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EFFECTS OF NAAG AND MPEP ON RAT CORTICAL SPREADING DEPRESSION

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Cortical spreading depression (CSD) is a pathophysiological phenomenon. There are sufficient evidences to prove that CSD plays an important role in some neurological disorders. However, exact mechanisms of its initiation and propagation are still unclear. Previous studies showed that glutamate receptors could be concerned with CSD, but those studies were mostly performed oriented to ionotropic glutamate receptors (iGluRs). There is relatively little report about effects of metabotropic glutamate receptors (mGluRs) on CSD. Here, we applied optical intrinsic signal imaging (OISI) combined with direct current (DC) potential recording to examine influences of some mGluRs antagonist (or agonist) on CSD propagation in rat's brain, to indirectly validate actions of some mGluRs on CSD. We found that *N*-acetyl-L-aspartyl-L-glutamate (NAAG, an agonist at mGluR3) inhibited the propagation of CSD, and the inhibition was gradually developed with time. However, 6-methyl-2-phenylethynyl-pyridine (MPEP, an antagonist of mGluR5) did not produce any significant alterations with the CSD propagation. Our findings suggest that mGluR3 could play an important role in the CSD propagation, but the activity of mGluR5 was comparatively weak. These findings can help to understand the propagation mechanism of CSD, and consider the therapy of some neurological diseases involved with CSD.

Keywords: Metabotropic glutamate receptors; cortical spreading depression; optical intrinsic signal imaging; direct current potential; rats.

1. Introduction

Cortical spreading depression (CSD) is a propagating wave of neuronal hyper-excitability followed by depression.[1] It is an important pathophysiological phenomenon, because not only may it underlie certain clinical neurological conditions, but also understanding its mechanism is essential for a complete picture of general neurophysiology.[2] However, a specific mechanism of CSD is still unclear so far.

Glutamate is a primary excitatory neurotransmitter in nervous systems, and it plays important roles through its actions on ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).[3] Previous researches[4,5] showed that the propagation of CSD was promoted by neuronal release of glutamate and subsequent activation of its receptors on neighboring neurons. Some evidences have been provided that *N*-methyl-D-aspartate (NMDA) receptor (one type of iGluRs) activation could contribute to CSD initiation and propagation.[6] NMDA receptor antagonists could block CSD initiation and slowed down its propagation.[4,7] Except for NMDA receptor, there are two other types of iGluRs, which are α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

(AMPA receptor) and Kainte receptor (KA receptor). A few investigations have also indicated that neither AMPA nor KA receptor activation appeared to be important for the initiation or propagation of CSD.[4,8,9] Those studies,[4,6,8,9] as reviewed above, were mostly performed oriented to ionotropic receptors. To the best of our knowledge, there is little report about effects of mGluRs on CSD, except for that Mitsikostas[10] reported that mGluR4 receptors could serve as potential targets for the development of new anti-migraine drugs. It is well known that CSD is a putative electrophysiological event underlying migraine aura, which may provide indirectly evidence to support potential roles of mGluRs in CSD.

N-Acetyl-L-aspartyl-L-glutamate (NAAG), as an agonist at mGluR3, has neuroprotective actions in a variety of neurological disorders and it is also a neurotransmitter released by stimulated neurons[11–14] 6-methyl-2-phenylethynylpyridine (MPEP) is a potent and selective noncompetitive antagonist of mGluR5. Over the past several years, MPEP has been used to study potential role of the mGluR5 in neuroprotection.[15] CSD is believed to have negative impact on tissue injury during various neurological disorders including migraine, cerebrovascular diseases, and head injury.[16] This study was designed to identify whether neuroprotective roles of NAAG and MPEP reported in previous studies[13,15] would be achieved by inhibiting CSD, which may help to understand potential roles of mGluRs on CSD.

Here optical intrinsic signal imaging (OISI) combined with direct current (DC) potential recording was applied to examine influences of NAAG and MPEP on CSD propagation in rat brain. OISI enables visualization of CSD at cortical surface with fair temporal and spatial resolutions.[17] Changes of optical intrinsic signals (OIS) during CSD were observed at visible wavelengths (550 ± 10 nm), where hemoglobin absorption is prominent, and the dominant contribution to the OIS is thought to be changes in blood volume.

2. Materials and Methods

2.1. *Surgical operation*

Thirty adult male Sprague-Dawley rats (weight: 200–300 g) were used for experiments. All experimental procedures were approved by the Committee for the Care and Use of Laboratory Animals of Huazhong University of Science and Technology. The rats were deeply anesthetized with a mixture of α-chloralose (50 mg/kg) with urethane (600 mg/kg) injected intraperitoneally. Body temperature of each rat was kept constant at 37.0 ± 0.5°C with a feedback-controlled heating pad. Left femoral artery of the rat was cannulated for serial measurements of arterial pressure and blood gases.

Each rat was placed on a stereotaxic apparatus. Parietal bone was removed unilaterally with a high-speed dental drill (Fine Science Tools, USA) under constant normal saline cooling, and an imaging window (6.0 mm × 8.0 mm) was formed, and the dura over the parietal cortex in the window was torn off. A burr hole (Ø 2 mm) was drilled in ipsilateral frontal bone as an induction window of CSD. The imaging window was bathed with artificial cerebrospinal fluid at 37 ± 0.5°C, and there was a recording electrode to sample the DC potentials in this window (4–5 mm lateral from midline and 3–4 mm posterior from Bergman). A stimulating electrode was inserted carefully into the induction window to induce CSD (Fig. 1(d)). Prior to imaging, all rats were allowed to recover at least one hour after above-mentioned surgery.

All rats were divided into three groups, normal saline group ($n = 10$), NAAG group ($n = 10$), and MPEP group ($n = 10$). NAAG and MPEP were both purchased from American Sigma Company, USA. NAAG (1.5 mg/rat) and MPEP (1.2 mg/rat) were injected intracerebrally into the cortex of the imaging window respectively. When NAAG is administered exogenously at low or moderate doses (2–10 mg/kg i.p.), its prevalent effect is as an activator of mGluR3, and is neuroprotective. When NAAG is used at high doses (20 mg/kg i.p.), however, its effect as a precursor of glutamate becomes predominant, which results in toxicity.[18] The NAAG dose range (1.5 mg/rat) used here was based on a reference.[18] In this reference, it was reported that NAAG protects against focal ischemia brain injury in adult rats, when it was administered at a dose of 10 mg/kg, i.p., which was equivalent to 2–3.5 mg/rat i.p. (weight: 200–300 g).[18] Since we used injections intracerebrally in our experiments, a little small dose range of NAAG (1.5 mg/rat) was injected. MPEP was also administered intracerebrally at 10 mg/kg (1.2 mg/rat) according to a reference.[19]

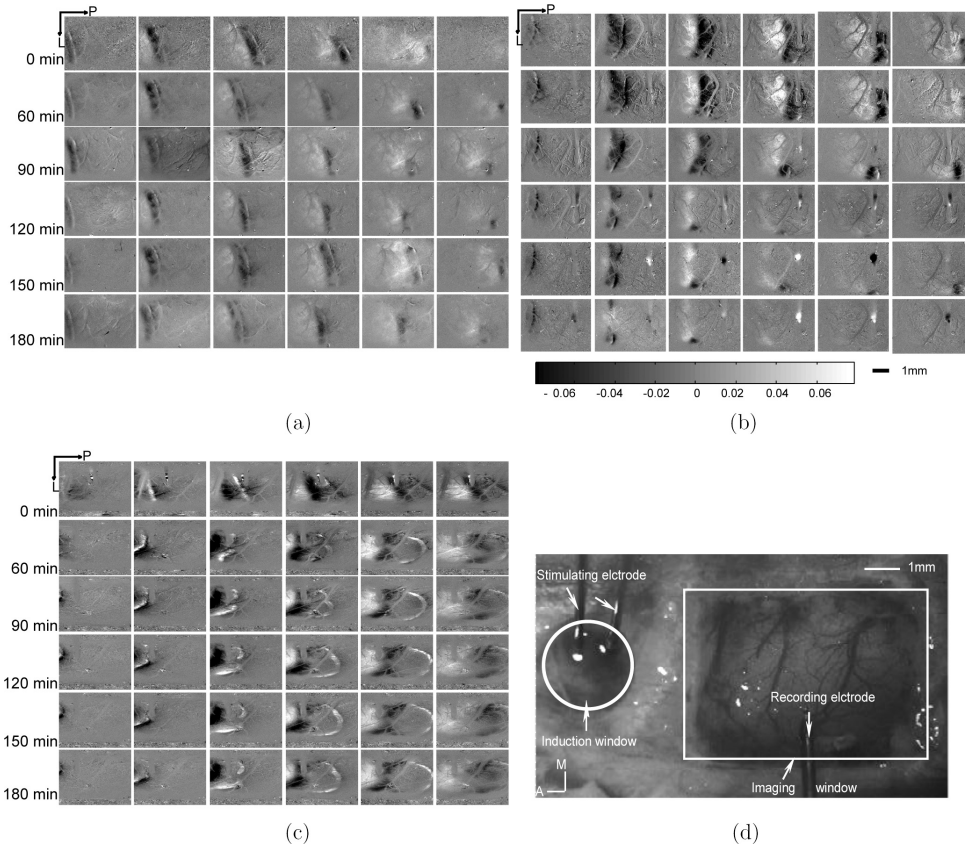

Fig. 1. Spatiotemporal evolutions of CSD waves elicited were revealed by subtracting consecutive images in the normal group (a), the NAAG group (b), and the MPEP group (c). Each row denotes a single CSD wave. The interval between consecutive images is 32 s. Generally, the CSD wave showed first as a dark, broad band and then as dispersed light area. Grayscales represent the change in optical reflectance signal intensity. Direction: P, posterior and L, lateral. Scale bar: 1 mm. Figure 1(d): White light images of the cranial window and the induction window. The induction window (circle shown, Ø 2 mm) with stimulating electrode in the frontal cortex and the imaging window (rectangular indicated, 6.0 mm × 8.0 mm) with recording electrode in the parietal region were denoted.

2.2. *CSD induced by electrical stimulation*

Each CSD wave was induced by electrical stimulation (5 mA, 1 s duration, 1 pulse) into the cortex of the burr hole. The first CSD was induced before administrations (0 min), and the remaining CSD waves were repetitively elicited at 30 min intervals after administrations.

2.3. *Optical imaging and DC recording during CSD*

Optical intrinsic signal imaging (OISI) for CSD was performed as described previously.[20] In brief, a halogen light (LG-PS2, Olympus, Japan) with a green filter (550 ± 10 nm) was used to illuminate the cortical surface of the brain of rats. A 12-bit charge coupled device camera (Pixelfly, 480 × 640 pixel array, PCO Computer, Germany) attached to a stereomicroscope (SZX12, Olympus, Japan) was employed for image acquisition. The final digitized images were captured after setting a mode of the charge coupled device camera with an exposure time of 25 ms and online average of 16 frames. The images of 1000 frames in each time were continuously collected and then stored in a computer for further processing as described.[20]

OISI were performed respectively in conjunction with DC potential recording during CSD in the NAAG group and the MPEP group. Electrical signals were digitized and stored concurrently with the optical images for further analysis.

2.4. *Data analysis*

Subtracted image analysis was used as described by Chen and colleagues[20] to characterize

spatiotemporal pattern of CSD propagation. Generally, a representative CSD wave appeared first as a dark, broad band and then as a dispersed light area.

Within the image sequence of each time, small regions of interest in cortical parenchyma close to the recording electrode were selected. The average intensity of optical reflectance of each region in each time was calculated. Choosing the intensity of optical reflectance before CSD induction as control, relative changes of the intensity of optical reflectance during CSD were calculated in each region at each time point after administrations. Values of the relative changes are expressed as Mean ± S.D., and Student's t-test was used for statistical analysis, and values were considered statistically significant at $p < 0.05$. In addition, the DC signals were shown in the same plot with the relative change of the intensity of optical reflectance in the region of interest near the recording electrode, sharing the same time axis in the same time point.

3. Results

All physiological parameters were maintained within normal limits throughout the experiments.

A representative experiment in the normal saline group was selected, and spatiotemporal patterns of propagating of CSD waves were shown in Fig. 1(a). By electrical stimulation, a single CSD wave was induced each time. Two minutes later, it invaded the ipsilateral parietal cortex. Elicited CSD wave can propagate successfully from anterior to posterior in the imaging window at each time (Fig. 1(a)).

3.1. *The inhibition of NAAG on CSD propagation*

The results from the representative experiment in the NAAG group were shown as in Fig. 1(b). Sixty minutes after NAAG treatment, CSD wave still has similar spatiotemporal pattern as that before NAAG administration. There was always single CSD wave smoothly propagated from anterior to posterior in the imaging window. However, 90 min after the administration, the propagating patterns of CSD wave began to change, the CSD wave only propagated into the anterior and middle imaging window, and it did not invade the posterior imaging window. Furthermore, the brain region invaded by CSD wave got smaller at 120 min and 150 min than that of 90 min after NAAG administration; the CSD wave only reached the anterior in the imaging window. Finally, CSD wave could not at all be observed in the imaging window at 180 min after NAAG.

DC potential recording was used simultaneously to sample the electrical signals during CSD. Figure 2(a) shows the results from the selected regions of interest near the recording electrode in a representative experiment. The relative changes of optical reflectance during CSD were calculated, and characteristics of optical intrinsic signal (OIS) related to CSD wave were a strong negative peak in

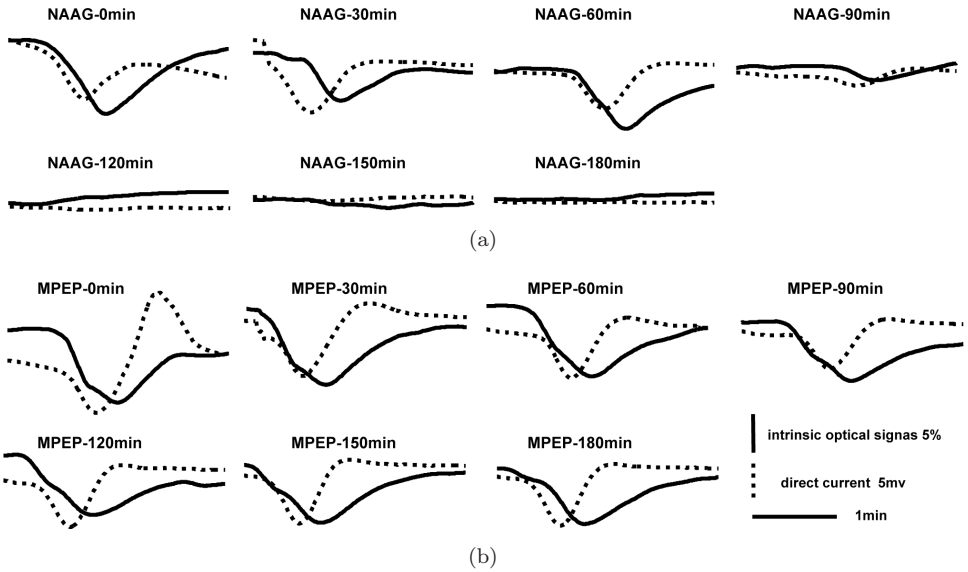

Fig. 2. Relative changes of optical intrinsic signals and changes of DC potentials were recorded simultaneously to characterize CSD waves elicited before and after administrations in the NAAG group (a) and the MPEP group (b). Changes of DC potentials and optical reflectance are both shown using the same time axis and the two kinds of signals were corresponding closely.

the optical reflectance at each time. The amplitudes of the negative peak were respectively $-9.71\pm1.43\%$ (0 min), $-7.38 \pm 0.45\%$ (30 min), $-8.37 \pm 0.33\%$ (60 min), and $-5.41\pm0.74\%$ (90 min). There was no significant difference among these three points (0, 30, and 60 min, $p > 0.05$), but the amplitude of the negative peak was declined at 90 min after NAAG treatment ($p < 0.05$). Here, we set the threshold as -5%, and some negative peaks would not be able to be detected according to the threshold. Changes of DC potentials were highly correlated with relative changes of optical reflectance. A CSD wave appeared as negatively DC shifts with -7.5 mv (0 min), -7.4 mv (30 min), -6.5 mv (60 min), and -2.7 mv (90 min). There was also no significant difference among the changes of DC potential at 0, 30, and 60 min ($p > 0.05$), and the DC potential was also obviously decreased 90 min after NAAG administration ($p < 0.05$). However, the OIS and DC potentials related to CSD were not observed at 120, 150, and 180 min after NAAG administration. Similar results were found in other rats of NAAG group.

3.2. *MPEP making no difference on CSD*

After MPEP treatment, there was always a single CSD wave propagated across the imaging window at each time in a typical experiment. The entire parietal cortex was invaded by CSD waves (Fig. 1(c)). MPEP seems to make no difference on the propagation of CSD. Similar results were found in other four rats.

The relative changes of optical reflectance and DC potentials collected at the same time from a typical experiment in the MPEP group are shown in Fig. 2(b). The relative changes of optical reflectance during CSD were calculated. The amplitudes of the negative peak in the regions close to the recording electrode were respectively $-10.71\pm0.72\%$ (0 min), $-9.04\pm1.01\%$ (30 min), $-10.62 \pm 0.25\%$ (60 min), $-9.35 \pm 1.45\%$ (90 min), $-10.03 \pm 1.02\%$ (120 min), -10.14 ± 0.72 (150 min), and $-9.30\pm1.25\%$ (180 min). The amplitudes of the negative peaks were always kept constant during the experiment ($p > 0.05$). The corresponding DC potentials were -7.4 mv (0 min), -6.0 mv (30 min), -6.6 mv (60 min), -5.5 mv (90 min), -6.7 mv (120 min), -7.2 mv (150 min), and -7.3 mv (180 min), respectively. The DC potentials shift at 30, 60, and 90 min after MPEP were weakly descent. At 150 min and 180 min after MPEP, the potentials restored to the value before MPEP treatment. Similar results could be found in other rats on MPEP group.

4. Discussion

It has been previously reported that the OIS change consistently occurred with DC potential shift during CSD.[21] This consistency between optical and electrophysiological changes was observed both *in vitro* and *in vivo*.[22,23] Our results also confirmed this consistency. More important was the fact that NAAG could block spatial propagation of CSD wave, and the block action developed gradually with time. However, MPEP did not produce any significant alterations with CSD propagation.

NAAG is distributed widely in the nervous system of mammals and serves as a co-transmitter with several small amine transmitters, including glutamate, γ-aminobutyric acid (GABA) and acetylcholine. NAAG was found to be a highly selective mGluR3 agonist. NAAG activation of postsynaptic mGluR3 was known to reduce cAMP levels via an inhibitory G protein in neuron and astrocyte. It was also clear that NAAG acts on presynaptic receptors to inhibit the release of traditional amine transmitters including GABA and glutamate.[24]

NAAG could inhibit CSD propagation attributed to some reasons as described as following.

As CSD was evoked, a considerable quantity of endogenous NAAG was released cooperative with extraneous NAAG. These NAAG acted on pre-synaptic mGluR3, as a result, lower amounts of the glutamate were released.[24] The initiation and propagation of CSD was essentially considered to be the consequence of excessive releasing of excitatory neurotransmitter glutamate.[25] Therefore, the reduction of glutamate release induced by NAAG could contribute to the inhibition of CSD propagation.

Secondly, NAAG can inhibit voltage-gated Ca^{2+} channels.[1] Voltage-gated Ca^{2+} channels, $Ca_V2.1$, were involved in facilitation of *in vivo* induction and propagation of CSD and Ca^{2+}-dependent neurotransmitter release from cortical neurons and membrane excitability.[26] Loss-of-function of $Ca_V2.1$ channels led to a lower velocity of propagation of CSD wave.[27]

Furthermore, increasing metabolic activities were required during CSD for the restoration of

membrane potential.[28] NAAG could inhibit adenylate cyclase activity through activation of postsynaptic mGluR3, and contributed to an increase of ATP and reduction of cAMP through the inhibition of adenylate cyclase,[3] which could not meet the requirement for the increasing energy consumption associated with CSD. Therefore, the propagation of CSD waves could be suppressed by NAAG.

Finally, in the adult somatosensory cortex, Na^+, K^+-ATPases were located exclusively in glial cells.[29] Glial Na^+, K^+-ATPases played an important role in clearance of K^+ from extracellular space during neuronal activities.[28] NAAG could activate the K^+ channel through mGluR3, and consequently K^+ and glutamate were quickly cleared by glial cells during CSD. Glutamate and K^+ concentration in the synaptic cleft decreased rapidly, and neuron and glial cells can recover from over-excitation.

Metabotropic glutamate receptor 5 (mGluR5), a sub-type in the group I mGluRs, couples to phospholipase C through Gq protein.[30] MPEP noncompetitively inhibits mGluR5 through a novel allosteric site reducing the efficacy of glutamate-stimulated phosphoinositide hydrolysis.[31] It is noteworthy that MPEP does not act at the extracellular glutamate-binding site of mGluR5 receptor, which is common for all known competitive mGluR antagonists.[32] MPEP inhibits mGluR5 function without affecting binding of glutamate to the extracellular region of mGluR5,[33] which makes MPEP less sensitive to the ambient concentration of glutamate, and the excitatory neurotransmitter glutamate essentially contributes to the propagation of CSD.[25] MPEP could not influence the combination of extracellular glutamate with mGluR5 during CSD, leading to no significant effect on CSD propagation.

Taken together, this study demonstrated that NAAG had an inhibitory effect on CSD propagation, but MPEP seemed to have no effect on CSD, suggesting that mGluR3 could play an important role in the CSD propagation, and the action of mGluR5 was comparatively weak. These findings may lead to better understanding of the potential roles of mGluRs during the mechanism of CSD, and furthermore to stride forward the therapy of some neurological diseases involved with CSD.

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