Retinoic acid influences the development of the inferior olivary nucleus in the rodent

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Abstract

All-trans retinoic acid (atRA) is an endogenous morphogen that regulates gene transcription. Maternal exposure to atRA results in severe developmental abnormalities by disrupting normal patterns of atRA distribution. Previously, we have shown that the pontine nucleus, which originates from the rhombic lip, is severely atrophied in the mouse on exposure to atRA at gestational days 9 and 10. In this study, we show that this same period of atRA exposure has the contrary effect on the inferior olive and this rhombic lip derivative is expanded in volume and probably contains an increased number of cells. The posterior region of the inferior olive maintains a relatively normal shape but is significantly expanded in size. In contrast, the organization of the anterior inferior olive is severely disrupted. Because endogenous atRA levels are known to be higher in the region of the posterior inferior olive at the time of birth of inferior olivary neurons, these results suggest that endogenous atRA may promote the generation, or select the fate, of posterior neurons of the inferior olive. In support of this concept, a reduction in atRA resulting from vitamin A deficiency results in loss of cells of the posterior inferior olive.

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Introduction

All-trans retinoic acid (atRA) regulates gene expression in the developing nervous system by activating specific receptors that are members of the nuclear receptor superfamily of transcriptional regulators (Bastien and Rochette-Egly, 2004; Maden, 2002). The influence of atRA on the early nervous system has been well studied in the formation of the anteroposterior axis of the neural tube (Durston et al., 1989; Thaller and Eichele, 1987; Tickle et al., 1982). Compelling evidence points to an essential role for atRA in the determination of the segmental pattern of the posterior hindbrain (Dupe and Lumsden, 2001; Dupe et al., 1999; Kolm et al., 1997; Maden et al., 1996; Marshall et al., 1992; Morris-Kay et al., 1991; Niederreither et al., 2000; Sakai et al., 2001; van der Wees et al., 1998; White et al., 2000b). A function for atRA at later stages of hindbrain development has been proposed in the mouse (Parenti and Cicirata, 2004; Yamamoto et al., 1998, 1999, 2003) and recently in zebrafish (Begemann et al., 2004; Linville et al., 2004). In the mouse, this atRA may be provided by the synthetic enzyme RALDH2 in the meninges (Zhang et al., 2003). One set of neurons that may be regulated by atRA are those that make up the cerebellar system. During development of the cerebellar system, the precursors of cerebellar neurons and the precerebellar nuclei that send afferents to the cerebellum lie within the dorsal rhombic lip (Altman and Bayer, 1987;
Sotelo, 2004). This primodium generates neurons that migrate ventrally to their appropriate positions. The subset of these neurons that migrate circumferentially under the meninges and over the surface of the hindbrain are constantly exposed, and respond, to atRA (Zhang et al., 2003). It would be predicted that excess amounts of atRA at the wrong time, place, or concentration would result in aberrant development. Certainly, exposure to 13-cis RA during human pregnancy results in malformation of the cerebellum as well as the pontine nucleus and the inferior olivary nucleus (IO) (Lammer and Armstrong, 1992) and the 13-cis isomer of RA likely acts through its isomerization to atRA (Nau, 2001). Likewise, in rats, the cerebellum and inferior olive are both sensitive to atRA exposure (Holson et al., 1997a,b).

We have previously shown in mice that the neurons that migrate around the exterior of the hindbrain to form the pontine nuclei are highly sensitive to teratogenic atRA, which disrupts their migratory pathway resulting in the loss of these nuclei (Yamamoto et al., 2003). In this study, we focus on the effects of atRA on the developing inferior olive, the progenitors of which migrate along a submarginal stream (Bourrat and Sotelo, 1988). Contrary to atRA’s effect on the pontine nuclei, we show that atRA can increase the size of the IO when treatment is at gestational days 9 and 10, preferentially acting on the posterior IO. This may reflect the high endogenous atRA levels of the posterior hindbrain (Smith et al., 2001; Zhang et al., 2003) and possibly a normal requirement of the posterior IO for these amounts of atRA.

Materials and methods

Animals and atRA treatment

C57Bl6 pregnant mice were purchased from SLC (Tokyo, Japan). The day when the plug was confirmed was designated as gestation day 0. A stock solution of 0.1 M atRA was prepared in DMSO and diluted in SFM101 culture medium (Nissui) with 10% FCS in 1:10 ratio. Diluted atRA solution (30 mg/kg b.w.) was injected intraperitoneally on 1 day—embryonic days 8, 9, or 10, or on two consecutive days—embryonic days 9/10, 10/11, 12/13, 14/15, and 16/17, or for 5 days—12–17. At embryonic day 18, the animals were deeply anesthetized with ether and decapitated and the embryos removed. Controls consisted of either DMSO injected animals (with the DMSO dissolved in the same vehicle at 1:10 dilution) or untreated animals. This research conformed to the stipulations of the animal experimental committee of the University of Tsukuba and the University of Massachusetts Medical School.

Histology

Brains of embryonic day 18 embryos were fixed in 4% paraformaldehyde, embedded in paraffin, or embedded in agarose (2.3%), cut into serial sections (5 μm for paraffin and 100 μm for agarose), and stained with cresyl violet. The brainstem and the cerebellum were examined histologically.

The wet weight of the brain was measured from the posterior edge of the olfactory bulb to the anterior edge of the C1 nerve root. To measure the volume of the IO, it was traced by using a camera lucida on each section. The surface area of the traced IO from every section was measured. The total volume of the IO nucleus was calculated as the total surface area × 0.1 mm (thickness of a section).

Measurement of the cell density of the IO

To determine the average cell density through the IO, a single embryonic brain from each injection schedule was embedded in paraffin, cut in 5 μm sections, and analyzed every 100 μm. The border of the IO was traced and the surface area was measured in these sections and the total neuronal number was counted within the circumference of the IO by the dissector method (Coggleshall and Lekan, 1996) at 40× magnification. Only neurons that had nucleoli were counted. The density was calculated for each section as the total neuronal number contained within the outline of the IO, divided by the area of IO, and the average was determined for all counted sections of each embryo.

For all statistical analysis, the Student’s t test was used with P < 0.01 or P < 0.05 values considered significant.

DIG-RNA labeling

Mouse OL-protocadherin (Hirano et al., 1999), and human Brn 3a probes (a gift from Dr. Mengqing Xiang, UMDNJ-Robert Wood Johnson Medical School, NJ) were labeled following the method described in the Boehringer-Mannheim DIG labeling kit. The final concentrations of the probes were 0.47–1 ng/ml.

For whole mount in situ hybridization, embryonic brains were fixed in 4% paraformaldehyde in PBS (pH7.5) overnight and stored in 100% methanol at −20°C for up to 3 weeks until use. Brains were placed in PBS with 0.1% Tween-20 (PBT), transferred through a methanol gradient (75%, 50%, 25% in PBT), and treated with proteinase K (Boehringer-Mannheim) (10 mg proteinase K/ml PBT) for 15 min. This was followed by incubation with 2 mg glycine/ml PBT for 10 min, a PBT wash (2 × 5 min), and re-fixation with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS. After the PBT wash, each sample was prehybridized with 1 ml of 50% formamide 25% 20× SSC (pH4.5), 1% SDS with 50 mg yeast RNA/ml and 50 mg Heparin/ml for 1 h at 70°C, followed by incubation with hybridization solution (prehybridization buffer plus DIG-labeled RNA probe (1 mg/ml) overnight at 70°C. Samples were then washed with solution 1(50% formamide, 1% SDS, 25% 20× SSC (pH4.5), in d.w.) 3 × 30 min at 70°C
and with solution 3 (50% formamide, 10% 20× SSC (pH4.5), in d.w.) 3 × 30 min at 65°C, followed by a 3 × 5 min PBT wash at RT.

Samples were preblocked with 10% Normal Goat Serum (Chemicon) in PBT for 1–3 h and incubated with alkaline phosphatase (AP) conjugated anti-digoxigenin antibody (Fab fragments, Boehringer-Mannheim) (1 μl/mlPBT/sample) preabsorbed with mouse embryonic powder. For the wash with TBST (0.8% NaCl, 0.02% KCl, 25 mM Tris–HCl pH7.5, 2 mM Levamisole, 0.1% Tween-20), the solution was changed every hour, 8–10 times at RT, and rocked overnight at 4°C. To visualize AP reaction, samples were incubated with NTMT (100 mM NaCl, 100 mM Tris–HCl pH 9.5, 50 mM MgCl₂, 0.1% Tween-20, 2 mM Levamisole) 3 × 10 min, then with BM Purple (Boehringer-Mannheim) for 20 min to 2 h in the dark. The reaction was stopped with 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.9% NaCl and the samples were stored in 4% paraformaldehyde (modified from (Riddle et al., 1993)).

For section in situ hybridization, the sections were first hydrated in 100%, 90%, 75%, and 50% ethanol and then 2× SSC for 1 min each. Sections were treated with proteinase K 10 mg/ml in 100 mM Tris (pH8.0) with 50 mM EDTA for 37°C for 8–10 min, rinsed in d.w., and incubated with 0.1 M triethanolamine with acetic anhydride for 10 min, followed by dehydration through an ethanol gradient and air-dried for 1 h. Hybridization solution was composed of 50% formamide, 10% dextran sulfate, 1% blocking solution (Boehringer-Mannheim), 7.2% of TEN buffer (5 ml; 1.0 M Tris pH 7.5, 30 ml; 5 M NaCl, 1 ml; 0.5 M EDTA) with DIG-RNA probe (0.47–1 ng/ml) boiled 10 min at 68°C and cooled on ice.

Hybridization solution was placed on sections (1 ng/80 μl/each slide), sealed with DPX mount (Fluka), and incubated at 55°C overnight. After incubation, DPX mount was peeled off and sections were soaked in 2× SSC until the coverslips separated from the slides. The slides were washed in 2× SSC for 5 min at RT followed by a wash in 50% formamide in 2× SSC at 55°C for 30 min and rinsing twice in 2× SSC at 37°C for 10 min. 20 mg/ml of RNaseA was added and slides were incubated for 30 min at 37°C and washed in 55°C RNase buffer for 30 min. For immunocytochemical detection of DIG, slides were preincubated with 2× SSC with 0.05% Triton X-100 and 1% blocking buffer (from Boehringer-Mannheim Genius kit 3) for 1–2 h at RT. After washing with maleate buffer (0.1 M, pH 7.5) 3× for 10 min, the slides were incubated with alkaline phosphatase labeled anti-DIG antibody (1:1000 in maleate buffer with 1% blocking reagent and 0.3% Triton X-100) overnight at 4°C. After washing in maleate buffer followed by alkaline phosphatase buffer (0.1 M Tris pH 9.5, 0.1 M NaCl and 50 mM MgCl₂) 3 times each for 10 min, the sections were reacted with BM purple (Boehringer-Mannheim) for 1–5 h at RT. After the reaction was stopped with 10 mM Tris pH 7.5, 1 mM EDTA, 0.9% NaCl for 30 min, sections were mounted in glycerol.

Generation of Vitamin A-deficient rats embryos

It is difficult to generate vitamin A-deficient mice because of their efficient use and storage of vitamin A. Embryonic vitamin A deficiency models however have been well defined in the rat (Clagett-Dame and DeLuca, 2002). Rats were rendered vitamin A-deficient (VAD) from weaning at 20–21 days, employing a purified diet devoid of vitamin A activity (Suda et al., 1970) but supplemented with vitamins D, E, and K (Council, 1995). The animals were maintained on this diet for 70–120 days before the first signs of deficiency were evident. When symptoms of vitamin A deficiency appeared (body weight plateau and xerophthalmia), the animals were supplemented with atRA at 12 μg/g diet and mated as described (White et al., 1998).

The vitamin A-deficient pregnant rats were maintained on an atRA sufficient diet of 12 μg/g atRA up to embryonic day 8.5. Dietary atRA was then increased to 250 μg/g between embryonic day 8.5 to 10.5, which is essential for heart development over that period (White et al., 2000a). After this period, atRA was reduced to 1.5 μg atRA/g diet to examine the effects of vitamin deficiency on hindbrain development. The control vitamin A-sufficient (VAS) pregnant rats were treated in one of two ways, either receiving vitamin A (retinyl palmitate, 500 units/day) together with 12 μg/g atRA in the diet from embryonic day 0.5 (VAS #1); alternatively, to match the 250 μg/g pulse of atRA used in the VAD treatment, a second control was used in which the rats were treated with 12 μg/g atRA in the diet from embryonic day 0.5 to 8.5 (VAS #2). This was followed by 250 μg/g of atRA between embryonic day 8.5 to 10.5 and retinyl palmitate from embryonic day 10.5 onwards.

Results

Morphological change

To determine the critical period for atRA’s interference with IO development, atRA was injected i.p. at 30 mg/kg into pregnant dams at a range of gestational time points, and the embryos examined at embryonic day 18. The number of animals used for each experiment is shown in Table 1.

Embryonic day 8 injection

The total brain weight (from olfactory bulb to the level of the exit of the C1 nerve) was smaller than the controls (Fig. 1). Furthermore, the length of the medulla in the anteroposterior axis appeared shorter than normal (Fig. 2) and therefore the relative ratio of anterior to posterior length of the IO versus the whole medulla seemed larger than normal. However, despite this significant alteration in the morphology of the medulla, the overall structure of the
IO did not show a drastic change (Fig. 2)—although slightly elongated in the anterior direction, the lamella structure was preserved and the total volume of the IO was about 118% that of controls (untreated and DMSO injected, Fig. 3). In contrast, the facial nucleus was diminished in size versus the controls (Fig. 2).

Embryonic day 9–10 injection

atRA exposure over this period gave the most obvious deformation of the IO. In the anteroposterior axis, the IO elongated from its usual position to nearly reach the level of the pontine nucleus (about 2.5 times the normal length) (Fig. 2). The total volume of the IO was 143% of that of the controls (Fig. 3). The overall shape of the posterior portion of the IO (posterodorsal and posteroverentral olive) resembled that of the controls but was significantly elongated over the anterior/posterior axis while the size of each lamella appeared somewhat smaller (Fig. 2). In contrast, the anterior region of the IO (anterior convoluted olive and anteromedial olive) was severely disrupted and the lamella structure was unidentifiable. The facial nucleus appeared to be entirely absent (Fig. 2).

Single day injections on embryonic days 9, 10, or 11

In order to identify the day when atRA was most effective, we injected atRA at either embryonic days 9, 10, or 11 (results not shown). Exposure to atRA at these single time points did not induce as significant an effect as the dual injections at embryonic day 9 and 10, although we observed slight elongation (by a factor 1.5 compared to 2.5 when injected over consecutive days) and lamella disarrangement of IO in embryonic day 9 and in embryonic day 10 atRA injected animals. Therefore, prolonged exposure to atRA over a 2-day period was necessary to induce the maximum change in the IO. This would indicate that the atRA sensitive molecular events that guide the normal development of the IO take place over a period beyond that of a single day.

Embryonic day 10–11 injection

The IO was anteroposteriorally elongated approximately twofold and the changes were, in essence, the same as found for atRA injections at embryonic day 9–10, with an elongated posterior region of relatively normal organization and highly disorganized anterior IO (Fig. 2). The magnitude of the changes, however, was smaller and the total IO volume did not differ from that of controls (Fig. 3). The shape of the facial nucleus appeared elongated in the anteroposterior axis but occupied a smaller area in each section.

Embryonic day 12–13 injection

Following this time of atRA treatment, the total IO volume decreased to 68% of that of controls (Fig. 3). Whereas the posterior IO exhibited relatively normal morphology, the anterior IO was severely underdeveloped in its lamella structure suggesting the majority of the cell loss may be focused to the anterior IO (Fig. 4). The facial nucleus appeared normal.

Embryonic day 14–15 injection

The change in the IO was similar to that following atRA exposure at embryonic day 12–13 but with less irregularity (Fig. 4). The total IO volume was about 84–90% of the control (Fig. 3) and the facial nucleus did not exhibit an obvious change.

Embryonic day 16–17 injection

No obvious change was observed (Fig. 4).
Embryonic day 12–17 (long-term) injection

The changes in the IO following extended atRA treat-
ment (Fig. 5) were similar to that observed after atRA
injections at embryonic day 12–13 (Fig. 4), including a
decline in the total volume to 68% (Fig. 3) of the control,
i.e. prolonged exposure to atRA did not augment the change
in the IO as compared to atRA exposure at embryonic day
12–13. When the long-term (more than 3 days) exposure to
atRA commenced at earlier stages, such as embryonic day
9, the embryos died in utero.

Cell density

To determine whether cell density of the IO changed with
RA treatment, the average density was determined through-
out the IO in a single embryo from the control and RA
treatment groups. The cell density of the IO was unaltered by
atRA injection at any age when compared to a control. The
cell density of the atRA injected group (mean cell density
among sections ± SD) was 8353 ± 460/mm² (embryonic
day 8), 7401 ± 926/mm² (embryonic day 9–10), 8111 ±
844/mm² (embryonic day 12–17), while the IO cell density
of untreated animals was 7849 ± 1205/mm². Therefore, the
volume change of the IO may largely reflect a change in cell
number. Thus, following atRA injections at embryonic day 8
and embryonic day 9–10, the IO volume was likely elevated
because the total cell number increased, whereas injections at
embryonic day 12–13 resulted in a decrease in the total IO
cell number.

IO projection to the cerebellum

The axons of the IO project to the contralateral cerebellum
by crossing the ventral midline and it was determined
whether the projection pattern was preserved in the atRA
injected animal (Fig. 6). DiI was injected in one side of the
embryonic day 18 cerebellum and labeled cells were
observed in the IO. In normal animals, the projection is
contralateral, and this pattern was preserved in atRA-injected
animals even in severely deformed cases (embryonic day 9–
10 injection), i.e. ipsilateral cells were never labeled.

The expression pattern of OL-protocadherin and Brn 3a

In order to confirm the identity of the cell mass that we
identified as the IO, Brn 3a expression by these cells was
examined. Brn3a and 3b are expressed in the proliferating
cells along the ventral wall of the fourth ventricle, and in IO
cells by gestational day 15.5 in the rat (Turner et al., 1994). Because Brn3b is down-regulated by RA (Turner et al., 1994), while Brn3a is unaffected, Brn3a was used in the study as a marker for the IO. In control animals, Brn3a labeled the entire IO in the gestation day 18 embryo (Fig. 7A). In embryonic day 9/10 RA injected animals, the anterior cell mass was also labeled (Fig. 7B), indicating that the distribution of cells of the IO extended up to the level of the pontine nucleus.

To investigate the events preceding this altered pattern of Brn3a labeled IO cells, the medulla of younger embryos (embryonic day 14) was examined following exposure to RA at embryonic day 9–10 (Figs. 7C and D). In the posterior-most portion of the IO in control animals, Brn3a staining spread laterally from the midline, forming a triangular shape with the base of the midline (Fig 7C). In the atRA-injected animals, however, the morphology of the IO changed with the migratory cell mass spreading laterally in more anterior regions of the IO (Fig. 7D, arrowhead) as well as being elongated rostrally (Fig. 7D, double-headed arrow). The difference in the spread of the ventral Brn3a positive cells following atRA exposure may suggest some scattering of the migratory path of these cells along the anteroposterior orientation, spreading the IO to an abnormally high degree along this axis.

To further confirm that the atRA induced changes identified by histological stain (Figs. 2, 4 and 5) were changes in neurons of the IO, a second IO marker was employed, OL-protocadherin (Olpc), a member of the protocadherin family of adhesion proteins (Hirano et al., 1999). Its mRNA transcript is detected in the anteromedial olive (AMO) and posteroventral olive (PVO) from embryonic day 15 up to postnatal stages (Hirano et al., 1999) as well as in the cerebellum, pontine nucleus, and the principle trigeminal sensory nucleus. Using this probe, we could trace the distribution of particular groups of IO neurons in atRA-injected animals. In control animals, the expression pattern of Olpc in the embryonic day 18 mouse by whole mount in situ hybridization is shown in Figs. 8A and D. Following atRA injections at embryonic day 8 (Fig. 8B), there was no Olpc expression observed in the principle trigeminal sensory nucleus, whereas, as found by histological analysis (Fig. 2), the expression pattern in the IO was similar to that of controls (Figs. 8A and D). After atRA injections at embryonic day 9–10, in both PVO and AMO, the Olpc positive region extended rostrally but with fainter expression in the AMO (Fig. 8C). A similar change, although to a lesser extent, was seen after injection of atRA at embryonic day 10–11 (Fig. 8E). The change of Olpc expression was similar to that observed by cresyl violet staining (Fig. 2). Treatment with atRA from embryonic day 12–17 had only a minor influence on Olpc expression in the PVO but levels in the AMO were again reduced (Fig. 8F). It is evident that the changes in Olpc mRNA distribution (Fig. 8) parallel the
changes seen by histological staining of the medulla (Figs. 2, 4 and 5). Furthermore, the rostral distribution of both IO markers Brn3a and Olpc after atRA treatment at embryonic day 9–10 or 10–11 implies that the abnormally positioned rostral cell mass originally seen by histological stain is derived from IO precursors.

Fig. 4. Morphology of the IO and facial nucleus of embryonic day 18 mouse embryos following treatment of animals with atRA at embryonic days 12–13, 14–15, 16–17 or non-treatment with atRA. Inferior olive (violet) and facial nucleus (red) are depicted using camera lucida drawings. Abnormalities in the IO were only observed following treatment at embryonic day 12–13, and these were similar to those following RA treatment at embryonic day 9–10 or 10–11 but milder in extent.

Fig. 5. Morphology of the IO and facial nucleus of embryonic day 18 mouse embryos following treatment of animals over an extended period with atRA between embryonic day 12–17 or treatment with DMSO vehicle over the same period. The changes in IO and facial nucleus following this lengthened exposure were similar to that observed after briefer atRA injections at embryonic day 12–13.
Effects of Vitamin A deficiency on posterior IO development

If excess atRA can promote expansion of the posterior IO, it would be expected that a deficiency in atRA would diminish this region of the IO. Although it is difficult to generate vitamin A-deficient mice and the null mutant of the RA synthesizing enzyme in the vicinity of the trunk, RALDH2, is lethal by embryonic day 10.5 (Niederreither et al., 1999), a well characterized vitamin A deficiency model exists in the rat (Clagett-Dame and DeLuca, 2002; White et al., 1998, 2000b). To provide an initial test of this hypothesis, embryonic day 21.5 rat embryos, depleted of vitamin A (VAD) from embryonic day 10.5, were examined in methyl green stained coronal sections (Fig. 9). These were compared to vitamin A-sufficient embryos that had been treated in a similar fashion but supplemented with vitamin A from embryonic day 10.5 onwards. Two types of control VAD embryos were examined, as detailed in the methods (VAS #1 and #2) but morphology of the IO seemed identical between the two and the comparison with the VAD embryos in Fig. 9 was only made with VAS #2. The convoluted anterior IO was normal in morphology.
following VAD treatment (Figs. 9B, D, aIO) compared to the VAS #2 control (Figs. 9A, C). However, the posterior IO was much smaller in the VAD treated animal (Figs. 9F, H) compared to the control (Figs. 9E, G). At higher magnification, the posteroventral olive is seen to be entirely absent after VAD treatment (Fig. 9I) compared to the control (Fig. 9I, pvO).

Discussion

Effects of atRA excess and deficiency imply a role for RA in IO development

atRA is essential for the early patterning of hindbrain rhombomeres over the embryonic day 7.5 to 8.5 period in the mouse (reviewed in (Begemann and Meyer, 2001; Gavalas, 2002; McCaffery et al., 2003)) and starting at embryonic day 9 in the rat (reviewed in (Clagett-Dame and DeLuca, 2002)). atRA synthesized by RALDH2 in the paraxial mesenchyme of the trunk diffuses to the posterior hindbrain to organize rhombomeres 4–7 that will constitute the myelencephalon. Excess atRA from embryonic day 7.5 to 8.5 in the mouse disturbs the normal balance of high posterior and low anterior atRA and transforms anterior rhombomeres to take on posterior characteristics. We have previously identified a second critical period of hindbrain atRA sensitivity in the mouse between embryonic 9.5 and 10.5 (Yamamoto et al., 2003). Exposure at this time results in loss of the pontine nuclei in the anterior hindbrain due to altered migration of neurons from the precerebellar primordium. We now show that excess atRA has a very different influence on more posterior hindbrain nuclei. Focusing on the IO, we show that there is an elongation of this nucleus in the anterior direction. Cells of the IO identified by Brn3a expression were found to extend to the level of the pons. The time of atRA exposure that results in this expansion of the IO (embryonic day 9/10 or 10/11) is after the period at which rhombomere identity is set. For instance, the genes that mediate atRA induced posteriorization include Hoxb1 and Hoxb2 and anterior induction of neither Hoxb1, b2, or b4 occur after embryonic day 9 (Conlon and Rossant, 1992). This implies that the expansion of the IO is not simply a switch of anterior rhombomeres to a posterior fate but is part of a later event of determination.

Excess atRA results in an increase in IO cell number, primarily in the posterior IO

The finding that the total IO volume increased with apparently no change in cell density in the embryonic day 9/10 atRA-injected embryo implies that the total number of IO cells increased. The time of atRA injection that results in elongation of the posterior IO, between embryonic days 9/10 and 10/11, is just before or at the earliest point when the cells of the IO in the mouse become post-mitotic, at approximately embryonic day 10–12 (based on the embryonic day 12–14 period of birth in the rat, (Altman and Bayer, 1997), indicating that the period at which the progenitors are sensitive to atRA is around their initial point of birth and

Fig. 8. In situ hybridization of OL-protocadherin (Olpc) expression in the embryonic day 18 IO following atRA treatment. In either the DMSO vehicle injected control (A) or the un.injected control (D), Olpc is expressed in anterior (anteromedial olive, AMO) and posterior (posteroventral olive, PVO) divisions of the IO (labeled by two arrows). Olpc is also present in the trigeminal sensory nucleus (V). Treatment with atRA at embryonic day 8 resulted in little change in Olpc in the IO (B) whereas treatment at embryonic day 9–10 resulted in significant elongation of the divisions of the IO (C). A less pronounced elongation resulted from treatment at embryonic day 10–11 (E). Treatment with atRA at embryonic day 12–17 had only a minor influence on Olpc in the posteroventral olive but expression in the anteromedial olive was significantly reduced. All the panels are the same magnification and panel C scale bar is 1 mm.

migration. As already discussed, most studies on the teratological effects of atRA on the hindbrain have focused on the embryonic day 7.5 to 8.5 period when atRA disrupts rhombomere organization. However, the time of exposure used in this study was subsequent to the phase of rhombomere specification. Indeed, atRA exposure at embryonic day 8 has only a relatively mild influence on the morphology of the IO. It is evident that the hindbrain is still sensitive to atRA after embryonic day 9.5. If cells in the posterior hindbrain respond to atRA, this may suggest that endogenous atRA influences their normal development. Certainly, atRA is generated in the vicinity of the posterior hindbrain by the synthetic enzyme RALDH2 in mesenchyme along the trunk (Niederreither et al., 1997; Zhang et al., 2003). Although mesenchymal RALDH2 expression does not extend to parallel the posterior hindbrain birthplace of the

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**Fig. 9.** Reduction of the posterior inferior olive in the vitamin A-deficient rat embryo. The morphology of the anterior inferior olive (aIO) is relatively normal in both vitamin A-sufficient (VAS) conditions (A, C) as well as vitamin A-deficient (VAD) conditions (B, D). This contrasts with the posterior inferior olive (pIO), which is normal in the VAS rat embryo (E, G) but is severely diminished in the VAD embryo (F, H). At higher magnification, the posterodorsal (pdO) and the posteroverentral (pvO) olive are clearly evident in the VAS embryo (I) but the posteroverentral olive is entirely absent and the posterodorsal olive diminished in the VAD embryo (J). Panels A–H are the same magnification and panel H scale bar is 1 mm. Panels I and J are the same magnification and panel J scale bar is 200 μm.
IO, i.e. rhombomeres 7 and 8 (Ambrosian et al., 1996; Marin and Puelles, 1995), it has been shown by Smith et al. (Smith et al., 2001) that atRA diffuses anteriorly and is transcriptionally active in these posterior rhombomeres at least up to embryonic day 10.5. Thus, it is likely that atRA continues to play a function in posterior hindbrain development beyond embryonic day 8.5 and the IO and its progenitors will be exposed to endogenous RA. Results using a transgenic RA reporter mouse line indicate that subregions of the IO respond to endogenous RA (Zhang et al., 2003). The closer proximity of the posterior IO to the posterior source of RA from RALDH2 in the trunk mesenchyme might imply that this region of the IO has the highest requirement for RA. It is possible then that RA may promote specification of the fate of these neurons or may act as a trophic factor for posterior IO cells—excess RA applied at embryonic days 9 and 10 may lead to an over-generation or switch in fate of posterior IO cells and increase in number of cells. Supporting this hypothesis is the loss of the posteroventral olive and much of the posteroventral soma when the substrate for atRA, vitamin A, is depleted in the rat after embryonic day 10.5 (equivalent to approximately day 9.0 in the mouse). Vitamin A depletion will result in loss of atRA at the time the inferior olive progenitors are born in the rat at embryonic days 12–14 (Altman and Bayer, 1997), indicating that a vitamin A product, presumably atRA, is necessary for the formation of the posterior IO.

**Excess RA interferes with the morphology of the anterior IO**

Regional differences in the sensitivity of the IO to atRA not only exist between the anterior versus posterior IO but also in the dorsal versus ventral axis. On exposure to excess atRA, the expansion of the IO predominantly occurs in the posteroventral segment whereas the posteroventral olive can no longer be distinguished. When atRA is diminished in the VAD rat embryos, it is the posteroventral olive that is entirely eliminated. It may be relevant that the lamella of the anterior IO which, like the posteroventral olive, is born at around embryonic day 10–11 in the mouse (based on the embryonic day 12–13 period of birth in the rat, (Altman and Bayer, 1997)), is also severely diminished in size after exposure to excess atRA. Indeed, the anterior lamellar structure of the IO shows greatest sensitivity to atRA at embryonic day 10–11 suggesting that atRA interferes with differentiation of these cells at the point they become post-mitotic.

**Other hindbrain abnormalities result from excess atRA**

The increase in cell number of the IO does not account for its expansion along the anterior axis by a factor of 2–3 times that of the control animal implying that there are also abnormalities in the migration of the cells of the posterior IO, in particular along the anteroposterior axis. A change in the migratory pattern along this axis is one example of a similarity with the effects of atRA on the pontine nuclei (Yamamoto et al., 2003) in which case the normally compact stream of cells that migrate to form the pontine nuclei scatter over the anterior medulla.

The teratogenic effect of atRA on precerebellar nuclei such as the IO and pontine nuclei does not imply that RA only influences this subset of hindbrain neurons. Exposure to atRA at embryonic day 9–10 resulted in complete loss of the facial nucleus, the neurons of which derive from the floor of the fourth ventricle (Altman and Bayer, 1980; Auclair et al., 1996). The progenitors migrate caudally from their birthplace and it is interesting that exposure at E10–11 leads to elongation of this structure, suggesting RA may influence this migration. More intensive study of this nucleus however will be necessary to understand these two quite different effects of atRA but they imply that RA influences the facial nucleus. A recent study on zebrafish demonstrated that a deficiency of atRA, at a time that does not influence rhombomere patterning, interferes with migration of facial branchiomotor neurons (Linville et al., 2004). It is probable then that the development of the facial nucleus, like the IO, is regulated by endogenously synthesized atRA. In the zebrafish, the facial branchiomotor neurons depend on atRA synthesized by RALDH2 in the paraxial mesoderm (Linville et al., 2004) but in the chick the enzyme itself is expressed transiently in the facial/vestibulocochlear nucleus, so species difference may exist regarding the source of RA. Other derivatives of ventricular progenitors are also potential targets for regulation by RA, including the vagal nerve (Begemann et al., 2004; Linville et al., 2004) in which branchiomotor neurons are reduced in the atRA-deficient zebrafish and the vestibular nerve, which expresses RALDH2 in the mouse (Zhang et al., 2003). These will also of interest for future investigation comparing the effects of atRA excess and deficiency after the period of rhombomere patterning.

**RA teratogenicity in the human hindbrain**

Although animal models of atRA teratogenicity have emphasized the dramatic effects of early (embryonic day 7 and 8) exposure, it is notable that the teratogenic effects of 13-cis RA on the human brain, specifically abnormalities of the pontine nuclei and inferior olive (Lammer and Armstrong, 1992), more resemble the effects we describe following atRA exposure at embryonic day 9–10. An explanation for this is that early exposure of the human embryo to 13-cis RA will result in severe abnormalities resulting in early embryonic death whereas later exposure, resulting in pontine nuclei and inferior olive malformations (Yamamoto et al., 2003), may not result in abnormalities so severe as to trigger early embryonic death and hence these changes are more likely to be seen in the fetus. Postnatal death, however, frequently occurs presumably due to medullary malformations and degradation in the control of the autonomic nervous system. Similarities exist between atRA exposure, during these later...
development stages, and several human developmental brain disorders. One is Joubert’s syndrome, a relatively rare, autosomal-recessive disease defined by vermal agenesis and malformation of multiple brainstem structures, together with abnormal breathing patterns or abnormal eye movements (ten Donkelaar et al., 2000; Yachnis and Rorke, 1999). A second disorder is fetal alcohol syndrome, which has also been reported to include abnormalities in the cerebellar vermis (Archibald et al., 2001; Pierce et al., 1993), brainstem (Johnson et al., 1996), and inferior olive (Johnson et al., 1996; Swayze et al., 1997). It has been suggested that one mechanism by which alcohol interferes with embryonic development occurs through disruption of atRA signaling (Duester, 1991; Pullarkat, 1991; Zachman and Grummer, 1998). Our results suggest that the human gestation period equivalent to mouse embryonic day 9–10 will be a sensitive time for genetic or teratogenic disruption of hindbrain development.

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