Neuroscience 145 (2007) 974–980

TEMPORAL AND SPATIAL EXPRESSION OF PREPROTACHYKININ A mRNA IN THE DEVELOPING FILIAL MICE BRAIN AFTER MATERNAL ADMINISTRATION OF MONOSODIUM GLUTAMATE AT A LATE STAGE OF PREGNANCY

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Abstract—In the early stages of brain development, exposure of excessive monosodium glutamate (MSG) to neurons causes animal functional and behavioral disorders in adulthood. To investigate the effects of excessive MSG during pregnancy on the neurons in the developing brain, *in situ* hybridization was used. In mice, the expression of preprotachykinin A mRNA (PPT A mRNA) was assessed in neurons of in the brain after MSG treatment. Brain tissue sections were hybridized with specific digoxigenin-labeled RNA probes. The number of cells that expressed PPT A mRNA gradually decreased from 10-day-old (10d) to 60-day-old (60d) MSG-treated and normal animals. In the MSG-treated and normal mice, the PPT A mRNA-positive neurons almost disappeared in 90-day-old (90d) mice. The expression of PPT A mRNA significantly decreased at 10d in most of the brain regions of MSG-treated mice including the cerebral cortex (CC), hippocampal subregions of CA1, CA2 (CA1, CA2), habenula nucleus (HAB), hypothalamic periventricular nucleus (PE), hypothalamic arcuate nucleus (AR), median eminence (ME), amygdala nucleus (AMY), endopiriform nucleus (EN), and hypothalamic ventromedial nucleus (VMH) and dorsomedial nucleus (DMH). In the hippocampal CA4 subregions (CA4), paraventricular nucleus (PV) and caudate putamen (CPU), however, they were not significantly altered. Furthermore, in CC, hippocampal CA3 subregion (CA3), PE and EN regions the number of PPT A mRNA-positive neurons decreased at 20 days old (20d), but increased significantly in CA2 and CPU. At 30 days old (30d), the positive neuron number decreased in AMY, and they did not change in other regions. At 60d, the number of positive neurons significantly decreased in PV and ME, but increased in AMY. In the other observed regions, no changes were found. These results show that maternal administration of excessive MSG at a late stage of pregnancy significantly decreases PPT A mRNA expression in most of the brain regions of filial mice. This suggests that glutamate-induced excitotoxicity may affect the metabolism of precursors of substance P in developing brain neurons. The present study provides insights into the plasticity and vulnerability of neuron in different brain regions to glutamate excitotoxicity.

Key words: monosodium L-glutamate, excitotoxicity, preprotachykinin A, mRNA, fetal brain, development.

There is evidence suggesting that immature neurons of the brain may express neuroactive substances that are not found in the adult brain (Del Rio et al., 1991). During ontogeny, certain neurotransmitters may play roles in the interactions between neuronal populations and also may have different functions at different stages of ontogeny and adulthood, possibly playing a trophic role during development (Brené et al., 1990). Substance P (SP) has been shown to be one such neurotransmitter (Jones et al., 1988; Del Rio et al., 1991). SP and neurokinin A, members of the tachykinins family, are encoded by preprotachykinin A (PPT A) genes. PPT A mRNA has been detected in adult rat and mice brains (Li and Ku, 2002; Varga et al., 1998), however the temporal and regional expression of PPT A mRNA during brain development (Brené et al., 1990) is not clear. The expression of PPT A mRNA could be regulated by some neurotransmitters.

Glutamate (Glu), an important excitatory amino acid (EAA), is also a neurotransmitter distributed ubiquitously in the mammalian brain. This EAAs may play a key role in the induction of neuronal cell death occurring in several neurological disorders including Alzheimer's disease (Akbarian et al., 1995; Kim et al., 2000; Lawlor and Davis, 1992; Mattson et al., 1999), Huntington disease (Akbarian et al., 1995; Tang et al., 2005) and Parkinson's disease (Akbarian et al., 1995; Mattson et al., 1999). Glu elicits neurotoxic effects via distinct receptor and non-receptor-mediated mechanisms (Aarts and Tymianski, 2003; Cendes et al., 1995; Choi, 1992; Nacher et al., 2002; Lipton, 2004). Glu receptors play broad roles in neural plasticity, neural development and neurodegeneration, while *N*-methyl-D-aspartate (NMDA) receptor activity mediates the expression of neuropeptides (Angulo et al., 1995; Lipton, 2004).

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Abbreviations: AMY, amygdala nucleus; AR, arcuate nucleus of hypothalamus; CA1, hippocampal CA1 subregion; CA2, hippocampal CA2 subregion; CA3, hippocampal CA3 subregion; CA4, hippocampal CA4 subregion; CC, cerebral cortex; CPU, caudate putamen; DMH, hypothalamic dorsomedial nucleus; EAA, excitatory amino acids; EN, endopiriform nucleus; Glu, glutamate; HAB, habenula nucleus; ME, median eminence; MSG, monosodium L-glutamate; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PE, hypothalamic periventricular nucleus; PPT A, preprotachykinin A; PV, hypothalamic paraventricular nucleus; SP, substance P; VMH, hypothalamic ventromedial nucleus; 10d, 10 days old; 20d, 20 days old; 30d, 30 days old; 60d, 60 days old; 90d, 90 days old.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.12.029

Several studies indicate that Glu and tachykinins or their receptors coexist in the same neurons and interact in the brain (Hu et al., 2004; Otsuka and Yoshioa, 1993; Noailles et al., 1996). The interaction between SP and Glu was supported by the fact that application of MK801, a Glu receptor antagonist, could increase the level of PPT A mRNA in some brain regions (Angulo et al., 1995). However, there is little known about the effect of Glu-induced excitotoxicity on the metabolism of SP in the developing brain. The purpose of the present study was to investigate whether the maternal administration of excessive monosodium glutamate (MSG) during pregnancy has any effect on expression of PPT A mRNA in the fetal mouse brain.

EXPERIMENTAL PROCEDURES

Animals

Kunming female mice, 8 weeks old, were purchased from the Experimental Animal Center, School of Medicine, Xi'an Jiaotong University (Xi'an, China). The experimental procedures were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University. All experiments were carried out in accordance with the U.S. National Institutes of Health *Principles of Laboratory Animal Care* (NIH Publication No. 86-23, revised 1985). All efforts were made to minimize suffering and reduce the number of animals used. Animals were housed two female mice and one male mouse to each cage provided with standard mouse food and running water *ad libitum*, under a 12-h light/dark cycle. Ambient room temperature was kept at 23±1 °C. Embryonic day 1 was defined as the first day of pregnancy. The maternal mice were removed from the cages 20 days after delivery.

Administration of MSG

MSG was purchased from Sigma (St. Louis, MO, USA). Maternal mice were given per os MSG (4.0 g/kg) at 17~21 days of pregnancy. Offspring behaviors were studied at 10 days old (10d), 20 days old (20d), 30 days old (30d), 60 days old (60d) and 90 days old (90d).

Tissue preparation

At 10d, 20d, 30d, 60d or 90d offspring mice were anesthetized intraperitoneally with pentobarbital (50 mg/kg). They were perfused intracardially with 100 ml 0.01 M phosphate-buffered saline (PBS), (pH 7.4) for 5 min followed by 200 ml precooled (4 °C) 4% paraformaldehyde. Mouse brains were removed from the skull and placed into 4% paraformaldehyde at 4 °C for 4 h and then were placed in 30% sucrose at 4 °C until they sank (18~28 h). Brains were mounted in tissue embedding medium (O.C.T., Miles, Inc., Elkhart, IN, USA), quickly frozen on dry ice, and stored at −70 °C (Medical Freezer, Sanyo, Japan) until processed.

0 Days old (0d) was defined as the first 24 h after delivery. Three mice were processed from each of the litters at 10d, 20d, 30d, 60d and 90d.

In situ hybridization histochemistry

Coronal sections (12–14 μm) of the filial mice brains were cut on a cryostat microtome (Cryocut 1800, Leica Microsystems, Wetzlar, Germany) at −20 °C, thaw-mounted onto slides which were pretreated with amino propyltriethoxy silane, and stored at −70 °C until processing. Sections were dried at room temperature and rinsed twice in 0.1 M PBS solution for 3 min. Sections next were rinsed in 0.2 N HCl at room temperature for 10 min, treated or digested with proteinase K at 37 °C for 10 min, 0.2% glycine for 5 min followed by 0.1 M PBS for 5 min. Sections were post-fixed in 4% paraformaldehyde (in 0.1 M PBS) for 10 min and rinsed one more time in 0.1 M PBS for 10 min. Then the sections were rinsed in 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min followed by three washes in 0.02% diethylpyrocarbonate solution. All the sections were dehydrated in graded ethanols and air-dried. Prior to hybridization, B solution (containing ss-DNA 10 mg/ml, Poly A 10 mg/ml, 10× Denhardt's and 8× SSC) was added to each slide and slides then were coverslipped and sealed with DPX at 42 °C for 2 h. Hybridization mixture consisted of one portion of mRNA probe, four portions of B solution and five portions of A solution (20% dextran sulfate in deionized formamide). All sections were treated with 30 μl of hybridization mixture and were then coverslipped and sealed with DPX. Hybridization was carried out overnight in sealed humid chambers at 42 °C for 16–20 h. After hybridization, slides were washed with 2× SSC at 40 °C twice 20 min each; 0.5× SSC at 40 °C twice, 20 min; each followed by a final wash with 0.1× SSC at room temperature for 30 min. Slides then were soaked three times in buffer I (1.16% maleic acid and 0.88% NaCl, pH 7.5) for 2 min. Buffer I, containing 2% goat serum and 0.3% Triton X-100, was then added to tissue sections at 37 °C for 1 h. This was followed by an incubation with anti-digoxigenin-AP conjugate (1:500, diluted with 1% normal goat serum and 0.3% Triton X-100 in buffer I) at 37 °C for 2 h. Slides were rinsed in buffer I for 30 min and then equilibrated with buffer III (50 mmol/l $MgCl_2$, 100 mmol/l NaCl and 100 mmol/l Tris–HCl, pH 9.5) at room temperature for 5 min. The color reaction was developed in NBT-BCIP at 4 °C overnight and produced a dark-blue reaction product. The slides then were rinsed in buffer IV (0.12% Tris–HCl and 0.03% EDTA, pH 8.0) to terminate the reaction. After a short wash in distilled water, the sections were dehydrated in graded ethanol and coverslipped with resin.

The following 48-mer oligonucleotide probe was applied to detect the expression of PPT A mRNA: 5′-dTCG GGC GAT TCT CTG AAG AAG ATG CTC AAA GGG CTC GGG CAT TGC CTC-3′ which corresponded to amino acids 124–171 of the mouse proprotein. This probe was synthesized by Boster Biological Engineering Corporation Ltd. (Wuhan, China). To label the oligonucleotide probe, digoxigenin-dUTP was added to the 3′ terminal of the amino acid fragment (Boehringer Mannheim, Mannheim, Germany).

Control test

Adjacent control sections were incubated with either a hybridization mixture without an oligonucleotide probe or hybridization mixture containing unlabeled-probe. The adjacent sections also were stained with 0.1% Cresyl Violet for morphological identification of brain subregions.

Quantification and statistical procedures

A computer-assisted morphometric system (Image-Pro Plus) and Olympus microscope equipped with a digital camera were employed to analyze the data and take bright-field photomicrographs. Three coronal levels were examined: rostral (Bregma +0.74 mm), middle (Bregma −1.70 mm) and caudal (Bregma −2.80 mm). An atlas of the mouse brain (Franklin and Paxinos, 1999) was used to outline these levels for each slide.

The number of PPT A mRNA positive neurons is represented by the mean value per 0.0156 mm^2 in one brain region. Data were assessed by one-way ANOVA. All statistical analyses were performed with the aid of SigmaStat 2.0. Significance is defined as $P<0.05$.