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Original article

Maternal viral infection during pregnancy impairs development of fetal serotonergic neurons

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Abstract

Background: Maternal viral infection during pregnancy induces morphological abnormalities in the fetus and may cause emotional and psychological problems in offspring through unknown mechanisms. We have previously shown that prenatal exposure of rats to chemicals such as thalidomide causes an autistic-like phenotype in offspring, indicating that prenatal events affecting serotonergic development may cause developmental disorder. *Methods:* We investigated whether prenatal viral infection altered the expression of neurotransmitters involved in the emotional or psychological status of offspring. We here took advantage of the polyriboinosinic:polyribocytidylic acid (poly I:C) system, the synthetic double-stranded RNA, which is often used in animal models of viral infection. *Results:* Ten mg/kg of poly I:C was intraperitoneally injected on gestational day (GD) 9 and counted the numbers of serotonin-immunopositive cells on GD15 using flat whole-mount preparation method, resulting 11.1% of increase in the number of serotonergic neurons in poly I:C group. Furthermore, there was a significant decrease in hippocampal serotonin content in offspring by postnatal day 50 following poly I:C administration by high-performance liquid chromatography. *Discussion and Conclusion:* Since serotonin is known to link with behavior and emotion after birth, these results suggest that maternal viral infection might cause, in addition to morphological abnormalities, serotonin-related pathogenesis such as neurodevelopmental disorders including autism spectrum disorders.

Keywords: Maternal viral infection; Autism; Poly I:C; Serotonin

1. Introduction

Maternal viral infection during pregnancy induces morphological abnormalities in the fetus [1,2]. For example, congenital rubella syndrome caused by rubella virus infection during pregnancy, induces fetal cataracts, cardiac defects, and deafness [3]. Cytomegalovirus infection during pregnancy induces intrauterine growth retardation, microphthalmia, microcephalia, and cerebral calcification [4]. In addition to morphological abnormalities, maternal infection may also induce emotional problems in offspring such as schizophrenia [5,6] and mental retardation [7]. However, the mechanisms responsible for these disorders are unknown.

Autism spectrum disorders (ASD) are neurodevelopmental disorder including cognitive and emotional problems [8,9]. The etiologies of ASD are unknown; however, several lines of evidence suggest that disturbances in the embryonic development of serotonergic

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neurons may be the cause [10–12]. We have shown that embryonic disturbance of serotonergic development induced by thalidomide or valproic acid may cause an autistic phenotype in the offspring of treated rats [13,14] as well as by viral infection.

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter involved in emotional and mood control [13,15]. Clusters of serotonergic neurons are located in the raphe nuclei of the brainstem. Rostral raphe nuclei start to express the serotonergic neuron phenotype on approximately gestational day (GD) 12 [16]. Ascending serotonergic neurons are first observed on the same day as rostral raphe nuclei, and then they promptly develop diverse axonal projection networks throughout the brain until birth [17].

In the present study, we investigated whether prenatal viral infection changes the serotonergic system. We took advantage of the polyriboinosinic:polyribocytidylic acid (poly I:C) system, which is often used as an animal model of viral infection [18–20].

2. Materials and methods

2.1. Administration of poly I:C

Pregnant Wistar rats were obtained from CLEA Japan, Inc. (CLEA Japan, Inc., Japan). All animal experiments were approved by the Committee of Laboratory Animal Research Center at Mie University. Ten mg/kg of poly I:C (Sigma Aldrich, MO), which is dissolved in sterile phosphate-buffered saline (PBS), or sterile PBS alone was intrapenitorially (i.p.) injected to GD 9 or 10 pregnant Wistar rats. The dose of poly I:C was determined according to a previous study [21].

2.2. Measurement of monoamines and their metabolites using high-performance liquid chromatography (HPLC)

Measurements of monoamines and their metabolites on postnatal (P) day 50 were performed as described previously [22]. Experiments were performed independently twice, and consistent results were obtained from these two experiments. Statistical evaluation was performed using independent Student's t tests. Statistical significance was defined as $p < 0.05$ (two-tailed).

2.3. Flat whole-mount preparation of rat brain and determination of the number of serotonin-immunopositive cells

Flat whole-mount of hindbrains on GD15 was prepared as described previously [23]. GD9 pregnant rats were i.p. injected with poly I:C, and the GD15 fetuses were surgically removed. The embryonic brains were cut along the dorsal midline, opened, and whole-mounted flat with the ventricular side down. The flat, whole-mounted brain was fixed with 4% paraformaldehyde (PFA) overnight at 4 °C and probed with an anti-serotonin antibody as described previously [23]. The numbers of all serotonin-immunopositive cells localized in rostral and caudal clusters were blindly counted to exclude the possibility of bias.

2.4. Quantitative analysis of gene expression during the development of serotonergic neurons

Cranial regions of GD12 embryos were exactly cut just posterior to the forth ventricle, and five embryos were collected from four of each of the pregnant mothers in the two groups. Total RNAs were prepared using TRIzol Reagent (Invitrogen, CA) according to the manufacturer's instructions. One microgram of each total RNA preparation was reverse-transcribed using QuantiTect Reverse Transcription Kit (QIAGEN, Germany) according to the manufacturer's instruction. Real-time PCR reactions using Power SYBR Green PCR Master Mix (Applied Biosystems, CA) were performed using a StepOne Real-Time PCR System (Applied Biosystems, CA). Data were analyzed using the comparative cycle threshold method. *Gapdh* was used as an internal control. Oligonucleotide sequence used for real-time PCR were as follows: *sonic hedgehog* (*Shh*) forward primer:5′-AGCTTCGAGTGACTG AGGGC-3′*Shh* reverse primer: 5′-GTCCCTGTCAG ACGTGGTGA-3′, *fibroblast growth factor 8* (*Fgf8*) forward primer: 5′-CGCAAAGCTCATTGTGGAGA-3′, *Fgf8* reverse primer: 5′-ACACGCAGTCCTTGCCT TTG-3′, *Gata2* forward primer: 5′-AGCCTTGTGCC GCCATTAC-3′, *Gata2* reverse primer: 5′-ACCTGTC CATTTTGCTCTCCA-3′, *Pet-1* forward primer: 5′-TC CCACGACCTACTCAAAC-3′, *Pet-1* reverse primer: 5′-AGGGATGGACAACAGCAGAG-3′, *Gapdh* forward primer: 5′-CAAGTTCAACGGCACAGTCAA G-3′, *Gapdh* reverse primer: 5′-ACATACTCAGCACC AGCATCAC-3′.

2.5. Whole-mount in situ hybridization

Eight embryos from three mothers were used for each group. Whole-mount *in situ* hybridization of GD12 embryos using digoxigenin-labeled complementary RNA (cRNA) probes was performed following Wilkinson's method [24]. In brief, dissected embryos were fixed with 4% PFA in PBS overnight at 4 °C; PFA was removed with a graded-concentration ethanol series, and the samples were stored at −20 °C. Samples were gradually rehydrated and immersed in 0.1% Tween 20 (Sigma–Aldrich) in PBS (PBST). Next, samples were treated with 10 μg/ml proteinase K (Roche Applied Science, IN) for 30 min at 37 °C, fixed again in 0.2% glutaraldehyde, 4% PFA in PBS for 20 min. After two washes with PBST, samples were prehybridized for one hour at