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

Miyuki Yamamoto[a,1], Masahiro Fujinuma[a,1], Shinji Hirano[b], Yoshika Hayakawa[a], Margaret Clagett-Dame[c], Jinghua Zhang[d], Peter McCaffery[d,*]

[a]*Inst. Basic Medical Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan*
[b]*RIKEN Center for Developmental Biology, Chuou-ku, Kobe 650-0047, Japan*
[c]*Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706-1544, USA*
[d]*UMMS/E.K. Shriver Center, 200 Trapelo Road, Waltham, MA 02452, USA*

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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.

Keywords: Hindbrain; Migration; Teratogenesis; Hyperplasia; Vitamin A deficiency; Facial nucleus

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;

* Corresponding author. Fax: +1 781 642 0116.
E-mail address: peter.mccaffery@umassmed.edu (P. McCaffery).
[1] These authors contributed equally to this work.

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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 (Coggeshall 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