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Analytical methodology

Changed expressions of *N*-methyl-D-aspartate receptors in the brains of rats and primary neurons exposed to high level of fluoride

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ABSTRACT

Expressions of *N*-methyl-D-aspartic acid receptors (NMDARs) in the brains of rats and primary neurons exposed to high fluoride were investigated. Sprague-Dawley rats were divided randomly into a fluorosis group (50 ppm fluoride in the drinking water for 6 months) and controls (< 0.5 ppm fluoride) and the offspring from these rats sacrificed on postnatal days 1, 7, 14, 21 and 28. The primary cultured neurons from the hippocampus of neonatal rats were treated with 5 and 50 ppm fluoride for 48 h. NMDAR subunits at protein or mRNA levels were quantified by Western blotting or real-time PCR. The phosphorylated calmodulin-protein kinase II (CaMKII) was determined by Western blotting, concentration of Ca^{2+} in neurons by laser confocal microscopy and apoptosis by flow cytometry. In the brains of adult rats and pups as well as in primary neurons exposed to high fluoride, the mRNAs encoding GluN1 and GluN2 B subunits and the corresponding proteins were elevated, the GluN3A lowered and the GluN2A unchanged. In addition, the level of phosphor-CaMKII was reduced, and Ca^{2+} influx and apoptosis enhanced in the brains of rats and cultured neurons exposed to high fluoride. The results indicate that such modifications may involve brain damage induced by chronic fluorosis.

1. Introduction

Many reports indicate that prolonged ingestion of excessive amounts of fluoride can exert chronic toxic effects on the central nervous system (CNS) [1–3]. Under such circumstances excessive fluoride can cross the blood-brain barrier, accumulate in the brain and damage the CNS of experimental animals [4,5], promoting oxidative stress and apoptosis, reducing expression of cholinergic acetylcholine receptors and impairing learning and memory [6–9].

Interestingly, there is compelling evidence in which the neuronal destruction and synaptic injury caused by chronic fluorosis involves excitotoxicity [10], a common factor connecting to numerous neurological disorders including stroke, ischemia, CNS infections and neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease [11–14]. In addition, excitotoxicity induced by excessive activation of *N*-methyl-D-aspartate receptors (NMDARs) may enhance the vulnerability of local neurons in a manner similar to the neuropathology of AD [15].

NMDARs, which are widely expressed in the CNS and possess unique biophysical and pharmacological properties [16], play key roles in neurotransmission and in connection with neurological diseases. These belong to the family of ionotropic glutamate receptors (iGluR), which contains the α-amino-3-hydroxy-5-methyl-4-isoxazalepropionic acid, kainite and delta receptors [17]. To date, seven different subunits of NMDARs, i.e., the GluN1 subunit, the distinct GluN2 subunits (A-D) and a pair of GluN3 subunits (A and B), can be divided into three groups on the basis of sequence homology, have been identified [18]. The NMDARs are heterotetramers that typically contain GluN1 and GluN2 subunits or a mixture of GluN2 and GluN3 subunits [19,20].

It is becoming increasingly clear that the NMDARs may play a key role in many fundamental functions, such as mediating calcium influx, promoting neuronal synaptic plasticity, and participating in the processes of learning and memory [21,22]. At the same time, excessive excitatory of NMDARs can cause calcium influx and consequent intracellular calcium overload, activating a series of intracellular processes that induce neurotoxicity and apoptosis [18]. Such disturbance of NMDAR regulation could be involved in numerous CNS disorders, including neurodegenerative diseases, epilepsy and ischemic cerebral injury. Available evidence indicates that excessive fluoride can destroy neurons and injure synapses via production of free radicals and lipid

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peroxidation, which may magnify the vulnerability of neurons to excitotoxicity [10].

Since the mechanism by which chronic fluorosis damages the CNS remains elusive, the present investigation was designed to characterize alterations in the expression of NMDARs and associated neurotoxicity in adult rats and their offspring exposed to chronic fluorosis. In addition, primary hippocampal neurons were exposed to high levels of fluoride and alterations in their receptors and correlations with molecular injury examined.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies directed towards GluN1 (Cell Signalling Technology, USA), GluN 2A and Glu2B (Abcam, English); rabbit polyclonal anti- GluN3A antibody, mouse polyclonal anti-calcium calmodulin-protein kinase IIα (CaMKIIα) antibody, rabbit polyclonal anti-(Thr286) phosphor-CaMKIIα (pCaMKIIα) antibody, anti-rabbit or −mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., USA); Hyper Performance Chemiluminescence film and Enhanced Chemiluminescence (ECL) Plus reagent (Amersham, Sweden); QPCR SYBR Green Mix (Infinigen Biotechnology Inc., USA); Trizol reagents (Invitrogen, USA); Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson Biosciences, USA); Fluo-4 NW Calcium Assay Reagent (Thermo Fisher Scientific, USA); and sodium fluoride (NaF, analytical grade), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), mouse monoclonal anti-β-actin antibody and all other general chemicals (Sigma-Aldrich, USA) were purchased from the sources indicated.

2.2. Experimental animals

Forty-two parental Sprague-Dawley (SD) rats with one month-old (from the Experimental Animal Center in Guizhou, China) were housed in stainless steel cages suspended in stainless steel racks with a humidity of 30–55% and temperature of 22–25 °C for one week before treatment. These animals were divided randomly into two groups of 15 each (7 male and 6 female), one of which was served as the fluorosis group (50 ppm fluoride in drinking water, prepared by sodium fluoride) and another as the control group (with drinking water containing less than 0.5 ppm fluoride). Both received a normal diet containing no more than 6 ppm fluoride. At the end of six months, dental fluorosis, the fluoride content of the urine and body weight were determined.

Subsequently, another 6 rats with chronic fluorosis or 6 in the control group were housed in two cages (two female and one male rats for each) and the female animals allowed to become pregnant by natural mating. After birth, the pups received the same drinking water as their parents. On postnatal days 1, 7, 14, 21 and 28, 10 pups from each group were sacrificed.

The animals we used here were pre-approved by the Animal Care Welfare Committee of Guizhou Medical University, Guizhou, P.R. China (Permission number: 1403026). All methods in the study were performed in accordance with the relevant guidelines and regulations.

2.3. Exposure of primary cultured neurons to fluoride

In brief, nerve cells were isolated from rat embryos (E19) as follows. The hippocampus of brains were minced into pieces about 1 mm^3 in size with a sterile scalpel, washed with cold DMEM-high glucose solution, incubated with 0.25% trypsin at 37 °C for 20 min, and then gently dispersed by pipetting. Following addition of fetal calf serum to inhibit trypsin activity, the suspension was centrifuged at 1000 x *g* three times for 5 min each for complete removal of blood cells.

Finally, the neurons were plated onto poly-L-lysine-coated culture dishes in DMEM/F12 supplemented with 10% fetal calf serum, allowed to attach for 6 h, and then grown in Neurobasal A medium containing B27 supplement, with replacement of half of this neurobasal medium once every 3 days. These cells were exposed to various concentrations of fluoride (0.5–100 ppm) for 48 h. On the basis of their viability as evaluated by MTT reduction (data not shown), 5 and 50 ppm fluoride were selected as the low and high dose, respectively[9].

2.4. Assessment of dental fluorosis, determinations of ***fluoride contents in urine and body weight of rats***

Following 6 months of exposure, dental fluorosis in the rats was assessed as white or pigmented bands (I°), gray enamel (II°) and even defect or loss of tooth structure (III°)[43]. The fluoride content in urine was determined with a CSB-F-I fluoride ion electrode and the body weight detected.

2.5. Histopathological observations on rat brains

The brains were removed and the right hemispheres fixed with 4% paraformaldehyde for 24 h, the tissue embedded in paraffin, and 6 μm serial sections cut for HE or Nissl staining.

2.6. Quantitation of NMDAR subunits at the protein and mRNA levels in rat brains and primary cultured neurons by Western blotting and quantitative real-time PCR

A mixture of brain tissues including the hippocampus and cortex of rats or primary neurons were homogenized in buffer containing 2% Triton X-100 on ice with a Teflon homogenizer. After centrifugation at 14,000 x *g* for 30 min at 4 °C, the protein concentration of the supernatants thus obtained was determined using the BCA protein assay kit. The proteins were then separated by 8% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes employing a transfer unit (Bio-Rad Inc., USA).

For quantification of GluN1, GluN2A, GluN2 B and GluN3A subunit proteins, these PVDF membranes were thereafter incubated with different antibodies (diluted 1:200-500) at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated anti-goat IgG (1:5000) for 60 min. Finally, these membranes were incubated in ECL Plus reagent for 5 min and the signals thus produced visualized by exposure to Hyper Performance Chemiluminescence film.

Total RNA was isolated from brain tissue or primary neurons with Trizol reagents. For each sample, 3 μg of this RNA was converted into first-strand cDNA with the first-strand cDNA synthesis kit (Promega, USA) and oligo-d(T) primers. The PCR primers for target transcripts were designed on the basis of the complete cDNA sequences deposited in GenBank (accession numbers: NM-001270606.1 for GluN1, NM-012573.3 for GluN2A, NM-012 574.1 for GluN2B, NM-138546.1 for GluN3A and NM 031144 for β-actin) (Table 1).

Table 1
Sequences of the primers employed for amplification of the mRNAs encoding GluN1, GluN2A, GluN2B and GluN3A as well as β-actin by quantitative real-time PCR.

mRNA	Primer sequences	Product length (bp)
GluN1	5′-GCTGGGATTTTCCTCATTTTC −3′ 5′-GGCTCTGCTCTACCACTCTTTC-3′	140
GluN2A	5′-TCTAAACCAGAACCCAGTCTCC-3′ 5′-GTCAACATCGCTACAGTCCTTG-3′	232
GluN2B	5′-GCGAGGTAGAGAGAACATTTGG-3′ 5′-GTCACAGTCATAGAGCCCATCA-3′	131
GluN3A	5′-TTCTCTGGACTCTTCGTGCTGTT-3′ 5′-GCTTGGATTTGTTTTTGATGCG-3′	115
β-actin	5′-CACCCGCGAGTACAACCTTC-3′ 5′-CCCATACCCACCATCACACC-3′	207