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Altered expression of metabotropic glutamate receptor 1 alpha after acute diffuse brain injury

Effect of the competitive antagonist 1-aminoindan-1, 5-dicarboxylic acid☆

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Abstract
The diffuse brain injury model was conducted in Sprague-Dawley rats, according to Marmarou's free-fall attack. The water content in brain tissue, expression of metabotropic glutamate receptor 1α mRNA and protein were significantly increased after injury, reached a peak at 24 hours, and then gradually decreased. After treatment with the competitive antagonist of metabotropic glutamate receptor 1α, (RS)-1-aminoindan-1, 5-dicarboxylic acid, the water content of brain tissues decreased between 12–72 hours after injury, and neurological behaviors improved at 2 weeks. These experimental findings suggest that the 1-aminoindan-1, 5-dicarboxylic acid may result in marked neuroprotection against diffuse brain injury.
Key Words: diffuse brain injury; *in vivo*; animal model; metabotropic glutamate receptor 1 alpha; 1-aminoindan-1, 5-dicarboxylic acid

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INTRODUCTION

Brain injury can cause excessive release of glutamate, and activation of glutamate receptor, ultimately leading to neuronal death. The glutamate receptors include ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs). Group I mGluRs (mGluR1 and mGluR5) can enhance excitatory neurotoxicity and mediate N-methyl-D-aspartate (NMDA) receptor, while groups II and III mediate presynaptic inhibition and reduce the release of glutamate to produce a protective effect[1-3]. A number of studies have shown that the expression of mGluRs changes in three groups, especially the mGluR1, which can lead to neuronal damage[2, 4-17]. mGluR1 has subtypes a to f[18], and their effects can differ[19]. Animal experiments have shown that mGluR1α may be a key factor in the aggravation of head injury[20]. However, little is known about the expression pattern and the effect of mGluR1α after diffuse brain injury (DBI).

In this study, the expression of mGluR1α in cerebral cortex neurons was tested at different time points after injury in a DBI rat model. The neuroprotective effect of its antagonist, 1-aminoindan-1, 5-dicarboxylic acid (AIDA), was also investigated. The purpose of our study was to clarify the pathophysiology of mGluR1α during the process of DBI and to provide a new concept of neuroprotective treatment for DBI.

RESULTS

Quantitative analysis of experimental animals

A total of 136 Sprague-Dawley rats were randomly divided into four groups: sham-surgery (n = 40), DBI (n = 40), normal saline-treated (NS-treated, n = 28), and AIDA-treated (n = 28). With the exception of the sham-surgery group, the DBI model was established in rats in the DBI group, and the normal saline (NS)-treated and AIDA-treated groups. The rats that died within 12 hours after free-falling attack or whose respiratory depression exceeded 5 minutes were excluded from this study and then replaced with other rats. The rats in the NS- and AIDA-treated groups were stereotaxically injected with 10 μL AIDA (100 nmol) or normal saline in the lateral ventricles 30 minutes before injury. In the final analysis, 136 rats were involved in the study results.

Histological change of brain tissue in control and DBI rats

Gross morphology

In order to verify the validity of the DBI model, morphologies of brain tissues in the control and DBI groups were observed at 48 hours after injury. The brain meninges of the rats in the control group were intact and smooth, and the vessels at the surface of

the cerebral cortex were visible. The color of the brain tissue was normal without bleeding. The brain tissue of the rats in the DBI group was swollen and the vessels were blurred. There was no obvious focal cerebral contusion on most of the brain surface.

Hematoxylin-eosin staining results (Figure 1)

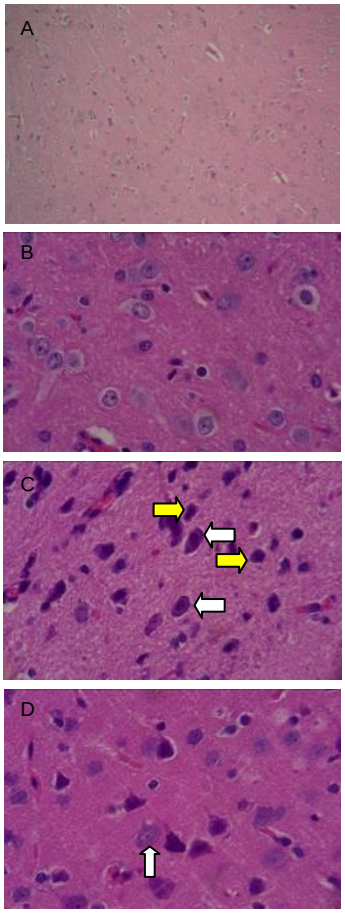

Figure 1 Morphology of brain tissue in rats with diffuse brain injury (hematoxylin-eosin staining).

(A, × 200; B, × 400) The brain tissue in the control group.

(C) The brain tissue at 48 hours after injury (× 400): The color of the neurons was deep, the shrinking nucleus was deeply stained with no evidence of nucleoli (white arrows), and swollen glial cells are evident (yellow arrow).

(D) The brain tissue at 48 hours after injury in the AIDA-treated group (× 400): The staining of the neurons was less compared with the cells in C (white arrow).

The frontal and parietal brain tissues of the rats were stained with hematoxylin-eosin and observed under a microscope at 48 hours after injury. The structures of the brain tissue in the control group were normal and the staining was uniform. The cortical neuronal shapes and structures were normal, with lightly stained round nuclei and clearly evident nucleoli. The structures of the brain tissue in the DBI group were disordered with shrinkage of neurons. The color of the neurons was deep, and the shrinking nuclei were deeply stained with no presence of nucleoli. Gaps and swollen glial cells surrounded the neurons. These changes were evident in the cerebral cortex at the supraventricular area, especially under the area that was directly struck to induce the brain injury. The change was negatively correlated with the distance from the injury site. There also were disordered structures, shrunk nuclei and swollen glial cells in the NS- and AIDA-treated groups, but the magnitude of these changes was less in the AIDA-treated group than that in NS-treated group.

Effect of DBI on water content in the brain tissue of rats

Compared with the control group, the water content in the DBI group was increased at 1 hour after injury ($P < 0.05$), and reached a peak at 24 hours after injury. The water content in the DBI group gradually decreased from 24 hours after injury, and returned to normal at 1 week after injury (Figure 2).

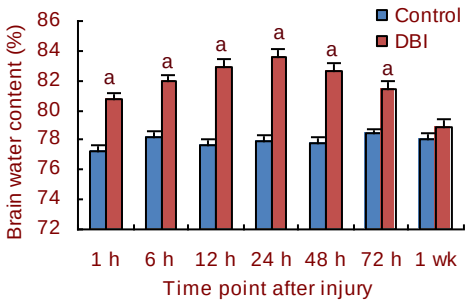

Figure 2 The water content in brain tissue at different time points after injury in control and diffuse brain injury (DBI) groups.

Water content (%) = (wet weight – dry weight)/wet weight × 100%. Data are expressed as mean ± SD, and Student's *t*-test was applied to the analysis of significant differences. [a]$P < 0.05$, *vs*. control group. h: Hour; wk: week.

Effect of DBI on the expression of GluR1α mRNA in brain tissue of rats

The expression of GluR1α mRNA was less in the control group compared with the expression of GluR1α mRNA in the DBI group, which increased noticeably from 1 hour after injury and reached a peak at 24 hours. The expression of GluR1α mRNA in the DBI group increased compared with the control group from 1 hour to 48 hours after injury ($P < 0.05$; Figure 3).

Effect of DBI on mGluR1α-positive neurons in the cerebral cortex of rats

The mGluR1α-positive neurons in the cerebral cortex in the DBI group increased from 1 hour after injury and reached a peak at 24 hours. The positive neurons decreased from 24 hours and appeared normal at 2 weeks after injury. Compared with the control group, the number of mGluR1α-positive neurons in the DBI group significantly increased from 1 hour to 1 week after injury ($P < 0.05$; Figures 4, 5).

Effect of AIDA on edema and behaviors of DBI rats

The therapeutic effects of AIDA can be assessed through the determination of water content and the neurological severity score.

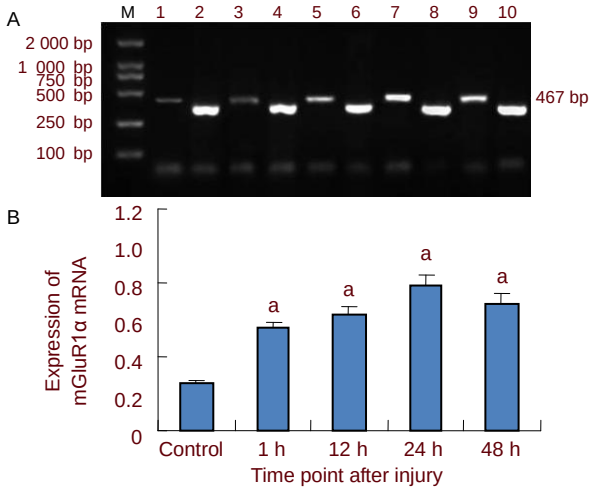

Figure 3 The expression of metabotropic glutamate receptor 1α (mGluR1α) mRNA in brain tissue at different time points after injury (reverse transcription-PCR).

(A) 1, 3, 5, 7, 9: mGluR1α mRNA of the control group and 1, 12, 24, and 48 hours after injury in the diffuse brain injury group; 2, 4, 6, 8, 10: GAPDH mRNA of the control group at 1, 12, 24, and 48 hours after injury in the diffuse brain injury group.

(B) Quantification of mGluR1α mRNA expression. Data are expressed as mean ± SD of absorbance ratio of mGluR1α mRNA to GAPDH mRNA, and paired Student's *t*-test was applied to the analysis of significant differences. [a]*P* < 0.05, *vs*. control group. h: Hour.

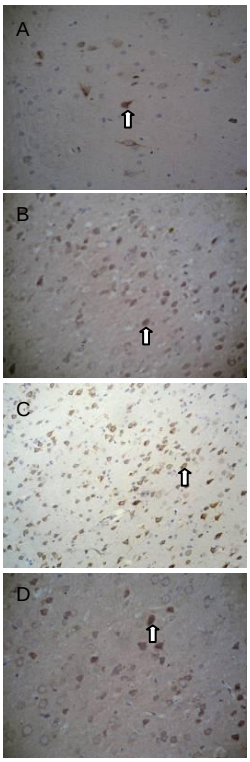

Figure 4 Metabotropic glutamate receptor 1α-positive neurons in the cerebral cortex after injury (immunohistochemical staining). Arrows show positive neurons.

(A) 1 hour after injury (× 200).
(B) 12 hours after injury (× 100).
(C) 24 hours after injury (× 100).
(D) 1 week after injury (× 200).

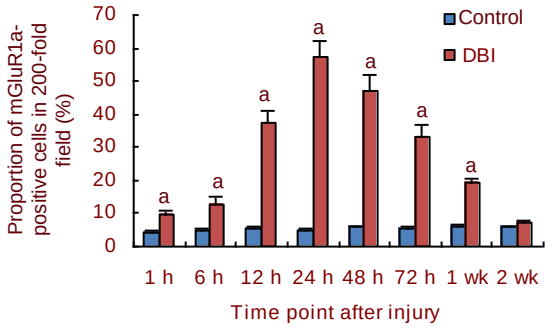

Figure 5 Number of metabotropic glutamate receptor 1α (mGluR1α)-positive neurons in the cerebral cortex at different time points after diffuse brain jinjury (DBI).

Data are expressed as mean ± SD and Student's *t*-test was applied to the analysis of significant differences. [a]*P* < 0.05, *vs.* control group. h: Hour; wk: week.

The water content in the AIDA-treated group decreased at 12–72 hours after injury ($P < 0.05$). The neurological severity score demonstrated that neurological function was not improved significantly at 1 day and 1 week after injury compared with the NS-treated group ($P > 0.05$), but the neurological function significantly improved at 2 weeks after injury in the AIDA-treated group ($P < 0.05$; Figures 6, 7).

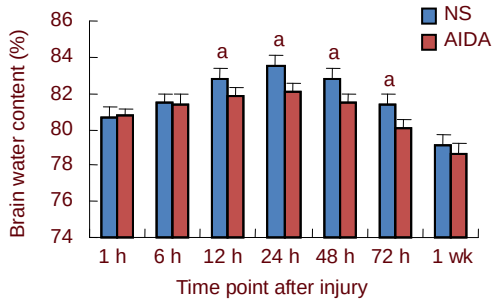

Figure 6 The water content in brain tissue at different time points after injury in normal saline (NS)-treated and 1-aminoindan-1, 5-dicarboxylic acid (AIDA)-treated groups.

Water content (%) = (wet weight – dry weight)/wet weight × 100%. Data are expressed as mean ± SD and Student's *t*-test was applied to the analysis of significant differences. [a]*P* < 0.05, *vs*. NS-treated group. h: Hour; wk: week.

DISCUSSION

Traumatic brain injury can be classified into primary injury and secondary injury. The secondary injury induces molecular and physiological changes following the initial trauma to the brain. The secondary injury mechanisms include a wide variety of processes, such as depolarization, disturbances of ionic homeostasis, and release of neurotransmitters (such as excitatory amino acid), lipid degradation, mitochondrial dysfunction, and free-radical generation, blood-brain barrier disruption, ischemic injury, edema formation, and intracranial hypertension[21]. One theory is that excitatory

amino acids (*e.g.*, glutamate) are released, and there is calcium influx in neurons and other brain cells following traumatic brain injury[22-23].

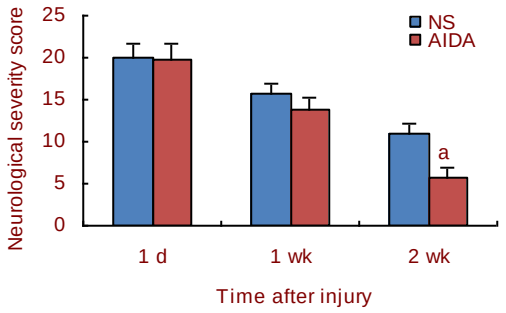

Figure 7 Neurological severity score of rats in normal saline (NS)-treated and 1-aminoindan-1, 5-dicarboxylic acid (AIDA)-treated groups at different time points after injury.
A score of 0 represents normal neurological function and a higher score represents more severe disturbance. Data are expressed as mean ± SD and Student's *t*-test was applied to the analysis of significant differences. $^{a}P < 0.05$, *vs*. NS-treated group. d: Day; wk: week.

Disruption of calcium homeostasis by glutamate-mediated ion channels, depolarization, or other cellular processes are key aspects in the progression of secondary injury in traumatic brain injury[22]. Whether mGluR1α induces intracellular Ca^{2+} overload is not entirely clear, but possible avenues include: activation of ionotropic receptors, which open receptor-gated Ca^{2+} channels, leading to a large Ca^{2+} influx and intracellular Ca^{2+} overload, and adjustment of phosphoinositide metabolism, which promotes the release of Ca^{2+} stores from the endoplasmic reticulum and increases cytosolic Ca^{2+}[3, 12, 24]. In the *in vitro* experiment, the antagonism of group I mGluR blocks the depletion of calcium stores and reduces potentiated capacitative calcium entry in strain-injured neurons and astrocytes[8]. Besides, most secondary brain injury is caused by brain swelling, an increase in intracranial pressure, and a subsequent decrease in cerebral perfusion leading to ischemia[25]. The intracranial hypertension induces brain herniation, which increases brain damage and morbidity. Brain edema is usually worst at 24–48 hours post-injury[22]. Our study showed that the number of mGluR1α-positive cells increased within 1 week post-injury compared with the control group, and reached its peak value at about 24 hours after injury. Reverse transcription-PCR results demonstrated a similar change in mGluR1α mRNA levels after injury as the protein expression. Neuronal death significantly increased at 1 hour after injury, and reached a peak at 24 hours after injury. These experimental results confirmed that mGluR1α took part in the neuronal death after DBI.

A number of studies showed that the blockade or down-regulation of mGluR1α reduces neuronal death and improves neurological function[20, 26-27]. AIDA is a selective competitive antagonist of mGluR1, and it can decrease the neuronal damage caused by mGluR1α after DBI. AIDA can block the depletion of calcium stores and reduce potentiated capacitative calcium entry in strain-injured neurons and astrocytes[8]. In this study, the antagonist AIDA significantly decreased the water content in brain tissues at 12–72 hours, indicating that AIDA has an antihydropic effect. Furthermore, AIDA significantly improved neurological function at 2 weeks after injury, which suggests that it may be a neuroprotective treatment for DBI.

In summary, our experimental findings revealed a change in mGluR1α after DBI in mRNA and protein levels, and demonstrated a neuroprotective effect of AIDA.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiments were performed at a laboratory in the First Affiliated Hospital, School of Medicine, Zhejiang University, China from August 2009 to November 2010.

Materials

A total of 136 healthy, male, Sprague-Dawley rats, of specific pathogen free grade, weighing 300–350 g, were provided by the Experimental Animal Center, Zhejiang University in China (certification No. SYXK (Zhe) 2007-0098). The rats were housed at 25 ± 2°C in 50–60% humidity, under a 100-watt filament lamp. All experimental protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[28].

Methods

Establishment of the DBI rat model

The DBI model was established according to the Marmarou free-falling attack method[29]. Briefly, rats were intraperitoneally anesthetized with 20% pentobarbital (70 mg/kg). After disinfection of the head, the rats were fixed in a prone position. A 3-cm incision was cut in the scalp along the median line. A round metal clout (diameter 15 mm, thickness 2 mm) was then fixed between the coronal suture and lambdoid suture. A 400 g metal stick was dropped freely to impact the metal clout from a 50 cm height, producing the DBI. The rats were immediately moved away to avoid a second injury by the bounce of the metal stick. The control group underwent all the manipulations except for the free-drop impact.

AIDA intervention in DBI rats

The rats in the NS-treated and AIDA-treated groups were stereotaxically punctured at the lateral ventricles 30 minutes before injury. The manipulations were as follows: the head of the rat was fixed in a prone position in the stereotaxic apparatus (Stoelting Corp., Wood Dale, IL, USA) after anesthesia. The scalp was cut in a sagittal plane to expose the right parietal bone. The puncture

point laid 1 mm posterior to bregma and 1.5 mm lateral to the midline. The skull was then drilled through the puncture point with a dental drill. The diameter of the drill hole was about 1 mm. A micro-syringe was punctured into the brain through the hole, until cerebrospinal fluid was sucked into the micro-syringe (Ito Corp., Shizuoka, Japan). Then 10 μL AIDA (100 nmol; Sigma, Santa Clara, CA, USA) and NS, respectively, were injected into the brain at a rate of 2 μL/min. Finally, the scalp was sewed closed.

Hematoxylin-eosin staining

Two rats selected from each group and were anesthetized at 48 hours after injury to obtain the frontal and parietal brain tissue[30] through decapitation. Tissue sections (6 μm thick) were stained with hematoxylin-eosin and the shape of neurons in each section was observed under a microscope (Leica, Wetzalar, Germany).

Water content in the brain tissue

Pentobarbital (20% solution, 70 mg/kg) was injected into the peritoneal cavity of rats in the control and DBI groups at different time points after injury (two rats at each time in each group). The brain obtained after decapitation was washed three times with cold saline (4°C), and the water on the surface was absorbed by filter paper. The left hemisphere was separated on an ice tray. Brain tissue was cut from 5 mm to 10 mm behind the frontal pole in a coronal plane. The brain tissue was weighed by electronic scale (PB303-S; Mettler-Toledo Industrial, Zurich, Switzerland), then dried in an oven (Galanz Corp., Shunde, Guangdong, China) with a constant temperature at 80°C, until the weight did not change (the brain was weighed twice with an interval of 24 hours). The water content in the brain tissue was calculated by the formula: water content (%)=(wet weight−dry weight)/wet weight × 100%[31].

Expression of mGluR1α mRNA in brain analyzed by reverse transcription-PCR

Pentobarbital solution (20% solution, 70 mg/kg) was injected into the peritoneal cavity of the control and DBI rats at different time points after injury (two rats at each time in each group), followed by decapitation. The reverse transcription-PCR (RT-PCR) product was obtained through the abstraction of total RNA by TRIzol, RT-PCR[32-33].

The primer sequence is as follows:

Primer	Sequence	Product size (bp)
mGluR1α	Upstream: 5’-GAT GAG AAG GAT GGG CTG AA-3’ Downstream: 5’-CCA CCC TCA GGA AGT ATT TG-3’	467
GAPDH	Upstream: 5'-TTC CAG TAT GAC TCT ACC CAC G-3' Downstream: 5'-TGA GCC CTT CCA CGA TGC-3'	385

Five microliters of the product was analyzed in the following steps: electrophoresis on a 2% agarose gel containing the appropriate amount of ethidium bromide, ethidium bromide staining, observation under an ultraviolet lamp, images captured by a gel imaging system (Bio-Rad, Benicia, CA, USA), densitometry of the PCR product from the experimental group and the loading control GAPDH. The images were analyzed by Scion Image 4.02 (Scioncop, Betheda, MD, USA), and relative gene expression (absorbance of target gene segment/GAPDH gene segment) was calculated[32-33].

mGluR1α-positive neurons in the cerebral cortex detected by immunohistochemistry

Two rats in the control and DBI groups were anesthetized with 20% pentobarbital (70 mg/kg) at different time points after injury (1, 6, 12, 24, 48, 72 hours, and 1 and 2 weeks), followed by heart perfusion with 100 mL saline and fixation with 300 mL paraformaldehyde (40 g/L). The brains were fixed with paraformaldehyde, dehydrated with ethanol, and embedded in paraffin (6 μm). The expression of mGluR1α was examined by immunohistochemical staining with a kit (rabbit polyclonal antibody, 1: 200; Sigma, CA, USA). After mounting with neutral resin, the number of mGluR1α-positive neurons in ten slides was counted under an optical microscope (200 ×; Leica, Wetzalar, Germany), then the proportion of positive cells was calculated.

Neurological severity score of the rats

Neurological severity score (supplementary Table 1 online) was used to evaluate the neurological function of rats in NS-treated and AIDA-treated animals at different time points after injury (1, 7 and 14 days). The score ranged from 0 to 25[34].

Statistical analysis

All data were expressed as mean ± SD. Using the SPSS 10.0 software package (SPSS, Chicago, IL, USA), paired Student’s *t*-test and two-sample *t*-test were applied to the analysis of significant differences, and *P* value less than 0.05 was considered statistically significant.

Author contributions: Fei Cao was responsible for the study design, establishment of the mechanical injury model, performance of the RT-PCR assay and immunohistochemical staining. Mantao Chen was responsible for the mechanical injury model, hematoxylin-eosin staining, and water content determination in the brain tissue. Gu Li conducted the experiment with AIDA. Ke Ye conducted the RT-PCR and immunohistochemical staining. Xin Huang was responsible for data analysis and statistical analysis. Xiujue Zheng was responsible for the study design and wrote the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animals Ethics Committee of Zhejiang University, China.

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Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 2, 2012 item after selecting the “NRR Current Issue” button on the page.

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