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Morphology of the facial motor nuclei in a rat model of autism during early development

Akiko Oyabu, Yasura Tashiro*, Takahiro Oyama, Kensaku Ujihara, Takeshi Ohkawara, Michiru Ida-Eto, Masaaki Narita

Department of Anatomy II, Graduate School of Medicine, Mie University, Japan

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ABSTRACT

The development of facial nuclei in animal models of disease is poorly understood, but autism is sometimes associated with facial palsy. In the present study, to investigate migration of facial neurons and initial facial nucleus formation in an animal model of autism, rat embryos were treated with valproic acid (VPA) *in utero* at embryonic day (E) 9.5 and their facial nuclei were analyzed by *in situ* hybridization at E13.5, E14.5 and E15.5. Signals for *Tbx20*, which is expressed in early motor neurons, appeared near the floor plate at the level of the vestibular ganglion and extended caudolaterally, where they became ovoid in shape. This pattern of development was similar between control and VPA-exposed embryos. However, measurements of the migratory pathway and the size of the facial nuclei revealed that exposure to VPA hindered the caudal migration of neurons to the facial nuclei. Signals for *cadherin 8*, which is expressed in mature facial nuclei, revealed that exposure to VPA caused a significant reduction in the size of the facial nuclei. Our findings provide the first quantitative description of tangential migration and nucleus formation in the developing hindbrain in a rat model of autism.

1. Introduction

Neural migration is one of the pivotal first steps in precisely establishing the neural network, and both radial and tangential migrations contribute to the organization of the cortex into layers and nucleus formation in the developing brain. Numerous recent studies comparing the time courses of neural migration in wild-type and transgenic animals have revealed that a combination of molecules regulates the direction of neural migration (Bloch-Gallego et al., 2005; Chédotal and Rijli, 2009; Chédotal, 2010; Hatten, 1999; Huang, 2009).

Developing facial nuclei migrate tangentially within the hindbrain. During early development, neurons that will eventually form the facial nuclei are generated in rhombomere 4 (r4) then migrate caudally to r6 to the site of the facial nuclei (Chandrasekhar, 2004; Hatten, 1999; Noden, 1993; Yamamoto and Schwarting, 1991). Studies using transgenic mice, both *in vivo* and *in vitro* with cells derived from these mice, have identified some of the molecules that are responsible for regulating tangential migration (Chédotal and Rijli, 2009; Huang, 2009). However, the development and migration of neurons to the facial nuclei are poorly understood in animal models of disease.

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairments in social interaction and communication, and are associated with repetitive behaviors and interests (Charman and Baird, 2002; Filipek et al., 1999). An association of autism with facial nerve (7th cranial nerve) palsy has been described in several cases of thalidomide embryopathy, Möbius sequence, CHARGE association and Goldenhar syndrome (Gillberg and Winnergård, 1984; Miller et al., 2005; Ornitz et al., 1977). Given this association, a more precise understanding of the development of the facial nuclei, from which facial nerves originate, in ASD patients, could potentially be indispensable for elucidating the pathogenesis of autism with facial palsy.

Rodent models of autism have been particularly useful for elucidating the association of autism with embryonic development of the nervous system. Epidemiological studies had revealed that exposure to thalidomide (THAL) or valproic acid (VPA) during the first trimester of pregnancy causes a higher incidence of autism in human offspring (Strömland et al., 1994; Williams et al., 2001); based on this, a rat model of autism was generated by prenatal THAL or VPA exposure (Narita et al., 2002). In studies using this model, behavioral, biochemical and neuroanatomic similarities between human cases of autism and rats exposed to VPA *in utero* were observed (Ingram et al., 2000; Miyazaki et al., 2005; Narita Naoko et al., 2002; Narita Masaaki et al., 2010; Rodier et al., 1996, 1997).

* Corresponding author at: Department of Anatomy II, Graduate School of Medicine, Mie University, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel.: +81 59 231 9938; fax: +81 59 231 9938.
E-mail address: ytashiro@doc.medic.mie-u.ac.jp (Y. Tashiro).

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Thus, our rat models of autism are well suited for investigations of the embryonic development of the nervous system.

Although the embryonic development of facial nerves in our rat model of autism has not been fully characterized, embryonic VPA treatment has been shown to reduce the number of adult motor neurons, including those of the facial nuclei (Rodier et al., 1996), resulting in anatomical anomalies within the cranial motor nuclei. Moreover, we previously reported on morphological abnormalities in the peripheral facial nerves in an embryonic rat model of autism (Tashiro et al., 2011). In these rats, peripheral facial nerves became truncated and defasciculated (Tashiro et al., 2011). In addition, Ornoy (2009) reviewed the relationship between VPA and autism, including experiments using VPA to generate animal models of autism, and concluded that experimental animal models generally mimic the effects of VPA in man, although animals seem to be more resistant to VPA than humans. Taken together, these findings suggest that the development of facial neurons, not only in the peripheral nerves but also in the central hindbrain, including the facial nuclei, should be examined after VPA exposure.

In the present study, to examine the caudal migration of neurons and the initial formation of facial nuclei in a rat model of autism, VPA was administered to pregnant rats at E9.5. This enables delivery to the embryos *via* the placenta and subsequent distribution to the facial nuclei. We utilized a combination of flat whole-mount preparations and *in situ* hybridization for molecular markers expressed in the cranial motor neurons to allow us to clearly identify the facial nuclei and quantify their size for statistical analysis.

2. Material and methods

2.1. Animals and teratogen exposure

All experiments involving animals were approved by the Community of Laboratory Animal Research Center at the University of Mie, Japan. Details of the teratogen administration have been previously described (Miyazaki et al., 2005; Narita Naoko et al., 2002; Narita Masaaki et al., 2010). In brief, female Wistar rats (2–6 months old) were mated overnight and the day of insemination was designated as embryonic day (E) 0.5. On E9.5, at 1:00 p.m., 800 mg/kg VPA was administered orally without sedation to dams in each group using an infant feeding tube (Atom Medical, Tokyo, Japan) attached to a 2.5 ml disposable syringe. We referred to prior animal experiments using these teratogens to determine the doses of VPA (Ingram et al., 2000). Because our previous study showed that VPA exposure after E9.5 induced morphological abnormalities of facial nerves (Tashiro et al., 2011), we adopted E9.5 as the day of administration. VPA was prepared by dissolving the drug in 5% arabic gum in distilled water. No pregnant mothers died from the dose of VPA, and most embryos survived.

2.2. Flat whole-mount preparations of rat hindbrain

Embryos were removed from the dams at E13.5, E14.5 or E15.5, and the crown–rump length (CRL) was measured at each stage. The portion of the head containing the midbrain and hindbrain was dissected out in cold phosphate-buffered saline (PBS). To obtain flat whole-mount preparations of the hindbrain, the dorsal midline of the neural tube was completely cut and the 4th ventricle was opened. The isthmus of the midbrain–hindbrain boundary was cut, and the hindbrain was carefully freed from the meninges and surrounding tissue while keeping the trigeminal ganglion and vestibular ganglion, as well as their roots, intact.

Flat whole-mounted hindbrains were fixed in 4% paraformaldehyde in PBS for 7–10 h at 4 °C. Tissues were then washed twice with Tris-buffered saline (TBS), dehydrated through a graded methanol/TBS series at 4 °C, and stored in 100% methanol at −20 °C.

In observing tissues under a microscope, to ensure equal pressure across whole-mounted hindbrains, multiple layers of plastic tape were inserted as supports between the slide and coverslip. Because the layered plastic tape supports the load from the coverslip, the preparations received equal pressure in all locations and were not transformed by unequal pressure.

2.3. In situ hybridization

A DNA fragment corresponding to a portion of rat *Tbx20* (nucleotides 51–885, GenBank NM_001108132) or *cdh8* (nucleotides 1909–2435, GenBank NM_053393.2) cDNA was cloned into the pGEM-T Easy vector (Promega, La Jolla, CA). Using this plasmid as a template, sense and antisense single-strand RNA probes were synthesized using a digoxigenin labeling kit (Roche Diagnostics, Tokyo, Japan). Whole-mount *in situ* hybridization using sense probes detected no signal except background noise, demonstrating specific hybridization to the target sequences.

In situ hybridization on flat whole-mounted hindbrains was performed according to the methods of Nieto et al. (1996), with minor modifications. Tissues were treated with 0.3% H_2O_2/methanol for 30 min and rehydrated through a graded methanol/PBST series. The hindbrains were then treated with 10 μg/ml proteinase K/PBST and fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBS. After rinsing three times for 5 min in PBST, they were prehybridized in prehybridization solution (50% formamide; 5 × SSC; 50 μg/ml yeast RNA (Roche); 50 μg/ml heparin (Roche); 5 mM EDTA; 1% SDS). Tissue was then incubated in hybridization solution (prehybridization solution containing 1 mg/ml probe) overnight at 60 °C. After high-stringency washes, the tissues were blocked for 2 h in blocking reagent (Roche) and incubated in a 1/500 dilution of anti-digoxigenin-AP conjugate (Roche) in blocking reagent for 3 h. After an overnight wash with TBST, the signals were visualized in NTMT (0.1 M Tris–HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, and 0.1% Tween 20) containing nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche).

2.4. Statistical analysis

Embryos from at least three different dams were analyzed for each condition. Bilateral facial nuclei were analyzed in each embryo and the number of analyzed facial nuclei pairs is referred to in the text as "*n*=". Digital images were captured using a light microscope equipped with a CCD camera (DXM1200F, Nikon, Tokyo, Japan). The intensities of *Tbx20* or *cdh8* signals and the sizes of the areas circumscribed by these signals were measured using Image J software. Signal intensity was defined such that complete white was scored as "0" and complete black as "255". The relative level of signal intensity was determined by comparison with the signal intensity in the control group. The boundaries of these regions were determined based on the signal threshold, excluding background noise. The mean and standard deviation (SD) was calculated, and differences among developmental stages were evaluated using *t*-tests.

3. Results

3.1. Development of Tbx20-positive facial motor neurons

To elucidate the development of the cranial motor nuclei within the hindbrain, in relation to the rostrocaudal and dorsoventral axes, flat whole-mount preparations (Fig. 1) from E13.5, 14.5 and 15.5 embryos were analyzed. Flat whole-mount embryonic hindbrain preparations were subjected to *in situ* hybridization using an RNA probe for *Tbx20*, which is expressed in early cranial motor neurons (Figs. 2 and 3).

In whole-mount preparations from control embryos at E13.5, intense *Tbx20* signals were observed near the floor plate between the vestibular ganglia, corresponding to r4 at the rostrocaudal level (Fig. 2A). Weak *Tbx20* signals were observed in the ventral column lateral to the floor plate (data not shown). In whole-mount preparations from embryos exposed to VPA *in utero*, the pattern of *Tbx20* expression was similar (Fig. 2B).

At E14.5 in the control group, *Tbx20* signals extended from r4 through r5–6 in a caudolateral direction (Fig. 2C, arrows). Toward the caudal end of the *Tbx20* expression domain, the signal faded out (Fig. 2C, arrowheads). These signal patterns were similar to those observed in the VPA-exposed group (Fig. 2D).

Because flat whole-mount preparations are much thicker at E15.5 than they are at earlier stages, *Tbx20* signals could be observed more clearly from either the ventricular or pial sides (Fig. 3). In the control group, despite limited *Tbx20* expression near the floor plate on the ventricular side (Fig. 3A, arrows), the caudal end of the *Tbx20* signals was located on the pial side and had an ovoid appearance (Fig. 3C, asterisks). In addition, intense *Tbx20* signals were also observed between the trigeminal ganglia, corresponding to r2 at the rostrocaudal level on the pial side (Fig. 3C and D, arrowheads).

In the VPA-exposed group, the pattern of *Tbx20* expression was similar (Fig. 3B and D). However, the signals near the floor plate were more intense than those in the control