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Effect of subarachnoid nerve block anesthesia on glutamate transporter GLAST and GLT-1 expressions in rabbits

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ABSTRACT

Objective: To observe the effect of subarachnoid nerve block anesthesia on glutamate transporter glutamate-aspartate transporter (GLAST) and GLT-1 expressions in rabbits, and to investigate the effect of peripheral nerve anesthesia on the morphology and function of the spinal cord.

Methods: Twenty healthy New Zealand white rabbits were randomly divided into two groups: the experimental group and control group; with 10 rabbits in each group. For spinal nerve anesthesia, 5 g/L of bupivacaine was used in the experimental group, and sterile saline was used in the control group. After 30 min of cardiac perfusion, GLAST and GLT-1 protein expression in spinal neurons were detected by immunohistochemistry and immunofluorescence staining.

Results: GLAST and GLT-1 protein-positive cells increased in neurons in the experimental group, compared with the control group ($P < 0.05$).

Conclusions: After subarachnoid nerve block anesthesia, rabbit glutamate transporter GLAST and GLT-1 expression is increased; and spinal cord nerve cell function is inhibited.

1. Introduction

Subarachnoid nerve block anesthesia (lumbar anesthesia) is an anesthetic widely used in clinical practice. This method is simple, provides reliable results, and has few side effects. However, its effect and mechanism in blocking nerve cells remains unclear. Amino acid neurotransmitters are important neurotransmitters in the central nervous system of mammals, and the most important excitatory neurotransmitters are glutamate (Glu) and aspartate (Asp) [1]. When the nerve is excited, glutamate concentrations in the synaptic cleft elevate and act on the postsynaptic membrane of the glutamate receptor; and glutamate in the synaptic cleft could be uptake by glutamate transporters to terminate its efficacy of synaptic transmission.

Glutamate transporters accurately regulates glutamate to ensure proper nerve excitatory synaptic transmission [2,3]. The current study shows that glutamate transporters is related to various brain and spinal pathological processes and play an important role in the process of glutamate transporters [4,5]. In the present study, New Zealand white rabbits were used in preparing the spinal anesthesia model to observe the expressions of astrocytes and microglial cells in the two types of glutamate transporters, Glutamate-aspartate transporter (GLAST) and GLT-1, after anesthesia and to investigate the potential regulatory mechanisms of anesthesia in spinal nerve cell function for guidance in clinical practice.

2. Materials and methods

2.1. Animals and groupings

Twenty male 6–8 month New Zealand white rabbits, weighing 2.5–3.5 kg, were provided by the Experimental Animal Center, Zhongnan Hospital of Wuhan University.

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2.2. Rabbit subarachnoid nerve block anesthesia

Rabbits were injected with 30 mg/kg sodium pentobarbital (20 g/L) from the ear. Rabbits were fixed in a prone position and a needle was inserted into the subarachnoid space at the side edges of the L6-L7 spinous process. After the needle was secured, 0.8 mL of 0.5% bupivacaine (Shanghai Harvest Pharmaceutical Co., Ltd.) was injected in rabbits in the experimental group, while saline was injected in rabbits in the control group.

2.3. Prepared sections after routine perfusion

After 30 min of anesthesia, rabbits were fixed on back and the chest was opened. After exposing the heart, the left ventricle was punctured from the apex with a syringe and then fixed with hemostatic forceps, cut a small opening in the right atrium for bloodletting, 500 mL normal saline was injected into the left ventricle until the right ventricle outflow of transparent liquid, reperfusion in 1L of 4% paraformaldehyde, and the L6-L7 spinal cord segments were cut and obtained. Then, corresponding segments of the spinal cord were taken out from rabbits in the normal control group as described above.

2.4. HE staining and observation

The L6-L7 spinal cord segment was obtained, embedded in paraffin, cross-section sliced with a thickness of about 5 μm , HE-stained, and the form of soft meninges of the spinal cord segments were observed under a microscope.

2.5. Immunohistochemical staining and analysis

L6-L7 spinal cord segments were placed and fixed in 4% paraformaldehyde for three hours, and dehydrated in 30% sucrose solution overnight. After tissue masses were sunk to the bottom, a transverse plane was made by frozen section machine, and sliced with a thickness of about 6–7 μm . Slices were placed in 3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase, and washed three times with 1 \times PBS. Segments were divided into three groups: experimental group and control group. Segments were separately added in anti-GLAST (1:500, Beijing Bioss company) and GLT-1 (1:400, CST company), and incubated at 4 $^{\circ}\text{C}$ overnight; then, shaking and rinsed three times, every 5 min. Added second antibody (1:200, Beijing ZSGB-Biotechnology Co., Ltd.), incubated at 37 $^{\circ}\text{C}$ for 30 min, and washed three times with 1 \times PBS. Added SABC and incubated at 37 $^{\circ}\text{C}$ for 20 min. Then, DAB stained after washing

with PBS. Dehydrated by 70%, 90%, 100% alcohol, Xylene I,II,III transparent medium, mounted with neutral gum, and dried. Positive cell with brown granules were microscopically observed, and each section was randomly observed by a five high power field film. Image-Pro plus 6.0 software was used to analyze the integrated optical density (IOD) value of the positive expression; wherein, the higher the IOD value, the stronger the protein expression.

2.6. Immunofluorescence staining and analysis of astrocytes and microglial cells

After sections were stained with blocking serum, different primary and secondary antibodies were added: astrocytes were stained with GFAP antibody (1:100, Sigma Company) and microglial cells were stained with OX-42 antibody (1:100, AbD Serotec company). At the same time, sections were separately stained with GLAST (1:100, CST company) and GLT-1 (1:100, CST company) antibodies to detect GLAST and GLT-1 expressions in the two cells. Fluorescence microscopy film (Olympus, Japan) was used to take a photograph and Image-pro plus 6.0 software were used to analyze the double stained positive area of the region and double positive area (DPA), with its average value as the measurement value.

2.7. Statistical analysis

Data were measured using SPSS 13.0 software and were represented as mean \pm standard deviation, groups were compared using *t* test, and *P* < 0.05 was considered as significant difference.

3. Results

3.1. Observation of rabbit spinal pia mater morphology

Spinal cord sections in the experimental and control groups were detected by HE staining, and the morphology of the spinal pia mater was observed, as shown in Figure 1. Compared with the control group, the spinal pia mater in the experimental group had some stratification and slight edema.

3.2. Influence of spinal nerve block anesthesia in GLAST and GLT-1 expressions in spinal cord neurons

In the normal control group, immunohistochemical assay revealed that GLAST and GLT-1 were mainly expressed in the

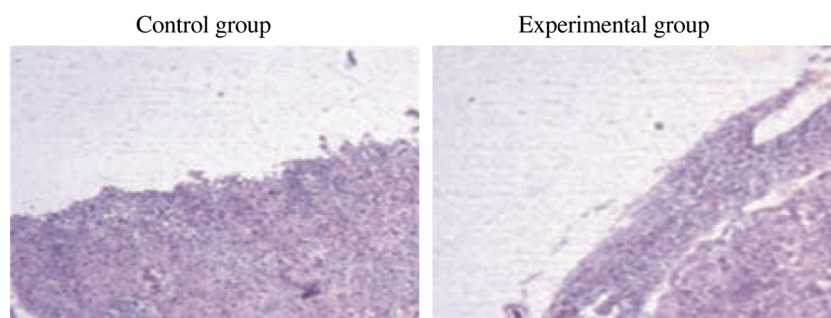


Figure 1. Rabbit spinal pia mater morphological result. HE staining ($\times 400$).

Table 1

Immunohistochemistry results of GLAST and GLT-1 in spinal cord neurons of rabbits (IOD, $\times 104$, mean \pm sd).

Group	No. of cases	GLAST	GLT-1
Experimental group (anesthesia)	10	8.27 \pm 1.4 ^a	9.66 \pm 1.6 ^a
Control group	10	2.25 \pm 0.8	1.78 \pm 0.5

^a Compared with control group, $P < 0.05$.

gray matter in the transverse section of the spinal cord. Brown particle density was evenly distributed, deeply stained, and the nucleolus was concentrated dark brown yellow. In the experimental group, GLAST and GLT-1 expressions significantly increased ($P < 0.05$), as shown in Table 1 and Figure 2.

3.3. Comparison of GLAST and GLT-1 expressions in astrocytes and microglial cells

GLAST and GLT-1 expressions in astrocytes and microglial cells were detected using immunofluorescence double staining method, as shown in Figure 3. Results revealed that the GLAST and GFAP double labeled positive area in the transverse section in the spinal cord between the experimental group and control group had no significant changes. However, GLT-1 and GFAP double positive areas significantly increased ($P < 0.05$); namely, GLT-1 protein expression was upregulated and GLAST expression remained unchanged in astrocytes in the anesthesia group. In microglial cells, GLAST and OX-42 double positive area expressions was up-regulated in the experimental group, compared with

the control group; and there was a statistical difference ($P < 0.05$). Further, the double positive area of GLT-1 and OX-42 significantly increased. Results revealed that GLAST and GLT-1 protein expressions in microglial cells increased in the anesthesia group.

4. Discussion

Glutamate is one of the most important neurotransmitters in the nervous system, and glutamate concentration in the synaptic cleft is precisely regulated mainly by the glutamate transporter. Under normal physiological conditions, glutamate is mainly distributed in the presynaptic vesicle area; and when the presynaptic membrane is released into the synaptic cleft excited due to depolarization, it plays a role by acting on postsynaptic membrane receptors. When glutamate is released into the synaptic cleft, it is necessary to be promptly removed to ensure the normal conduction of excitement [6,7]. Glutamate uptake mainly rely on it location in neurons, and high affinity glial cell membranes and force glutamate transporters. Glutamate transporters are mainly divided into five subtypes: excitatory amino acid transporter-1 (EAAT-1) (GLAST), EAAT2, EAAT3, EAAT4 and EAAT5 [8,9]. Among them, GLAST and GLT-1 are mainly expressed in astrocytes and microglia; and approximately 80%–90% of the total amount of their glutamate transporter are transported [10,11]. GLAST, also known as EAAT-1, is mainly expressed in the mammalian brains and spinal cord astrocytes and microglial cells. Some studies on surface excitatory amino acids, particularly glutamate, plays a key role in the central sensitization process, particularly in pain [12,13]; and its mechanism in the synaptic cleft of the spinal cord is to regulate the large number of glutamate accumulation. When

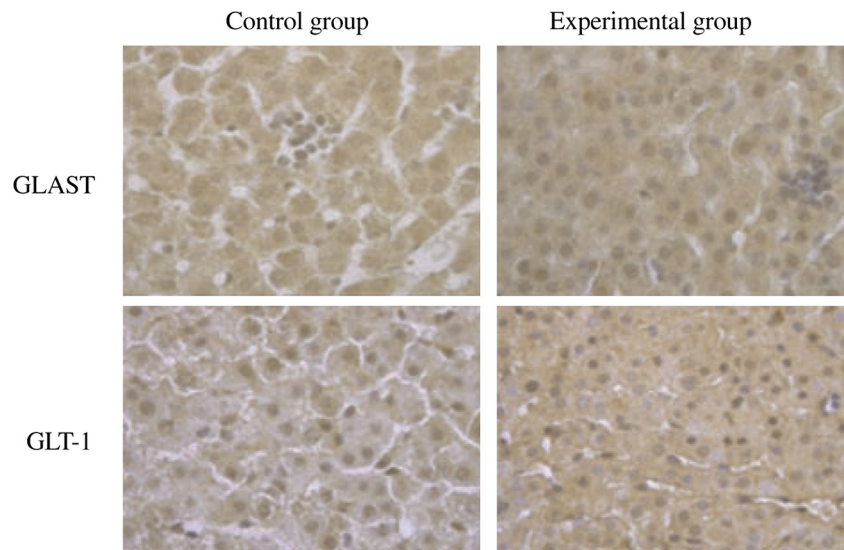


Figure 2. GLAST and GLT-1 expressions in rabbit spinal cord neurons ($\times 400$).

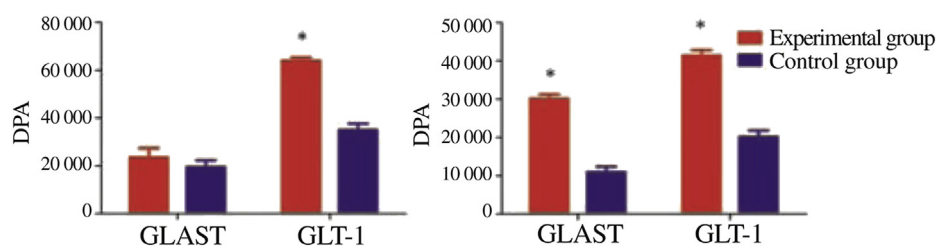


Figure 3. GLAST and GLT-1 expressions in astrocytes (A) and microglial cells (B) by immunofluorescence assay.

normal rats were intrathecally injected with glutamate transporter antagonists, rats screamed, trembled, showed behavioral disorders, as well as other pain responses in a dose-dependent manner [14]. Overall, glutamate transporters, with respect to excitatory amino acid circulation, are particularly important for terminating excitatory signals and protecting nerve cells.

Currently, the effect and mechanism of nerve blocks produced by anesthetics in nerve cells remains unclear; and there are some studies on the surface of local anesthetics for blocking nerve conduction through sodium channel inactivation. All five kinds of glutamate transporters are Na⁺ dependent. However, specifically, after intrathecal nerve block anesthesia, whether it can affect nerve cellular functions and glial transporter expressions remains unclear. After intrathecal nerve block anesthesia, by immunohistochemistry and immunofluorescence, we found that GLAST and GLT-1 expressions in microglial cells significantly increased; and in astrocytes, GLAST expressions did not have any significant change, while GLT-1 expressions were upregulated. These results suggest that intrathecal nerve block anesthesia could regulate glutamate transporter GLAST and GLT-1 expressions, upregulating these expressions and promoting synaptic cleft glutamate uptake, while promptly reducing glutamate acting on glutamate receptors; thereby, inhibiting the activation of nerve conduction and pain pathways. Anesthesia should be a safe and reversible process. In experiments, we found that after spinal anesthesia, there was a slight stratification in rabbit spinal cord meninges and phenomenon of edema. This prompted for strict control of anesthetic quality and concentration in clinical applications to avoid soft meninges caused by vasodilation and edema after anesthetics act on the spinal pia mater.

Results of this present study revealed that intrathecal nerve block anesthesia could promote the upregulation of transporter GLAST and GLT-1 expressions, and may contribute a role of avoiding excessive glutamate uptake in glutamate receptors; thereby, inhibiting nerve conduction. In this process, whether there is a corresponding cytokine or other regulatory molecules remains unclear. Studies have revealed that some pathological processes of the ERK1/2 signaling pathway after spinal cord injury could affect the expression of glutamate transporters [15]. Further research is needed to determine how GLAST and GLT-1 transporters are regulated.

Conflict of interest statement

We declare that we have no conflict of interest.

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