial and thoracic compartments revealed distinct malformations affecting specific embryonic tissues; these defects are summarized in Table 3 and detailed below. Dysmorphogenesis of the heart, visceral compartment and limb will be discussed separately (E. Dickman, S. Power, J. Lancman, and S.M. Smith, in preparation).

Eye abnormalities

A major event occurring during the retinoid deficiency window is lens and retina development (reviewed by McAvoy, 1980). At the time of the last RA dose (E11.5), the lens is induced by contact between the forebrain neuroectoderm and surface ectoderm. The optic vesicle invaginates (E12.5) to form the neural and pigmented retina. Simultaneously, the lens placode invaginates and fuses to form the lens (E13). Cells within the posterior lens differentiate and elongate anteriorly, forming long crystallin fibers which fill the lens lumen (Fig. 4A).

The primary ocular defect observed during RAD between E11.5-13.5 was bilateral microphthalmia. Lens induction, invagination, and fusion proceeded normally, as all RAD embryos had lens vesicles (Fig. 4B,C). However, all RAD lens were reduced in diameter (measured along its longest axis) and averaged 66% of control RAS values (Table 4). Lens development was normal in RAS controls; posterior lens cells elongated and extended anteriorly to form lens fibers with normal expression of α-, β-, and γ-crystallin proteins (Table 4; Fig. 4A,F,H and not shown). In contrast, RAD lenses were severely dysmorphic. Posterior lens cells failed to elongate (18/22), leaving the lens vesicle enlarged (22/22; Fig. 4B,C,G,I). RAD lens cells expressed α-, β- and γ-crystallin proteins at levels similar to controls, but remained rounded and did not elongate (Fig. 4G,I and not shown). These results are consistent with failed lens cell maturation. Additionally, RAD lenses (16/22) showed significant apoptosis particularly within the posterior lens (Fig. 4J,K; Table 4). Not surprisingly, apoptosis was strongest in those lenses with the least elongation and severest disorganization. Taken together, the

Table 2. Retinoid content of embryos and dams

Time after RA administration (hours)	Embryo retinol content		Embryo retinoic acid content		Maternal serum retinol		Maternal serum retinoic acid	
	ng/g wet wt.	nM	ng/g wet wt.	nM	μg/dl	nM	μg/dl	nM
12 (*n*=3)†	n.d.	n.d.	16.4	55	0.36	12.8	0.10	3.9
27 (*n*=2)	n.d.	n.d.	6.1	21	0.20	7.0	0.13	4.4
73 (*n*=3)‡	n.d.	n.d.	0.6	2.0	0.52	18.0	0.08	2.7
chow control (*n*=3)†	192	669	26.3	88	24.7	860	0.06	2.0
chow control (*n*=2)‡	296	1015	20.2	68	–	–	–	–

†35 somites; ‡44 somites; n.d., not detected.

Table 3. Major embryonic malformations following acute retinoid deficiency

	RA-deficient	RA-control	Chow
Crown-to-rump length (mm)	5.5±0.1*	6.4±0.3	6.9±0.2
Eye			
microphthalmia	13/13	0/12	0/8
delayed lens differentiation/maturation	11/11†	0/8	0/8
Craniofacial defects			
foreshortened snout	11/11†	0/12	0/8
hypoplastic frontonasal region	10/11†	0/12	0/8
hypoplastic mandibular arch	2/12	0/12	0/8
apoptotic frontonasal region	9/11†	0/4†	1/8
apoptotic mandibular arch	2/12	0/4†	0/8
failed mandibular arch fusion	1/12	0/4†	0/8
Cranial nerves			
cranial nerve delay	11/12	1/9	0/8
absent cranial nerve X	12/12	0/9	0/8
apoptosis	12/12	2/9	1/8
Pituitary			
open foramen of Rathke's pouch	9/10†	0/12	0/8
apoptosis of adrenohypophysis	11/11†	12/12	8/8
failed apposition of adreno- and neurohypophysis	5/12	0/12	0/8
Central nervous system (neural tube)			
hypoplastic dorsal root ganglia	5/12	0/12	0/8
deficient ependymal layer	10/12	0/12	0/8
deficient mantle layer	9/12	0/12	0/8
deficient marginal layer‡	9/12	0/12	0/8
underdeveloped hindbrain	4/13	0/12	0/8
Persistent laryngotracheal groove	12/12	0/12	0/8
Lack of cranial flexure	8/13	0/12	0/8
Abnormal limb bud	9/12	0/12	0/8
Straight tail	6/9†	0/11†	0/8

*P<0.05 for RAD vs. RAS; †not all embryos could be scored for all phenotypes; ‡also observed in brain.

Table 4. Ocular anomalies observed in acute retinoid deficiency

	RA-sufficient	RA-deficient
Lens diameter*	3.68±0.39	2.42±0.43 (P<0.001)
Lens induction	8/8	22/22
Crystallin expression†	6/6	6/6
Primary lens fiber elongation‡	++++ (8/8)	++ (4/22), + (10/22), – (8/22)
Retina ends apposed	8/8	14/22
Enlarged lens vesicle	0/8	22/22
Intraretinal gap	0/8	18/24
Apoptosis in lens	0/8	16/22 (6/22 modest, 10/22 strong)
Apoptosis in neural retina§	0/8	6/20
Apoptosis in pigmented retina	0/8	3/20

*Relative diameter along longest axis as measured on 20× photograph, in cm; †not all embryos were examined for phenotype. ‡+ indicates degree of elongation; §apoptosis largely confined to midline of anterior surface.

results suggest a maturational defect within retinoid-deficient primary lens cells which is prevented by RA.

Retinal development was also disrupted during RAD. Many exhibited a pronounced intraretinal gap between the neural and pigmented retina, with subsequent distortion of both layers (18/24; Fig. 4C); others appeared largely normal (Fig. 4B). RAS retinas had normal invagination of the neural and pigmented layers at the midline, and the ganglia cells, which are precursors of the optic nerve, can be seen as they begin the extension of their axonal processes toward the brain (Fig. 4D). All RAD retinas failed to invaginate and did not form the channel for the optic nerve (Fig. 4E); under high magnification, the retinal ganglial cells could not be identified (not shown). Instead, in some eyes a collection of apoptotic cells was present in that location (Fig. 4K).

The pigmented retinal epithelium appeared largely normal (apart from distortion by the intraretinal gap), with normal HNK-1 levels and occasional scattered apoptosis (not shown).

Craniofacial and upper airway development

Between embryonic days 11.5 through 13.5, the face is shaped through enlargement and fusion of the bilateral branchial arches. All RAD embryos exhibited a foreshortened snout (Fig. 3), which in cross-section had reduced size and cellularity (Fig. 5A vs B), suggesting insufficient growth or enhanced cell death. The frontonasal mass had a pronounced midline cleft, and mesenchyme surrounding the nasal placodes failed to expand, and the nasal cavity remained superficial. In contrast the mandible and hyoid had a more modest reduction in cellularity and shape and had properly fused (not shown). TUNEL analysis revealed a bilateral collection of apoptotic cells within the frontonasal mass and maxilla, restricted to the core mesenchyme lateral to the nasal cavity (Fig. 5C). This apoptosis was position-specific as few pyknotic cells were detected in the mandible, hyoid and adjacent mesenchymal and neuronal tissues (not shown). The pyknotic region reacted with antibodies directed against collagen I and not α-smooth muscle actin (not shown), suggesting they are primordia of the neural crest-derived facial cartilage, which initiates differentiation at this time.

Another common RAD defect was the lack of cranial flexure (Table 3; Fig. 3). By E12.5 embryos have undergone a flexure of the brain region which helps place embryos in the fetal position. Instead of curling inward toward the heart and thoracic cavity, the facial arches of many embryos remained upright and distended outward, resulting in a large cleft and distortion between the upper and lower presumptive jaws (Fig. 3). It cannot be determined if the facial cleft and lack of cranial flexure are direct or indirect consequences of retinoid deficiency, as the basis for flexure is ill-defined.

Tracheal and esophygeal development were also disrupted by retinoid deficiency. These structures arise as a single pharyngeal lumen which septates into two separate tubes (Fig. 5F-H). In all RAD embryos, separation never occurred and embryos displayed a persistent laryngeal-tracheal groove (Fig. 5I,J). Frequently the trachea did not terminate into lung buds and lung buds could not be identified (not shown), suggesting failed development of the future respiratory system.

Nervous system and pituitary

The cranial nerves originate from the cranial neural crest and ectodermal placodes (reviewed by Noden, 1991); during the retinoid-deficient period, neurons of sensory and motor nuclei begin extension toward their targets. Retinoid deficiency between E11.5 to E13.5 resulted in significant cranial nerve dysmorphogenesis. The RAD trigeminal ganglia were greatly hypoplastic (Fig. 5K-N); high magification revealed significant pyknosis within the ganglion core (Fig. 5L), confirmed as apoptosis via TUNEL staining (Fig. 5N). Ganglia VII (facial) and VIII (vestibulocochlear) are equally pyknotic (not shown).

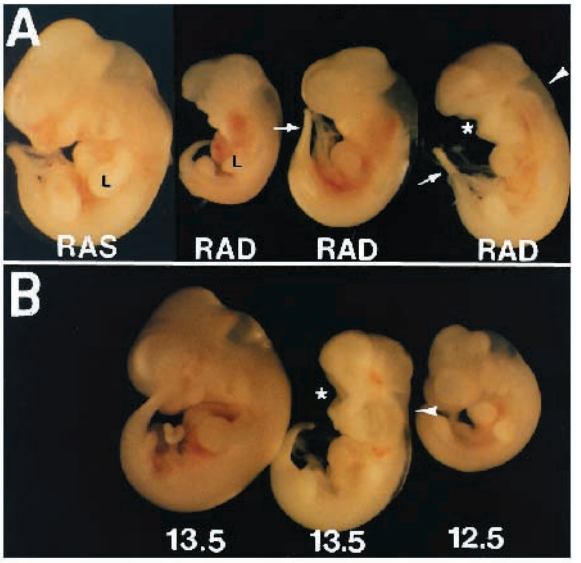

Fig. 3. (A) Gross morphology of RAD embryos given their last RA dose (50 μg) on E11.5 and examined at E13.5 compared with RAS embryos. RAD embryos exhibit an open facial orifice (*), lack of cranial flexure and hindbrain compression (arrowhead), straight tail (arrow), and limb delays (L). (B) Comparison of E13.5 RAD embryo (middle) with RAS embryos at E13.5 (left) and E12.5 (right) shows the RAD phenotype is distinct from that of an overall growth delay.

In some RAD embryos, the trigeminal motor neurons (Fig. 5M) arose from the medullary basal plate and extended into the boundary cap consistent with normal development; pyknotic cells are infrequently observed within this non-neural crest-derived compartment, suggesting specificity of sensitivity.

Within the upper thoracic region, the vagal nerves (X) were absent in all RAD embryos, confirmed by NCAM immunostaining (Fig. 5O-R). This loss was specific as the bilateral sympathetic trunks emerged normally from the neural tube and migrated correctly dorsal to the dorsal aorta (Fig. 5P,R). While sympathetic nerve identity appeared normal, many had reduced cellularity.

Central nervous system development itself also was impaired by RAD (Table 3). Within the neural tube, the mantle layer was significantly reduced in size and cellularity, as visualized by both hematoxylin staining (not shown) and NCAM immunolabelling (Fig. 5O,P). The outer marginal and inner ependymal layers were similarly thinned, as was the roof plate. In some embryos the dorsal root ganglia were reduced in size, while the neural roots exited normally (Fig. 5P,Q).

Similarly, the RAD brain had a striking reduction in differentiating neuronal populations, shown here as diminished HNK-1-labeling in the amygdala and striatum of the diencephalon and telencephalon, respectively; the inner neurepithelium appeared fairly normal albeit reduced in cellularity (Fig. 5D,E). There was little pyknosis (not shown), suggesting that reduced proliferation and/or differentiation, rather than increased cell death, might be responsible for the hypoplasia.

Pituitary gland morphogenesis is well underway in the stages examined here. Diverticula of the infundibulum (dien-

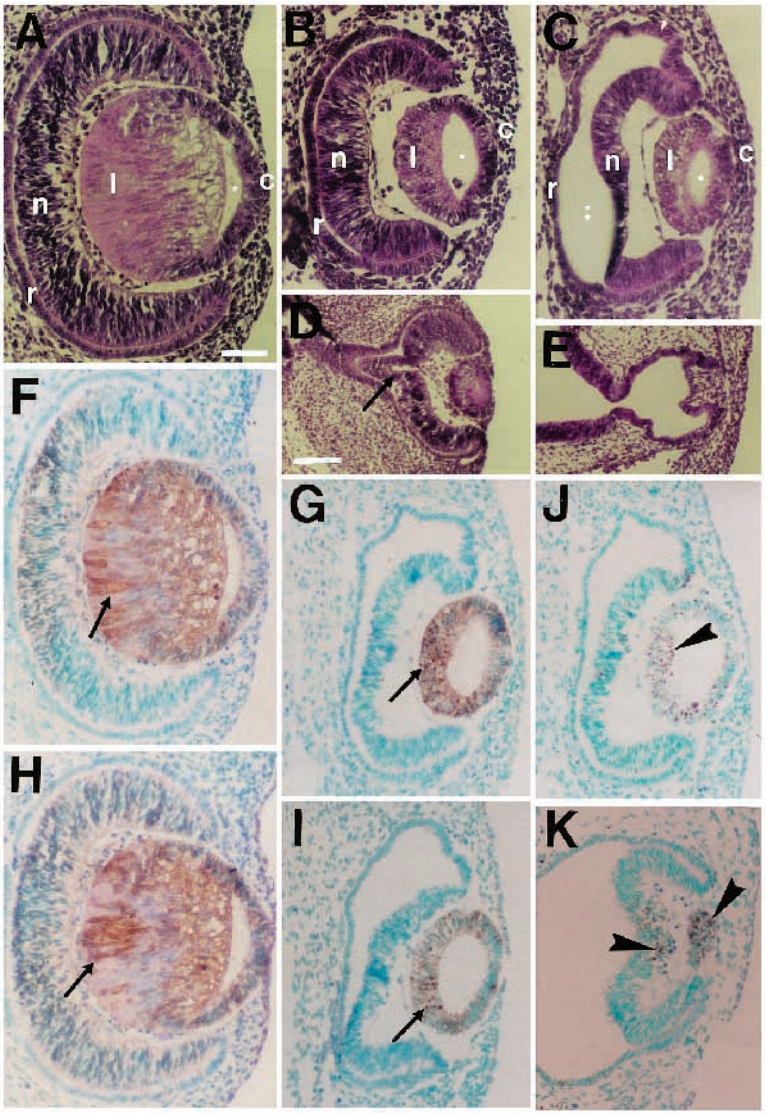

Fig. 4. Transverse sections of ocular development under retinoid-sufficiency and -deficiency. (A-C) Hematoxylin/eosin-stained eyes of E13.5 RAS (A) and RAD (B,C) embryos. RAD eyes are smaller than RAS eyes, and exhibit delayed lens maturation, failed lens cell elongation and prominent intralens vesicle (*). Note the pronounced intraretina gap and retina distortion in C (**), which extend through all sections. (D,E) The retinal groove for optic nerve passage forms normally in RAS (D, arrow) but is completely absent in RAD embryos and cannot be identified (E). (F-I) RAS lens exhibits normal expression of α-crystallin (F, brown signal) and γ-crystallin (H); RAD lens also expresses α-crystallin (G) and γ-crystallin (I) but do not appreciably elongate to form lens fibers. Note that while crystallin expression in RAS is seen in elongated cells (arrows F,H), in RAD crystallin-expressing cells remain rounded (arrows, G,I). (J,K) TUNEL staining revealed significant apoptosis (brown signal, arrowheads) in RAD lens (J,K) and at the midline of neural retina (K). c, precorneal stroma; l, lens; n, neural retina; r, pigmented retina epithelium. Scale bar, 25 μm for all panels except D,E (50 μm).

cephalon floor plate) and of Rathke's pouch extend toward each other between E11 and E13.5, apposing to form the neurohypophysis and adrenohypophysis, respectively (Fig. 6A; reviewed by Schwind, 1928; Simmons et al., 1990).

All RAD embryos formed Rathke's pouch, which extended normally to contact the infundibulum (Fig. 6B-D). In 5/12 of RAD embryos, the infundibulum failed to extend sufficiently and did not contact the posterior side of Rathke's pouch (future

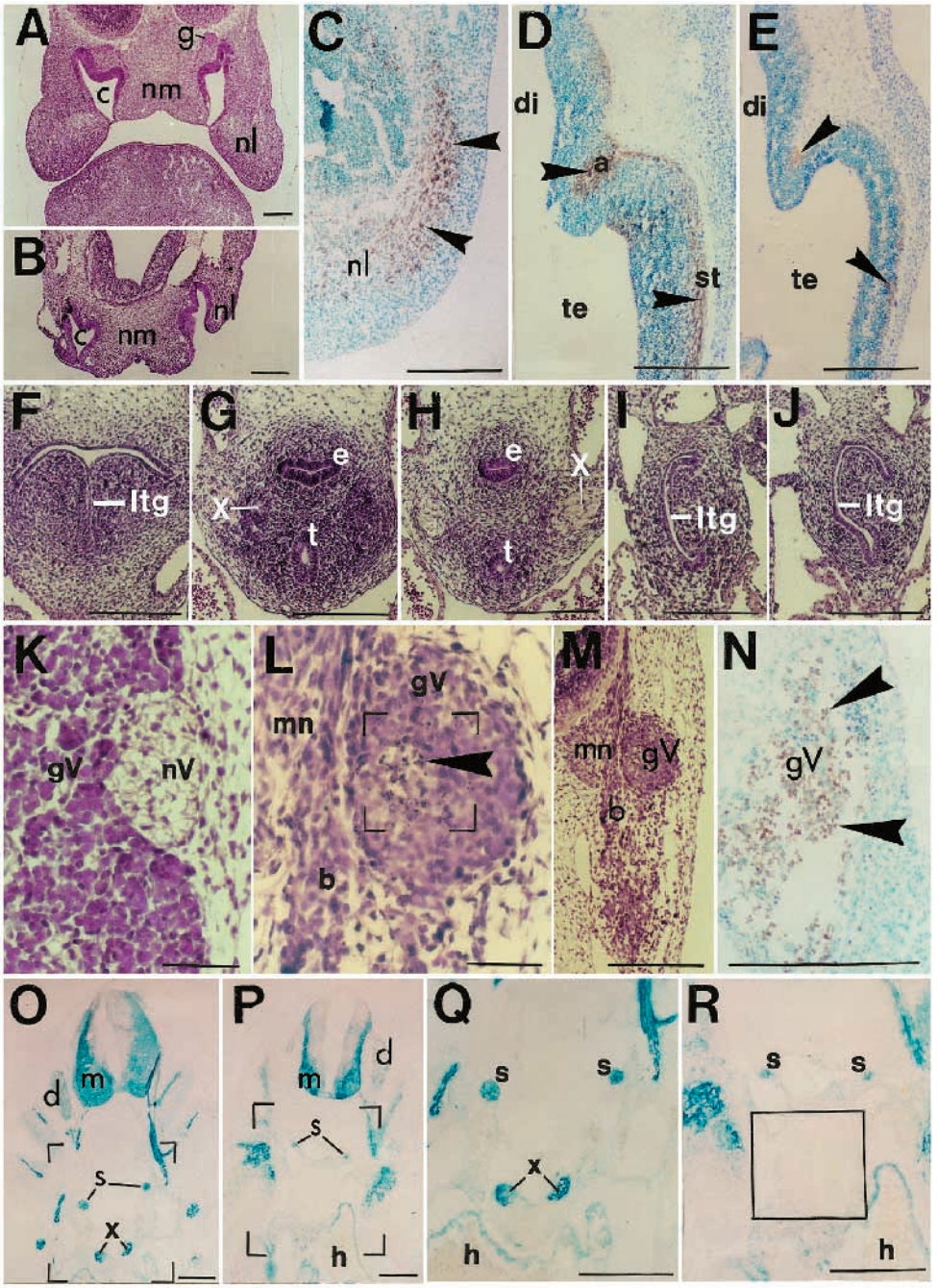

Fig. 5. Transverse sections showing development under RAS and RAD of face, cranial nerve, central nervous system and airway. (A-C) Frontonasal region development. (A) RAS frontonasal region shows normal expansion of midline (nm) and lateral (nl) populations, with cranial ganglia (g) adjacent the nasal cavity (c). (B) Hypoplasia of RAD midline (nm) and lateral (nl) frontonasal populations; note pronounced midfacial cleft and lack of ganglia innervation. (C) TUNEL staining of apoptosis (brown signal, arrowheads) restricted to lateral frontonasal (nl) population in RAD. (D,E) HNK-1 detection of differentiating neurons at the level of the neocortex and hypothalamus. (D) HNK-1 labels the differentiating neurons (brown signal, arrowheads) of the amygdala (a) and striatum (st) in the RAS diencephalon and ventral telencephalon. (E) These populations are greatly reduced in RAD embryos (compare brown signal at arrowheads, a, st). (F-J) Septation of esophagus and trachea in RAS and RAD. (F-H) Successive caudal sections showing septation of the RAS laryngotracheal groove (ltg) into the esophagus (e) and trachea (t); dorsal toward the top. (I-J) Successive RAD caudal sections showing the persistent laryngotracheal groove (ltg) and failed septation into esophagus and trachea; note the vagal nerve (X) is absent (compare with H and see R below). (K-N) Trigeminal nerve development in RAS and RAD embryos. (K) High magnification of RAS trigeminal ganglion and nerve showing lack of pyknotic fragments. (L) In RAD, significant pyknosis (boxed area, arrowhead) occurs in the trigeminal ganglion (gV) but not in the boundary cap (b) or motor nuclei (mn). (M) Low magnification of L showing the RAD boundary cap (b) and motor nucleus (m) appear largely normal. (N) TUNEL staining confirms the cell death in the trigeminal ganglion is apoptotic (brown signal at arrowheads). (O-R) Trunk nerve tracts at level of atria in RAS and RAD, visualized with NCAM. (O) RAS exhibits normal cellularity in mantle layer (blue signal, m) of neural tube, dorsal root ganglia (d) and nerve tracts; paired sympathetic ganglia (s) and vagal nerves (X) are indicated. (P) In contrast, in RAD the mantle layer (m) is much thinned while these dorsal root ganglia (d) and nerves appear largely normal with perhaps reduced NCAM signal. The sympathetic trunks (s) are small but present and the vagal nerves are not detected. (Q) Enlargement of boxed area in O, showing RAS sympathetic trunks (s) and vagal nerve (X) visualized by NCAM. (R) Enlargement of boxed area of P, showing presence of smaller sympathetic trunk (s) and complete absence of NCAM-labeled vagus nerve (boxed area) in RAD. Scale bar, 50 μm (A-J, M-R) and 10 μm (K,L). a, amygdala; b, boundary cap; c, nasal cavity; d, dorsal root ganglia; di, diencephalon, e, esophagus; g, ganglia; gV, trigeminal ganglion; h, heart atrium; ltg, laryngeotracheal groove; m, mantle layer; mn, motor nucleus; nl, nasolateral mesenchyme; nm: nasomedial mesenchyme; nV, trigeminal nerve; s, sympathetic trunk; st, striatum; t, trachea; te, telencephalon; X, vagal nerve.

Table 5. Comparison of RAD and retinoid receptor null mutant embryos*

	RAD	RARα/γ	RARα/β2	RARβ2/γ(2)	RXRα	RARα/RXRα	RARγ/RXRα
Eye							
microphthalmia	+	+†	–	–	+†	NR†	+†
lens elongation/fiber anomaly	+	+‡	–	–	–	–	–
lens cell apoptosis	+	NR	NR	NR	NR	NR	NR
neural retina apoptosis	+	NR	NR	+	NR	NR	NR
intraretinal gap	+	+§	–	+§	–	±§	±§
absent choroid fissure	+	–	–	–	–	–	–
thickened precorneal stroma	+	+	–	+¶	+	–	+
Heart defects	+	+	+	–	+	+	+
Abnormal limb shape	+	+	–	–	–	–	–
Absent tracheal-esophageal septum	+	–	+	–	–	–	–
Absent cranial flexure	+	–	NR	NR	NR	NR	NR
Head and nervous system							
hindbrain defects	+	+**	–	–	NR	NR	NR
frontonasal hypoplasia, cell death	+	+	–	–	–	–	–
mandibular hypoplasia, cell death	±	–	–	–	–	–	–
cranial nerve hypoplasia, absence	+	+††	–	–	NR	NR	NR
brain hypoplasia	+‡‡	+ §§	–	–	–	–	–

NR: not reported or assessed. *Data taken from this study and Grondona et al., 1996; Kastner et al., 1994; Lohnes et al., 1994 and 1995; Mendelsohn et al., 1994, and Sucov et al., 1994.†Also smaller ventral retina. ‡Not detailed, one instance of absent lens; § ± indicates retinal eversion, no obvious gap; ¶poorly differentiated; **open rhombencephalon; ††motor nuclei of CNVI only; ‡‡thinning of marginal layer; §§small cerebral hemispheres, collapsed ventricles.

pars intermedia; Fig. 6B); in other embryos the neurohypophysis had extended but compressed Rathke's pouch against the diencephalon floor plate (Fig. 6C,D). While the hypophyseal foramen was closed in RAS embryos (Fig. 6E,F), in 9/10 RAD embryos it remained wide open and suggested that the future anterior lobe of the pituitary would not detach from the stomodeal ectoderm (Fig. 6G,H). Interestingly, Rathke's stalk cells connecting the ectoderm and adrenohypophysis apoptosed in both RAS and RAD embryos (Fig. 6F,H), suggesting that signals mediating their ablation occurred regardless of whether the foramen had closed.

DISCUSSION

A model of acute targeted retinoid deficiency

The presented model permits analysis of retinoid-dependent events during defined gestational windows in mammals. Stringent retinoid-depletion creates dependence upon dietary retinoids; provision of a non-storage form such as RA allows for dietary withdrawal at any time to induce ligand deficiency, the rapidity of onset dictated by the supplement's halflife. Retinoid halflife, content and deficiency threshold varies with tissue, maternal retinoid status, gestational stage, species, strain, and intralitter (Ito et al., 1974; Takahashi et al., 1977; Wallingford and Underwood, 1987). Measurements suggest a halflife in the range of 14-16 hour in these RAD-VAD embryos; while it is possible that embryos were deficient for some fraction of time near the end of the 12 hour dose cycle, the fact that RAS controls were normal argues against this. Embryos are deficient by 27 hours after the last RA dose, with RA levels at 25% of control values. Extrapolation predicts RA levels at 48 hours (time of collection) in the range of 7.5 nM RA, roughly 10% of control values. The fractional clearance of RA is slower in RAD than in RAS rats (Napoli and McCormick, 1981) and in embryos as compared with maternal serum (Collins et al., 1995), and this may influence these embryos also. That we see deficiency symptoms (enlarged cardinal veins, reduced cranial flexure; S. Power, J. Lancman, S.M.S., unpublished observations) in embryos examined at 27 hours after their last dose, with RA content at 25% of controls, suggests there is a threshold effect for RA's functions in embryogenesis.

RA's apparent ability to support normal embryogenesis through E13.5 suggests RA and its derivatives are the major retinoid forms required for organogenesis. Retinol was not detected in these embryos, and the trace serum levels present in RAD/RAS dams (1-2% of chow values) argues against retinol derivatives making significant contributions to these embryos.

These RAD embryos exhibited defects observed in retinoid receptor null mutants but not previously displayed in retinoid deficiency, including craniofacial dysgenesis and limb malformations (Table 5; Lohnes et al., 1995). These defects may have been masked previously by lesser retinoid depletion and the inability to target developmental windows. Also, this model identified additional targets not previously observed in genetic mutants or prior deficiency models, such as cranial nerves, pituitary, limb buds, lens, and cranial flexure. Thus the conditional ligand 'knockout' presents a powerful approach for studies of retinoid roles throughout development and adulthood, as well as examination of ligand activity at the organismal level.

Retinoids are essential for cranial neural crest-derived populations

The RAD craniofacial dysmorphogenesis confirms a retinoid requirement for survival and differentiation of cranial neural crest derivatives. Both cranial ganglia cells and facial mesenchyme fail to expand and instead apoptose, suggesting an obligatory requirement for the hormone. Development and identity of cranial neural crest in quail is retinoid-dependent (Maden et al., 1996); here early patterning events occurred during retinoid-sufficiency and the observed dysplasia is at-

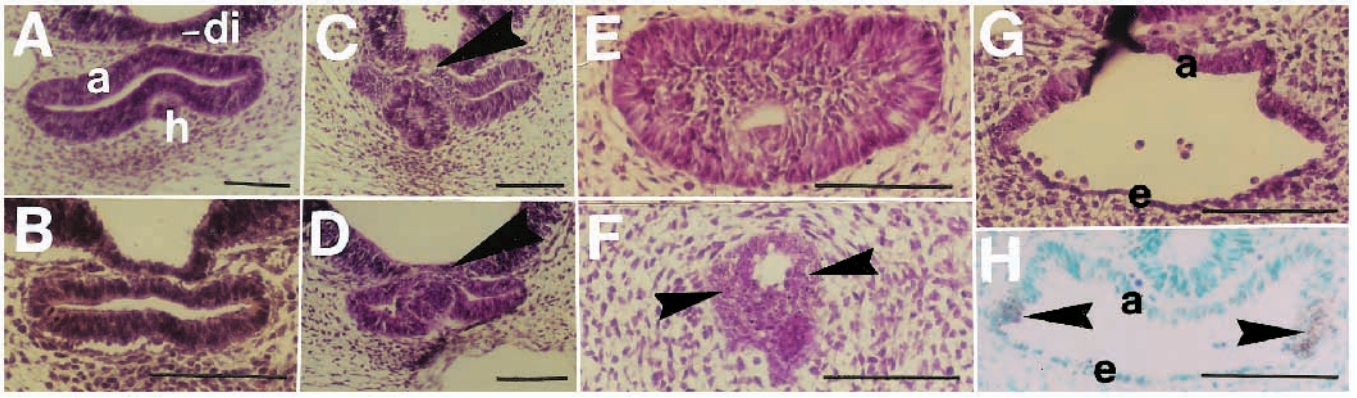

Fig. 6. Pituitary development in RAS and RAD. (A) Normal pituitary development in RAS, with appropriate contact between adrenohypophysis (a) and neurohypophysis (h) ventral to the diencephalon floor plate (di). (B) Absence of neurohypophysis extension to the posterior adrenohypophysis in RAD. (C,D) Aberrant adrenohypophysis compression against the diencephalon by the neurohypophysis (arrowhead). (E,F) Normal closure (E) and cell death (F, arrowheads) in Rathke's stalk of RAS. (G,H) Delayed hypophyseal foramen closure in RAD (G) although apoptosis of stalk cells connecting ectoderm (e) and adrenohypophysis (a) occurs on schedule (H), as detected by TUNEL staining (brown signal at arrowheads). Scale bar, 50 μm. a, adrenohypophysis; di, diencephalon floor plate; e, ectoderm; h, neurohypophysis.

tributed rather to an absolute requirement during their postmigratory expansion and differentiation. These findings also are consistent with previous pharmacologic and null mutant analyses, which strongly implicate retinoid involvement in cranial and trunk neural crest ontogeny (e.g. Morriss-Kay, 1993; Henion and Weston, 1994; Lohnes et al., 1994; Mendelsohn et al., 1994)

The retinoid-dependence of facial mesenchyme was position-specific and affected the upper and lower jaw populations disproportionately. Only the frontonasal/maxillary region was profoundly hypoplastic and apoptotic; no such derangements were detected in other facial arches; similar specificity was seen in RARαγ null mutants (Lohnes et al., 1994). The positional restriction of this response argues against an effect upon differentiation per se. While this model may have missed the susceptibility window for the mandibular and hyoid arches, in actuality these structures form in concert and have similar timing of outgrowth and fusion (Noden, 1991). The chick branchial arches also exhibit differing retinoid sensitivity; RA suppresses frontonasal mass outgrowth but does not inhibit mandibular development (Tamarin et al., 1984). The differential response described here may reflect a similar intrinsic property defined by facial segment identity.

Cranial nerve (CN) development is clearly retinoid dependent. Following retinoid deficiency CN X is absent and CN V and VII/VIII are dysmorphic and apoptotic; similar pyknosis occurs in dorsal root ganglia of retinoid-deficient quail (Maden et al., 1996), and RA promotes survival of neuronal derivatives of trunk neural crest (Henion and Weston, 1994). The impaired innervation of facial and thoracic targets might be attributed to CN cell death and delayed maturation, to a disrupted target environment, and/or to reduced cranial flexure, which increases the distance axons must travel to reach their targets (Altman and Bayer, 1982). The lack of cranial nerve defects in RAR and RXR null mutants (excepting CN VI deficits in RARα/γ mutants; Lohnes et al., 1994) suggests compensation by receptor redundancy. Thus quail and rat deficiency models detect a broader retinoid requirement for diverse events of cranial neural crest ontogeny (Maden et al., 1996).

RA is essential for primary lens cell maturation

Retinoid participation in primary lens cell maturation has not been reported previously; lens development in null mutants is largely normal. Normally, posterior lens cells activate crystallin expression and elongate anteriorly, forming crystallin fiber assemblies and closing the lens vacuole (reviewed by McAvoy, 1980). Instead, while RAD primary lens cells initiate α-, β- and γ-crystallin expression they do not elongate and instead apoptose, indicating failure in the lens differentiation pathway. Crystallin induction and lens elongation are separable events, the latter depending upon interactions with neighboring cells, crystallin assembly, cytoskeletal architecture, and neural retina signals (reviewed by Graw, 1996). Lens fiber assembly may also require correct proportions of the various crystallin isoforms; it is worth noting that γF-crystallin expression is RA-responsive (Tini et al., 1995). Abnormal lens fibers were noted in RARα$^{-/-}$/γ$^{-/-}$ but not described further (Lohnes et al., 1994). Whether RAD affects expression of crystallin isoforms must be determined.

Apoptosis is a common response to disrupted lens cell maturation, which normally depends on both internal and external signals (Graw, 1996). Lens expression of RARα, RARβ and CRBP suggests RA could contribute to its maturation and/or survival (Dolle et al., 1990). The adjacent neural retina synthesizes significant amounts of RA (McCaffery et al., 1992) and provides signals essential for lens survival; RA deficiency could disrupt retinal production of such signals. The similar phenotypes of RAD and growth factor-deprived lenses might represent retinoid involvement in these signalling events. Alternatively, failed lens fiber elongation may be sufficient to trigger lens cell apoptosis. Experiments are underway to distinguish such possibilities.

The disrupted retina development is consistent with ocular phenotypes observed in older VAD and retinoid receptor null mutants (Table 5), including retinal thinning, shortening and/or distortion, and for RARβ2/RARγ2 decreased neuroblast proliferation and increased apoptosis (Warkany and Schraffenberger, 1946; Kastner et al., 1994; Lohnes et al., 1994; Grondona et al., 1996). Retinal distortions observed in

our younger embryos are likely precursors to those later changes; that more differences are not observed may be due to these retinas' relative immaturity. The RAD retina also fails to invaginate and form the choroid fissure, through which the optic nerve exits, and the optic nerve precursors, the ganglion cell layer are not identifiable. Instead apoptotic cells are present where the ganglial cells ought to reside; whether these dying cells are ganglial cells cannot be determined except by their morphology, but their restricted position is highly suggestive. While the optic nerve does form in less-deficient rat embryos (Warkany and Schraffenberger, 1946) and RAR double-null mutants (Lohnes et al., 1994), it is reduced in diameter and subsequently degenerates; full penetration of the phenotype may only be observed in severe ligand deficiency.

Retinoids and pituitary morphogenesis

While pituitary differentiation is largely cell-autonomous, morphogenetic movements and contacts provide important contributions to its development (reviewed by Dubois and Hemming, 1991). Failured apposition between Rathke's pouch and the infundibulum can arrest anterior pituitary maturation. Observed delays in hypophyseal foramen closure and glandular/neural apposition suggest RAD could perturb pituitary development. The developing pituitary expresses RARα, RARβ, RXRγ and CRBP (Dolle et al., 1990; Mangelsdorf et al., 1992) and RARs comodulate a *pit-1* enhancer (Rhodes et al., 1993). Yet retinoid contributions to pituitary are poorly described. The pituitary protrusion into the pharynx observed in the RARα$^{-/-}$γ$^{-/-}$ could reflect a similar failure of Rathke's pouch closure and subsequent interruption of basisphenoid midline fusion (Lohnes et al., 1994). The combined findings suggest pituitary differentiation and function merits reexamination in RAD and receptor null mutants; such dysfunction could account for the reduced growth and impaired partuition observed in VAD (Rogers and Bieri, 1969; Mason, 1935; Bedo et al., 1989).

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