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Behavioral abnormalities of fetal growth retardation model rats with reduced amounts of brain proteoglycans

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ABSTRACT

Fetal growth retardation (FGR) is a critical problem in the neonatal period, because a substantial population of infants born with FGR go on to develop various developmental disorders. In the present study, we produced FGR model rats by continuous administration of a synthetic thromboxane A_2 analogue (STA_2) to pregnant rats. The FGR pups exhibited a significant delay in postnatal neurological development. Moreover, behavioral analyses revealed the presence of a learning disability in juvenile FGR male rats. To investigate the mechanism underlying the neurological disorders, histological and biochemical analyses of the brain of FGR rats were performed. The density of neurons in the cortical plate of an FGR brain was low compared with the brains of a similarly aged, healthy rat. Consistent with this finding, the density of TUNEL-positive cells was higher in the cortical plate of FGR brains. Western blot analyses showed that the levels of three brain-specific chondroitin sulfate proteoglycans (CSPGs), neurocan, phosphacan, and neuroglycan C, were all significantly reduced in the brain of neonatal FGR rats compared with those of the control. The reduction of CSPG-levels and morphological changes in the brain may be relevant to neurological dysfunction in FGR.

Introduction

Fetal growth retardation (FGR) is an important cause of prenatal and neonatal morbidity (Vik et al., 1997; Aucott et al., 2004). Furthermore, infants born with FGR often go on to develop various neurological disorders including mental retardation, and educational and/or behavioral problems (Peng et al., 2005; Bergvall et al., 2006; Walker and Marlow, 2008). However, the mechanism underlying these disabilities has not been elucidated.

In order to investigate FGR, various animal models have been designed. The classical FGR model is prepared by ligating or clamping the uterine artery (Wigglesworth, 1964; Van Geijn et al., 1980). Other models based on maternal malnutrition (Rosso, 1977) and fetal alcohol syndrome (Streissguth et al., 1980) have also been proposed. We developed a new animal model of FGR by continuous administration of a synthetic thromboxane A_2 analogue (STA_2) to pregnant rats (Hayakawa et al., 1999 and 2006). This model has an advantage over other classical models in that rats mimic the pathophysiology of pregnancy-induced hypertension (PIH)/pre-eclampsia, which is one of the major causes of FGR (Robinson et al., 2000, Xiao et al., 2003).

Although many studies have demonstrated the relationship between FGR and metabolic syndromes in animal models (Simmons et al., 2001; Alexander, 2003; Neitzke et al., 2008), only a few have demonstrated the relationship between neurological impairments and FGR. Using the FGR model rat prepared by passive smoking of pregnant rats, Xuelan et al. (2008) showed poor learning and memory abilities of young-adult FGR rats. Furthermore, it was shown that FGR could cause gender- and age-specific impairments of spatial learning and memory abilities (Huang et al., 2008). Using our FGR model, we demonstrated that neuronal migration was delayed in FGR brains in the early neonatal period (Sasaki et al., 2000). Decreased expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) has been also observed in this model (Fukami et al., 2000). Further investigation is needed to clarify both the nature of neurological impairment in this model and the mechanisms underlying neurological abnormality in FGR.

Chondroitin sulfate proteoglycans (CSPGs) exist abundantly in the brain, and neurocan (NC), phosphacan (PC), and neuroglycan C (NGC) are the major CSPGs in the developing brain (Oohira et al., 2000;

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Bandtlow and Zimmermann, 2000). They are involved in various developmental events including proliferation of neural stem cells (Yanagisawa and Yu, 2007), neuronal migration (Ohyama et al., 1998; Kawano and Kawamura, 1999), and neurite elongation (Hartmann and Maurer, 2001) as microenvironmental factors. In addition, their levels of expression change in the damaged central nervous system (Morgenstern et al., 2002).

To elucidate the mechanism underlying neurological disorders in FGR, we assessed the STA_2-induced-FGR model rats with regard to 1) behavior in both the early postnatal and juvenile periods; 2) morphology of the brain; 3) expression of the brain-specific CSPGs, neurocan, phosphacan, and neuroglycan C.

Materials and methods

Animals

Female Sprague Dawley rats (7 days pregnant) were obtained from SLC (Shizuoka, Japan) and maintained under a 12 h light/dark cycle (lights on from 8:00 A.M. to 8:00 P.M.) with *ad libitum* access to food and water. After acclimation to experimental conditions, rats underwent the procedure described below. Surgical operations and behavioral tests were performed during the light period. All experiments were conducted with protocols approved by Aichi Medical University Animal Experimental Committee.

FGR model rats

STA_2 (ONO-11113, 9,11-epithio-11,12-methano-thromboxane A_2; Ono Pharmaceutical Co., Osaka, Japan) was dissolved in 95% ethanol at a concentration of 20 ng/μL. Before use, 9 volumes of distilled water were added to this solution. STA_2-induced FGR model rats were produced by the method of Hayakawa et al. (1999). In brief, osmotic pumps (Model 2ML1; ALZET Osmotic Pumps Company, Cupertino, CA) were filled with 2 mL STA_2 solution, which provided continuous infusion at a rate of 20 ng/h. On day 13 of pregnancy, each rat was deeply anesthetized with ether and an osmotic pump containing the STA_2 solution or vehicle was implanted into the lower peritoneal cavity. Rats were allowed to deliver spontaneously, and the neonates born from the rats given continuous administration of STA_2 were considered as FGR rats if they were significantly smaller than control rats.

After birth, the body weight of each rat was measured daily and brain weight was measured on postnatal days 1 (P1) and 7 (P7).

Behavioral analyses

All behavioral tests were performed by the same researcher, starting at 10:00 A.M. In our preliminary experiments of the 3 neonatal reflexes described below, female rats showed a tendency of earlier achievement than male rats as described by Mesquita et al. (2007). However, since the differences in achievement between FGR and control rats were larger than those between female and male rats, we used a mixture of female and male rat pups in the neonatal reflex tests. In the behavioral analyses of juvenile rats, only male rats were used.

(i) Neonatal reflexes

We performed three reflex tests, namely the surface righting reflex, postural reflex, and negative geotaxis tests, which have frequently been used as a reflex test of early postnatal rodents. The neurological reflexes were evaluated daily during the day period from P1 to P19 as described by Bassan et al. (2005) and Mesquita et al. (2007). The methods of evaluating reflexes were modified in some experiments as described below.

(i-a) Surface righting reflex

Each rat was placed on its back and the time required for the rat to turn itself over and adopt the normal prone position was measured. The results were scored on a scale of 0 to 3; 0 = no response during 30 s, 1 = time to perform the task < 30 s, 2 = time to perform the task < 15 s, and 3 = time to perform the task < 5 s. If the pivoting limb of the rat remained under its abdomen after it had turned over and was not freed within 2 s, one point was subtracted from the original score as a penalty.

(i-b) Postural reflex

Each rat was placed in a small box and shaken at a constant rhythm. One cycle consisted of a movement left and right, back and forth, and up and down, and each rat underwent 5 cycles at most. The number of cycles during which the rat could maintain its original position by extending all four limbs was measured.

(i-c) Negative geotaxis

Each rat was placed on a board held at an angle of 30° with its face down, and the time required for the rat to turn around and start to climb up the slope was measured. The board had a rough surface to provide the rat with a reasonable grip. The results were scored on a scale of 0 to 5; 0 = no response during 60 s, 1 = time to perform the task > 60 s or the rat fell, 2 = time to perform the task < 60 s, 3 = time to perform the task < 45 s, 4 = time to perform the task < 30 s, and 5 = time to perform the task < 15 s.

(ii) Behavioral analyses of juvenile rats

After weaning, male rats were subjected to four types of behavioral test during the period from 3 to 6 weeks after birth. We usually use four rats from individual mothers (at least 3 mother rats) for each test. In some experiments, devices for mouse behavioral analysis were optimized in accordance with the body size of juvenile rats. The same animals went through the test battery consisting of the elevated plus maze, rotarod, and shuttle avoidance tests in this order. The other group of animals was used for the open-field test, because we wanted to reveal the intrinsic motor activity and habitual patterns of the FGR model rat without experiencing any stressful tests such as the shuttle avoidance test.

(ii-a) Open-field behavior

Each rat was put in a white cube of side 90 cm with a grid of 30 cm × 30 cm squares on its base, and its behavior was observed for 180 s. Since multiple trials of behavioral tests for a few consecutive days afford greater reliability and the opportunity of temporal analysis (Walsh and Cummins, 1976), the open-field test was performed once a day, for three consecutive days, according to Kihara et al. (2001). Motor activity and habitual patterns were recorded by a video camera and the following parameters were assessed; 1) *total movement* represented by the number of squares through which the rat passed; 2) *center entries*, namely the frequency at which the rat entered the center square; 3) *rearing* represented by the frequency at which the rat stood on its hind limbs and turned its nose upward; 4) *defecation* represented by the frequency at which the rat discharged urine or stools; 5) *movement time*, namely the sum time during which the rat continued to move. (If the rat did not move for more than 3 s, the period was regarded as resting time.)

(ii-b) Elevated plus maze

Each rat was placed in the center of an elevated plus maze (Med Associates Inc., St. Albans, VT), and its behavior was recorded by a video camera for 300 s. The test was performed once a day, for three consecutive days. The numbers of entries into the open arms and the closed arms were separately counted (Stork et al., 1999).