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Enhancement of inhibitory synaptic transmission in large aspiny neurons after transient cerebral ischemia

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Abstract

Large aspiny neurons and most of the GABAergic interneurons survive transient cerebral ischemia while medium spiny neurons degenerate in 24 h. Expression of a long-term enhancement of excitatory transmission in medium spiny neurons but not in large aspiny neurons has been indicated to contribute to this selective vulnerability. Because neuronal excitability is determined by the counterbalance of excitation and inhibition, the present study examined inhibitory synaptic transmission in large aspiny neurons after ischemia in rats. Transient cerebral ischemia was induced for 22 minutes using the four-vessel occlusion method and whole-cell voltage-clamp recording was performed on striatal slices. The amplitudes of evoked inhibitory postsynaptic currents in large aspiny neurons were significantly increased at 3 h and 24 h after ischemia, which was mediated by the increase of presynaptic release. Postsynaptic responses were depressed at 24 h after ischemia. Inhibitory postsynaptic currents could be evoked in large aspiny neurons at 24 h after ischemia, suggesting that they receive GABAergic inputs from the survived GABAergic interneurons. Muscimol, a GABA_A receptor agonist, presynaptically facilitated inhibitory synaptic transmission at 24 h after ischemia. Such facilitation was dependent on the extracellular calcium and voltage-gated sodium channels. The present study demonstrates an enhancement of inhibitory synaptic transmission in large aspiny neurons after ischemia, which might reduce excitotoxicity and contribute, at least in part, to the survival of large aspiny neurons. Our data also suggests that large aspiny neurons might receive inhibitory inputs from GABAergic interneurons.

Keywords

striatum; cholinergic interneuron; excitotoxicity; GAD; GABAergic interneuron; GABA_A

Stroke is the third leading cause of death and the number one cause of adult disability in the United States. Excitotoxicity, first coined by Olney (Olney et al., 1972), has been widely accepted as one of the major causes for post-ischemic neuronal death (Lipton, 1999). After the onset of cerebral ischemia, energy depletion results in ionic imbalance and neurotransmitter release. Extracellular glutamate concentration is dramatically increased. Through its interaction with N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-

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isoxazolepropionic acid (AMPA) receptors, glutamate triggers excessive calcium influx. Intracellular calcium overload activates an array of downstream cascades that will finally lead to post-ischemic neuronal death.

Transient cerebral ischemia, as a result of cardiac arrest in humans, induces selective neuronal death in vulnerable brain regions. In the striatum, medium spiny (MS) neurons show visible damage as early as 6 h after transient cerebral ischemia and most of them die in 24 h whereas interneurons including large aspiny (LA) neurons are spared (Pulsinelli et al., 1982, Chesselet et al., 1990). The mechanisms for such selective neuronal death after ischemia are under intensive investigation. Differential glutamatergic responses has been indicated to play a role in this differential vulnerability (Calabresi et al., 1998, Calabresi et al., 1999). Differential membrane responses to *in vitro* ischemia might also be involved. LA neurons undergo membrane hyperpolarization while MS neurons undergo depolarization (Centonze et al., 2001a). Lack of post-ischemic long-term potentiation (LTP) in LA neurons, which is present in MS neurons, is also suggested to be one of the mechanisms (Calabresi et al., 2002). However, the role of inhibitory synaptic transmission in this selective vulnerability remains unknown. Considering that both excitatory and inhibitory synaptic transmission contribute to excitotoxicity, we examined inhibitory synaptic transmission in LA neurons after transient cerebral ischemia.

In the striatum, MS neurons are the GABAergic projecting neurons, representing 95% of the neuronal population. Three kinds of GABAergic interneurons have been identified, parvalbumin (PV)-containing fast-spiking (FS) GABAergic interneurons, and neuropeptide Y, somatostatin (SOM)-containing GABAergic interneurons showing low-threshold spikes (LTS GABAergic interneurons), and calretinin-containing GABAergic interneurons (Kawaguchi et al., 1995). Therefore, LA neurons, which is cholinergic, are believed to be the only non-GABAergic neurons in the striatum (Wilson, 2007). It has been shown that LA neurons receive GABAergic inputs from the axon collaterals of MS neurons (Bolam et al., 1986, Martone et al., 1992). But it remains enigmatic whether LA neurons receive inhibitory inputs from GABAergic interneurons in the striatum. Because most of the survived neurons in the striatum are LA neurons and GABAergic interneurons at 24 h after transient cerebral ischemia, it could provide a unique system to investigate the synaptic connections between GABAergic interneurons and LA neurons. In the present study, morphological and electrophysiological approaches were used to examine the inhibitory inputs to LA neurons up to 72 h after ischemia.

Potentiation of inhibitory synaptic transmission has long been a concern for neuroprotection after ischemia since its contribution to the decrease of excitotoxicity by counterbalancing the excitability. In fact, ample evidence has demonstrated that the GABAmimetic drugs are neuroprotective after ischemia (Shuaib and Kanthan, 1997, Green et al., 2000, Schwartz-Bloom and Sah, 2001). Therefore, we hypothesized that the inhibitory synaptic transmission is enhanced in the ischemia-resistant LA neurons after ischemia.

EXPERIMENTAL PROCEDURES

Animal models of transient forebrain ischemia

Male Wistar rats of 200–250g (Charles River Laboratories, Wilmington, MA), corresponding to the age of 6–7 weeks, were used in the present study. Experimental protocols were institutionally approved in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We made our efforts to minimize both the suffering and number of animals used.

Four-vessel occlusion was used to induce transient forebrain ischemia (Pulsinelli and Brierley, 1979) with modifications (Ren et al., 1997). The rats were fasted overnight to provide uniform

blood glucose levels. During the surgery, the animals were anesthetized with a mixture of 1–2% halothane in 33% oxygen and 66% nitrogen via a nasal mask. The silicon tubing (0.025" I.D., 0.047" O.D.) was placed underneath each common carotid artery and passed through the two holes in a small Teflon button. With a plastic cylinder, a loop was formed loosely around each common carotid artery and a suture line was tied at the end of the loop to allow subsequent occlusion of these vessels. The vertebral arteries were then electrocauterized. Following craniectomy, a temperature probe (0.025" diameter, Physitemp, Clifton, NJ) was placed underneath the skull and the brain temperature was maintained at 36.5° C with a heating lamp (BAT-10, Physitemp, Clifton, NJ). A glass microelectrode (5–8 μ m in diameter of the tip) filled with 2M NaCl was advanced 3.0 mm below the dura mater into the striatum. When performing the occlusion, the silicone tubing was pulled through the cylinder, compressing the artery against the Teflon button. Ischemic depolarizations (DC potential shifts from 0 mV to about –20 mV), recorded by a neuroprobe amplifier (Model 1600, A-M systems, Carlsborg, WA), occurred approximately 2–3 min after the occlusion. The occlusion lasted for 20 min. Upon release of the tubing, cerebral blood flow resumed immediately but it took about 1–2 min for the DC potential to return to 0 mV.

Slice preparation and whole-cell voltage-clamp recording

Brain slices were harvested from control rats and the rats at 3 h, 8 h, and 24 h after ischemia as previously reported (Pang et al., 2002). The rat was deeply anesthetized with the Ketaset and then perfused transcardially with the cold sucrose solution oxygenated with 5% CO₂ and 95% O₂. The composition of the sucrose solution was as follows (in mM): Sucrose 230; NaHCO₃ 26; KCl 2.5; NaH₂PO₄ 1.25; CaCl₂ 0.5; MgSO₄ 10; Glucose 10. The rat was then decapitated and the brain was quickly removed from the skull. Coronal striatal slices (300 μ m) were cut using a vibratome (Leica VT1000, Leica Microsystems GmbH, Heidelberg, Germany) in the ice-cold sucrose solution. The slices were then incubated in the artificial cerebrospinal fluid (aCSF) equilibrated with 95% O₂ and 5% CO₂ for 30 min at 34 °C before being returned to room temperature. The aCSF was comprised as the following (in mM): NaCl 130; KCl 3; CaCl₂ 2; MgCl₂ 2; NaH₂PO₄ 1.25; NaHCO₃ 26; Glucose 10.

The electrodes for whole-cell voltage-clamp recordings were prepared using a horizontal micropipette puller (Framing/Brown 97, Sutter, Novato, CA) from the borosilicate glass capillaries (Warner, Hamden, CT). The internal solution contained (in mM): CsCl 43; CsMeSO₄ 92; TEA 5; EGTA 2; MgCl₂ 1; HEPES 10; QX314 3 and Mg-ATP 4. The inclusion of QX314 blocked the voltage-dependent sodium channels and GABA_B receptor activated potassium conductance (Andrade, 1991). The pipette resistance was 2–4 M Ω using this internal solution. Recordings were performed under an infrared-DIC microscope (BX50WI, Olympus, Tokyo, Japan) and a CCD camera in the aCSF at 30–32 °C (SF-28 in-line heater, Warner, Hamden, CT). The flow rate was adjusted to 2–3 ml/min. After the high-resistance seal (>1 G Ω) was formed, a negative pressure was used to rupture the membrane to obtain the whole-cell configuration. Recordings were made using Axopatch 200B (Molecular Devices, Sunnyvale, CA). LA neurons were held at –60 mV. Signals were filtered at 1–5 KHz and sampled at 5–10 KHz for on-line and later off-line analysis by the data acquisition program Axograph 4.9 (Molecular Devices, Sunnyvale, CA). Intrastriatal stimulation was evoked by using the bipolar tungsten electrodes (~5 M Ω , Micro Probe, Potomac, MD). The stimulus electrode was positioned 150–200 μ m subjacent to the recording electrode. Isolated stimulator (ISO-Flex, AMPI, Israel) was used and electrical currents with the pulse width of 100 μ s were given every 5 sec (Master-8, A.M.P.I., Israel). The threshold of stimulus intensity was determined when the amplitude of evoked inhibitory postsynaptic currents (eIPSCs) just appeared. Since a submerged chamber and the bipolar stimulating electrodes were used, the currents released from the stimulator may not represent the currents passing the tissue. Thus, we normalized the stimulus intensity as 2 times of the threshold (2T), 3T, 4T etc. For the paired-

pulse stimulation, 3T was always used and the interval between the two stimuli was 50 ms. Only monosynaptic transmission with consistent onset latencies was collected for data analysis. Access resistance was monitored throughout the recording, and cells that demonstrated the access resistance below 20 M Ω were included in the analysis. An episode of 5–10 min was collected for miniature IPSCs (mIPSCs) and spontaneous IPSCs (sIPSCs). Tetrodotoxin (TTX, 1 μ M) was applied during mIPSCs recording. Axograph 4.9 was used to detect and measure the amplitude and inter-event intervals of the synaptic events. IPSCs above a threshold (4.5–5.5 SDs above baseline noise) were automatically detected by a sliding template algorithm and manually checked off-line. For the Ca²⁺-free solution, CaCl₂ was removed from the aCSF and 3 mM ethylene glycol tetraacetic acid (EGTA) was added.

Doublelabeling immunofluorescence and histochemical processing of tissues

Under deep anesthesia, the rat was transcardially perfused with 0.01 M NaPBS (pH=7.4) followed by the cold 4% paraformaldehyde (pH=7.4) in 0.15 M NaPBS. The brain was removed and post fixed in the same fixative at 4 °C overnight. Coronal sections of 50 μ m were cut using a vibratome and they were collected in 0.01 M KPBS. Some of the sections were mounted on slides for standard haematoxylin and eosin (HE) staining. Sections for immunofluorescence studies were incubated in the blocking serum (5% normal horse serum in 0.01 M KPBS with 0.5% Triton X-100) for 1 h at room temperature. The sections were then incubated with either mouse anti-glutamate decarboxylase 65 (anti-GAD 65; 1:500; Chemicon, MAB 351; Temecula, CA) or mouse anti-GAD 67 antibodies (1:1,000; Chemicon, MAB 5406) in the blocking serum overnight at room temperature. After three washes in KPBS, they were incubated in the secondary antibody solutions, Fluorescein horse anti-mouse IgG (1:100; Vector, FI-2000; Burlingame, CA) for two hours. Again, they were in the blocking serum (10% normal goat serum in 0.01 M KPBS with 0.5% Triton X-100) and then incubated with the rabbit anti-choline acetyltransferase antibodies (anti-CHAT; 1:1000; Chemicon, AB 5042) overnight at room temperature. Subsequently, they were in the Texas Red goat anti-rabbit IgG (1:100; Vector, TI-2000). Following three washes, they were mounted on slides and cover slipped with vectashield (Vector, H-1000) for fluorescence observation. Images were taken with a CCD camera installed on the Olympus BX50 microscope.

Neurobiotin (1%) was included in the internal solution for intracellular staining of some recorded neurons. After successful recordings, neurobiotin was delivered into the cell by passing depolarizing pulses for about 5 min. The slice was placed in 4% paraformaldehyde (pH=7.4) at 4 °C overnight. Then, the slice was incubated in 0.1% peroxidase-conjugated avidin-D in KPBS (pH=7.4) with 0.5% Triton X-100 for 6–8 h at room temperature. Subsequently, the slice was detected with 3,3'-diaminobenzidine (DAB).

Drug application

γ -aminobutyric acid (GABA), (–)-bicuculline methiodide (BMI), DL-2-amino-5-phosphonopentanoic acid (D-APV), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), TTX and muscimol were purchased from Sigma (St. Louis, MO). Neurobiotin was purchased from Vector (Burlingame, CA). Drugs were dissolved in the aCSF and applied through bath perfusion except that GABA was applied through a Y tube system.

Statistics

Numerical data were expressed as mean $\pm$ SE. Unpaired or paired two-tailed Student's t-tests were applied using the Statview (Statview, Abacus Concepts, Cary, NC). The amplitudes and inter-event intervals of mIPSCs and sIPSCs from a single neuron were examined by constructing the distributions of cumulative probability and the Kolmogorov-Smirnov (K-S) tests were used to detect the difference. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 99 rats were used in the present study. Experiments were performed on the control rats (n=35), rats at 3 h (n=13), 8 h (n=10), 24 h (n=37) and 72 h (n=4) after transient cerebral ischemia.

Inhibitory synaptic transmission is enhanced in LA neurons after transient cerebral ischemia

To reveal the morphological changes of striatal neurons after ischemia, striatal sections were examined by the HE staining. A majority of the striatal neurons were medium-sized, possibly MS neurons and GABAergic interneurons (Fig. 1A). Only a few of the striatal neurons were large-sized, possibly LA neurons. At 3 h after ischemia, most of the striatal neurons showed normal morphology (Fig. 1B). But at 24 h and 72 h after ischemia, the medium-sized neurons exhibited pyknotic, shrunken nuclei, indicating post-ischemic degeneration while the large-sized neurons remained morphologically intact (arrows, Fig. 1C–D).

Under infrared-DIC microscopes, LA neurons could be easily identified by their large soma size (25–60 μm in diameter) compared to the medium-sized neurons (10–15 μm in diameter, Fig. 2A) (Kawaguchi, 1992). Some LA neurons were further confirmed by intracellular staining with neurobiotin after successful recordings. In LA neurons, primary dendrites formed many branches bearing few spines (Fig. 2B). LA neurons also have distinctive electrophysiological characteristics. Upon depolarizing current pulses, LA neurons showed prominent afterhyperpolarizations after action potentials. Injection of negative current pulses first produced a hyperpolarization and later a sag in the membrane potential as a result of the activation of I_h currents (Fig. 2C) (Jiang and North, 1991; Kawaguchi, 1993).

In the control animals, intrastriatal electrical stimulation evoked postsynaptic currents from LA neurons containing IPSCs mediated by GABA_A receptors and excitatory postsynaptic currents (EPSCs) mediated by glutamate receptors. They could be blocked by bicuculline (30 μM), the combination of D-APV (50 μM) and CNQX (10 μM), respectively (Fig. 2D). In the subsequent experiments, D-APV and CNQX were used to pharmacologically isolate GABAergic IPSCs. At a holding potential of -60 mV , inwardly directed eIPSCs were elicited.

Based on the morphological study, at 3 h after ischemia, the inhibitory synaptic transmission in LA neurons should be mainly from MS neurons. However, at 24 h after ischemia, the inhibitory synaptic transmission in LA neurons, if there is any, should be from the survived GABAergic interneurons. Therefore, IPSCs were evoked from LA neurons at 3 h and 24 h after ischemia, respectively, to reveal the inhibitory inputs from different sources. The amplitudes of eIPSCs were significantly enhanced in LA neurons after ischemia across the stimulus intensities (Figure 3A). For example, at a stimulus intensity of three times of the threshold stimulus intensity (3T), the amplitude was enhanced from $-47 \pm 7\text{ pA}$ in the control (n=12) to $-136 \pm 30\text{ pA}$ at 3 h after ischemia (n=9; $P < 0.05$) and $-183 \pm 30\text{ pA}$ at 24 h after ischemia (n=9; $P < 0.05$; Fig. 3B). In contrast, eIPSCs in MS neurons were significantly depressed after ischemia (Fig. 3C). For example, at 3T, the amplitude was depressed from $-91 \pm 14\text{ pA}$ of the control level (n=12) to $-52 \pm 10\text{ pA}$ (n=12; $P < 0.05$; Fig. 3D) at 8 h after ischemia.

The stimulus threshold was significantly increased from $0.31 \pm 0.02\text{ mA}$ of the control level (n=12) to $0.86 \pm 0.15\text{ mA}$ (n=9; $P < 0.01$) and $0.82 \pm 0.19\text{ mA}$ (n=9; $P < 0.01$; Fig. 3E) in LA neurons at 3 h and 24 h after ischemia, respectively. The increase of the stimulus threshold at 3 h after ischemia indicated that the excitability of presynaptic terminals might be decreased or the terminals might be damaged even though most of the MS neurons were morphologically normal (Fig. 1B). The increase of stimulus threshold at 24 h after ischemia was consistent with the decreased availability of GABAergic terminals as a result of extensive degeneration of MS neurons (Fig. 1C). The threshold also showed greater variability after ischemia, ranging from

0.35 mA to 2 mA. To examine if this increase of eIPSCs resulted from the increase of the stimulus threshold, we plotted the absolute values of 3T against the amplitudes of eIPSCs at 3 h and 24 h after ischemia. No linear correlation was observed in both groups, indicating the increase of eIPSCs was not due to the increase of the stimulus threshold (Fig. 3F).

It is known that MS neurons mainly express GAD65 while GABAergic interneurons mainly express GAD67 (Kawaguchi et al., 1995). Therefore, the antibodies of GAD65 and GAD67 were used in the doublelabeling immunofluorescence studies to identify the terminals of MS neurons and GABAergic interneurons (Gonzales et al., 1992). In the control LA neurons (CHAT-positive), many GAD65-positive terminals were observed on the somata and proximal dendrites of LA neurons (Fig. 4A). A few GAD67-positive terminals were found on the somata and proximal dendrites of LA neurons (Fig. 4B). At 24 h and 72 h after ischemia, more GAD67-positive terminals were found on LA neurons (Fig. 4C–D).

The above results show that the inhibitory synaptic transmission is significantly enhanced in ischemia-resistant LA neurons while depressed in ischemia-vulnerable MS neurons after ischemia. The inhibitory inputs to LA neurons at 24 h after ischemia might come from the GABAergic interneurons.

The enhancement of eIPSCs after ischemia is mainly due to the increase of presynaptic release

To further determine whether the pre- and/or postsynaptic mechanisms were involved in the alterations of eIPSCs after ischemia, we performed paired-pulse tests. For LA neurons, the synapses exhibited paired-pulse depression, indicating a high initial release probability (Fig. 5A). Paired-pulse ratio (PPR) was 0.69 ± 0.03 under the control condition ($n=12$), 0.50 ± 0.05 at 3 h after ischemia ($n=8$; $P<0.01$) and 0.52 ± 0.02 at 24 h after ischemia ($n=10$; $P<0.01$; Fig. 5B), suggesting an increase of presynaptic release probability.

We also examined sIPSCs, which are composed of both the quanta-release events and the action potential-mediated events. The frequency of sIPSCs was significantly increased after ischemia. In the control, the frequency was 1.24 ± 0.15 Hz ($n=17$); at 3 h after ischemia, it was 2.01 ± 0.3 Hz ($n=12$; $P<0.05$); at 24 h after ischemia, it was 2.01 ± 0.26 Hz ($n=15$; $P<0.05$). Therefore, the excitability of presynaptic terminals is significantly increased after ischemia. The increase of the stimulus threshold at 3 h after ischemia is due to the damage but not the decreased excitability of GABAergic terminals. The amplitude of sIPSCs was slightly decreased. In the control, it was 20.02 ± 1.74 pA ($n=17$); at 3 h after ischemia, it was 16.99 ± 1.27 pA ($n=12$; $P>0.05$); at 24 h after ischemia, it was 16.52 ± 1.10 pA ($n=15$; $P>0.05$; Fig. 5C–E).

mIPSCs are believed to represent only the random quantal release of neurotransmitters. We couldn't detect any changes in the frequency of mIPSCs after ischemia (control: 1.52 ± 0.34 Hz, $n=11$; 3 h after ischemia: 1.03 ± 0.17 Hz, $n=10$; $P>0.05$; 24 h after ischemia: 1.30 ± 0.27 Hz, $n=12$; $P>0.05$). The amplitude of mIPSCs was significantly decreased at 24 h after ischemia indicating depressed postsynaptic responses (control: 15.08 ± 0.67 pA, $n=11$; 3 h after ischemia: 14.35 ± 0.94 pA, $n=10$; $P>0.05$ compared to the control; 24 h after ischemia: 12.16 ± 0.5 pA, $n=12$; $P<0.01$ compared to the control; $P<0.05$ compared to 3 h after ischemia; Fig. 6A–C).

A decrease in the number or function of postsynaptic GABA_A receptors might contribute to the depressed postsynaptic responses. To test this possibility, local application of GABA (300 μ M) was used to examine the postsynaptic responses. The current amplitude induced by exogenous GABA application was reduced at 24 h after ischemia. It was 566 ± 84 pA in the control ($n=5$), 510 ± 64 pA at 3 h after ischemia ($n=11$; $P>0.05$ compared to the control) and 305 ± 55 pA at 24 h after ischemia ($n=6$; $P<0.05$ compared to the control; $P<0.05$ compared to 3 h after ischemia; Fig. 6D–E).

The above studies indicate that both pre- and postsynaptic mechanisms are involved in the alterations of inhibitory synaptic transmission after ischemia. Presynaptically, the GABA release is increased. Postsynaptically, the GABA_A receptors show depressed responses at 24 h after ischemia. But in all, the presynaptic effects far exceed the postsynaptic effects, which results in the facilitation of inhibitory synaptic transmission after ischemia.

Presynaptic GABA_A receptors facilitate GABA release at 24 h after ischemia

It is well known that GABA_A receptors are located not only at the postsynaptic sites but also the extrasynaptic and presynaptic sites. GABA_A receptors are actively involved in the phasic inhibition, tonic inhibition and the modulation of presynaptic transmitter release (Farrant and Nusser, 2005). Presynaptic GABA_A receptors are of great interest because they could be excitatory or inhibitory depending on two factors. One is the difference between the resting membrane potential and chloride reversal potential (E_{Cl}), the latter of which is mainly dependent on $[Cl^-]_i$. The other is the shunting effect.

To explore whether presynaptic GABA_A receptors play a role in the modulation of inhibitory synaptic transmission, eIPSCs were recorded before and after muscimol (a GABA_A receptor agonist) application in both control LA neurons and LA neurons at 24 h after ischemia. Besides being an agonist for GABA_A receptors, muscimol is also reported to activate GABA_B receptors but at a higher EC₅₀ of 25 μ M (compared to the EC₅₀ of 1 μ M for GABA_A receptors) (Yamauchi et al., 2000). Therefore, at a concentration of 1 μ M used in the present study, muscimol should have only activated GABA_A receptors. Muscimol (1 μ M) was applied for 5 min via bath perfusion. Recordings were made after muscimol application. Baseline noise and holding current were increased in the presence of muscimol, indicating the activation of postsynaptic GABA_A receptors. In the control, muscimol depressed the amplitude of eIPSCs (at 3T) by 64 $\pm$ 10% (before: 53 $\pm$ 13 pA; after: 35 $\pm$ 10 pA; n=7; $P>0.05$; Fig. 7A1, B). After ischemia, muscimol significantly increased the amplitude of eIPSCs to 177 $\pm$ 15% of the pre-application level (before: 95 $\pm$ 34 pA; after: 140 $\pm$ 44 pA; n=8; $P<0.01$; Fig. 7A2, B). Paired-pulse tests resulted in a decrease of PPR (before: 0.51 $\pm$ 0.04; after: 0.40 $\pm$ 0.03; 76 $\pm$ 5% of the pre-application level, n=6; $P<0.05$) in ischemic group but no significant change of PPR in the control (before: 0.69 $\pm$ 0.04; after: 0.74 $\pm$ 0.12; 108 $\pm$ 17% of the pre-application level, n=7; $P>0.05$; Fig. 7B) was detected after muscimol application. The data from paired-pulse tests suggest that muscimol presynaptically increases GABA release after ischemia.

We also examined sIPSCs before and after muscimol application. In the control, the frequency of sIPSCs was decreased (before: 1.01 $\pm$ 0.16 Hz; after: 0.71 $\pm$ 0.12 Hz; 64 $\pm$ 9% of the pre-application level, n=7; $P<0.05$) and the amplitude was increased (before: 16.48 $\pm$ 1.80 pA; after: 22.21 $\pm$ 2.90 pA; 136 $\pm$ 8% of the pre-application level, n=7; $P<0.01$; Fig. 8A–C) after muscimol application. After ischemia, the frequency of sIPSCs was increased (before: 2.02 $\pm$ 0.36 Hz; after: 2.91 $\pm$ 0.28 Hz; 148 $\pm$ 14% of the pre-application level, n=6; $P<0.05$) and the amplitude showed no change (before: 16.43 $\pm$ 2.33 pA; after: 17.21 $\pm$ 1.68 pA; 107 $\pm$ 5% of the pre-application level, n=6; $P>0.05$; Fig. 8D–F) after muscimol application. Therefore, muscimol differentially modulates presynaptic GABA release in the control and ischemic neurons. Muscimol depresses GABA release in the control while it facilitates GABA release after ischemia.

To reveal the role of calcium in muscimol's action, sIPSCs were recorded before and after muscimol application at 24 h after ischemia in the Ca²⁺-free solution. Cadmium, which is a blocker of the voltage-gated calcium channels, was not used since cadmium itself is known to block GABA_A responses (Fisher and Macdonald, 1998). Furthermore, it is reported that GABA_A currents in LA neurons are zinc-sensitive (Yan and Surmeier, 1997). When the bathing solution was switched from the aCSF to the Ca²⁺-free aCSF, the amplitude (before: 16.89 $\pm$ 1.03 pA; after: 13.90 $\pm$ 0.62 pA; 87 $\pm$ 4% of the pre-Ca²⁺ removal, n=8; $P<0.05$) and frequency

(before: 1.54 ± 0.29 Hz; after: 0.89 ± 0.19 Hz; $61 \pm 10\%$ of the pre- Ca^{2+} removal, $n=8$; $P<0.05$; Fig.9A, B) of sIPSCs were greatly decreased. In the Ca^{2+} -free aCSF, muscimol application had no effect on the frequency (before: 0.94 ± 0.20 Hz; after: 0.77 ± 0.17 Hz; $87 \pm 12\%$ of the pre-application level, $n=7$; $P=0.15$) and amplitude (before: 13.18 ± 0.90 pA; after: 13.96 ± 0.76 pA; $113 \pm 7\%$ of the pre-application level, $n=7$; $P=0.25$; Fig.9A, C, F) of sIPSCs after ischemia. Therefore, muscimol's facilitation on presynaptic GABA release after ischemia is sensitive to the extracellular calcium.

Unlike sIPSCs, mIPSCs showed no significant changes in the frequency (before: 1.35 ± 0.50 Hz; after: 1.27 ± 0.56 Hz; $95 \pm 7\%$ of the pre-application level, $n=6$; $P>0.05$) and amplitude (before: 13.01 ± 1.01 pA; after: 13.91 ± 1.22 pA; $111 \pm 4\%$ of the pre-application level, $n=6$; $P>0.05$; Fig.9D, E, G) after muscimol application in the ischemic neurons. Therefore, muscimol's facilitation on presynaptic GABA release after ischemia is dependent on the voltage-gated sodium channels.

DISCUSSION

The main finding of the present study is that inhibitory synaptic transmission is facilitated in LA neurons after transient cerebral ischemia. Presynaptic GABA_A receptors facilitate inhibitory synaptic transmission by increasing the presynaptic GABA release after ischemia. The inhibitory synaptic transmission evoked at 24 h after ischemia might come from the GABAergic interneurons. The enhancement of inhibitory synaptic transmission in LA neurons might partially contribute to their resistance to transient cerebral ischemia.

Enhancement of inhibitory synaptic transmission in LA neurons after ischemia

Large accumulation of GABA in the extracellular space has been demonstrated by *in vivo* and *in vitro* studies during ischemia and early after reperfusion (Globus et al., 1988, Allen et al., 2004). But as to the alterations of GABAergic synaptic transmission, there are contradictory reports. Inhibitory synaptic transmission is depressed or disappeared in striatal MS neurons during ischemia and early after reperfusion *in vivo* (Xu, 1995, Gajendiran et al., 2001). The frequency of sIPSCs is increased in neocortical layer 5 neurons and hippocampal CA1 neurons during simulated ischemia *in vitro* (Fleidervish et al., 2001, Allen and Attwell, 2004). The present study observed an enhancement of inhibitory synaptic transmission in the ischemia-resistant LA neurons at 3 h and 24 h after transient cerebral ischemia. It is known that synaptic strength is the product of the number of the readily releasable vesicles, the probability of each vesicle being released and the quantal size. It is difficult to predict the number of the readily releasable vesicles on brain slices. As to the presynaptic release probability, we cannot make the conclusion that it is increased at 3 h after ischemia since there is no change in the frequency of mIPSCs, although we observed a decrease of PPR. However, we could infer that presynaptic release is increased, which is action potential-dependent, as the frequency of sIPSCs is increased. A possible explanation for this is that the excitability of the functional terminals might have increased at 3 h after ischemia. The increase of presynaptic release probability as inferred from the paired-pulse tests might be dependent on the increased excitability of the presynaptic terminals. The release probability from GABAergic interneurons to LA neurons might have increased at 24 h after ischemia considering the severe loss of inhibitory inputs from MS neurons but little change in the frequency of mIPSCs. Previous studies also suggest that presynaptic mechanisms contribute to the abnormal synaptic transmission after ischemia. For example, MS neurons show decreased inhibitory synaptic transmission and LA neurons show decreased excitatory synaptic transmission after ischemia due to the activation of presynaptic adenosine A1 receptors (Centonze et al., 2001b, Pang et al., 2002); MS neurons show increased excitatory synaptic transmission after ischemia due to the activation of presynaptic ATP P2X receptors (Zhang et al., 2006).

The quantal size exhibited a time-dependent decrease after ischemia since the mean amplitude of mIPSCs was unchanged at 3 h after ischemia but was significantly decreased at 24 h after ischemia. The same pattern was observed in the current amplitude induced by exogenous GABA application. Decreased number and/or depressed functions of postsynaptic GABA_A receptors might contribute to the depressed postsynaptic responses. We found muscimol application increased the amplitude of sIPSCs in the control group but not in the ischemia group, indicating that postsynaptic GABA_A receptors might have been saturated or the number of postsynaptic GABA_A receptors might have decreased at 24 h after ischemia. The functions of postsynaptic GABA_A receptors might have also depressed since GABA_A responses are very sensitive to intracellular ATP levels (Shirasaki et al., 1992). Decreased ATP production after ischemia induces a selective and rapid rundown of the postsynaptic GABA_A responses (Harata et al., 1997). Other factors induced by ischemia, like calcium overload and the production of arachidonic acids, also depress GABA_A receptor functions (Schwartz et al., 1992).

Slow synaptic inhibition mediated by GABA_B receptors is also a concern for neuroprotection. It has been revealed that increasing GABA_B receptor function will help to reduce excitotoxicity and promote neuronal survival after ischemia (Kuramoto et al., 2007). However, considering the activation of presynaptic GABA_B autoreceptors, the release of glutamate, GABA and a group of other neurotransmitters will be decreased and varying effects might occur. The present study demonstrates an enhancement of inhibitory synaptic transmission in the ischemia-resistant LA neurons after transient cerebral ischemia, which might contribute to the decrease of excitotoxicity by depressing the overall excitability. This is consistent with previous studies showing that LA neurons have decreased excitatory synaptic transmission (Pang et al., 2002) and excitability after ischemia (Deng et al., 2008). On the contrary, ischemia-vulnerable MS neurons show increased excitatory synaptic transmission (Zhang et al., 2006) and depressed inhibitory synaptic transmission after ischemia. The balance between excitation and inhibition is needed to maintain the normal brain function. Depressed excitability limits calcium influx and stops the cascades to post-ischemic neuronal death while increased excitability contributes to post-ischemic neuronal death. The differential changes of the synaptic transmission in LA neurons and MS neurons might contribute to their differential vulnerability to ischemia.

Modulation of inhibitory synaptic transmission by presynaptic GABA_A receptors

At 24 h after ischemia, we observed an increase of eIPSCs after muscimol application. This might be partly contributed by muscimol's postsynaptic effects since baseline noise were increased upon muscimol application. However, we believe that this is mainly due to the increase of presynaptic release because a decrease in PPR and an increase in the frequency of sIPSCs were observed at the same time. In the control, muscimol decreased presynaptic release as evidenced by the decrease in the frequency of sIPSCs although muscimol's postsynaptic action couldn't be ignored as no change in PPR was observed. Considering muscimol's totally opposite effects on the presynaptic release, the effect of presynaptic GABA_A receptor activation must have turned from inhibition in the control to excitation after ischemia. A more depolarized E_{Cl} relative to the resting membrane potential might have occurred after ischemia, which could be the consequence of an increase in [Cl⁻]_i. Studies have shown that [Cl⁻]_i is increased in hippocampal neurons after *in vitro* ischemia. Activation of GABA_A receptors (Inglefield and Schwartz-Bloom, 1998), downregulation of K⁺-Cl⁻ cotransporters (KCC2) (Galeffi et al., 2004) or activation of Na⁺-K⁺-Cl⁻ cotransporters (NKCC1) (Pond et al., 2006) has been suggested to be involved in the [Cl⁻]_i increase. Although the above results strongly indicate muscimol's presynaptic action, we cannot tell the exact location of the GABA_A receptors activated by muscimol. The GABA_A receptors could be on the presynaptic terminals, somata or the dendrites. Studies have shown that synaptic activities in the dendrite and soma control the generation of action potentials and the synaptic transmission that these action potentials initiate (Shu et al., 2006).

The present study showed that muscimol's facilitative effect on synaptic transmission after ischemia was dependent on the extracellular calcium and voltage-gated sodium channels. We speculate that after ischemia, activation of presynaptic GABA_A receptors by muscimol might depolarize the terminals to the extent to activate the voltage-gated sodium channels and calcium channels, which will cause an elevation of $[Ca^{2+}]_i$ and more GABA release. Studies have shown that voltage-gated calcium channels are involved in the GABA_A receptor-induced $[Ca^{2+}]_i$ rises (Leinekugel et al., 1995, Jang et al., 2001, Xiao et al., 2007). However, there are also studies showing that GABA_A receptor-induced $[Ca^{2+}]_i$ rises is driven by osmotic tension (Chavas et al., 2004).

It is known that reversed uptake through GABA transporters contributes to the large accumulation of GABA in the extracellular space after ischemia (Allen et al., 2004). From the other point of view, the large accumulation of GABA might be due to the constant activation of presynaptic GABA_A receptors located on the GABAergic terminals. More GABA release happens when $[Cl^-]_i$ is increased in the GABAergic terminals after ischemia. Thus, the present study provides a new way to explain the relationship among GABA, GABA_A receptors and GABAergic synaptic transmission after ischemia.

Inhibitory inputs from GABAergic interneurons to LA neurons

The inhibitory inputs from GABAergic interneurons to LA neurons have long been doubted though not substantiated yet (Bennett and Wilson, 1998, Koos and Tepper, 1999). The reason for the lack of competent evidence is obvious. The scarcity of these two types of neurons (2–3% of striatal population) makes it extremely difficult to catch their actions (Rymar et al., 2004), which explains why the electrophysiologists are daunted to perform the paired recordings. On the other hand, LA neurons actually receive very few inhibitory inputs. Coincident with our results, previous study shows that the frequency of sIPSCs in the control LA neurons is very low (Bennett and Wilson, 1999). Consistently, electron microscopic (EM) studies found that the somata and dendrites of LA neurons are poorly innervated by inhibitory terminals (Chang and Kita, 1992). Among the three types of GABAergic interneurons, the synaptic contacts from PV-containing GABAergic interneurons to LA neurons are not found although the contacts from LA neurons to GABAergic interneurons are frequently seen (Chang HT and Kita H, 1992). Reciprocal synaptic contacts are found between neuropeptide Y-containing GABAergic interneurons and LA neurons (Vuillet et al., 1992). One recent study speculates that the polysynaptic inhibitions from GABAergic interneurons to LA neurons are mediated by neuropeptide Y-containing GABAergic interneurons (Sullivan et al., 2008). No reports are found regarding the synaptic connections between calretinin-containing GABAergic interneurons and LA neurons. Therefore, neuropeptide Y-containing GABAergic interneurons are the candidates providing inhibitory inputs to LA neurons.

Although the question whether GABAergic interneurons provide inhibitory inputs to LA neurons could only be definitely answered by electrophysiological evidence such as the paired recording or anatomical evidence such as the EM study, our preliminary data at least indicates that these inputs exist at 24 h after ischemia. We speculate that the inhibitory contacts from GABAergic interneurons to LA neurons exist in the control condition but the terminals from GABAergic interneurons are functionally silent or masked by the overwhelming majority of the terminals from MS neurons. However, they are functional and unmasked after ischemia. Presynaptically silent synapses have been reported in a variety of brain structures and presynaptic unsilencing is very important for the expression of LTP (Atasoy and Kavalali, 2006). Some molecular mechanisms participated in the ischemia-induced neuropathology are also involved in the expression of long-term synaptic plasticity (Di Filippo et al., 2008). These common mechanisms might contribute to the presynaptic unsilencing after ischemia and the expression of post-ischemic LTP. It is possible that the surviving subpopulations of MS neurons

provide the inhibitory inputs to LA neurons after ischemia but they cannot account for the significant enhancement of eIPSCs. One other possibility is that new GABAergic synapses might be formed and contributes to the enhancement of IPSC at 24 h after ischemia. This is the least likely since most of the newly generated cells in the striatum are microglia in the first several days after ischemia and only very few newborn cells express GAD67 until several months later (Pforte et al., 2005, Tonchev et al., 2005). Further studies on the neuronal populations in the post-ischemic striatum will be the future interests.

Abbreviations

LA, large aspiny
 MS, medium spiny
 GABA, γ -aminobutyric acid
 eIPSCs, evoked inhibitory postsynaptic currents
 PV, parvalbumin
 FS, fast spiking
 aCSF, artificial cerebrospinal fluid
 sIPSCs, spontaneous inhibitory postsynaptic currents
 mIPSCs, miniature inhibitory postsynaptic currents
 TTX, Tetrodotoxin
 HE, haematoxylin and eosin
 GAD65, glutamate decarboxylase 65
 GAD67, glutamate decarboxylase 67
 CHAT, choline acetyltransferase
 D-APV, DL-2-amino-5-phosphonopentanoic acid
 CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium
 K-S test, Kolmogorov-Smirnov test
 PPR, paired-pulse ratio
 EM, electron microscope
 LTP, long-term potentiation.

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Fig. 1.

Striatal neurons show selective death after transient cerebral ischemia. (A) HE staining of the striatal slice (50 μ m) from the control rats. LA neurons were recognized by the large soma size among the other medium-sized neurons. (B) HE staining of the striatal slice from the rats at 3 h after ischemia. Most of the neurons remained intact. HE staining of striatal slices from the rats at 24 h (C) and 72 h (D) after ischemia. Most of the medium-sized neurons exhibited pyknotic, shrunken nuclei demonstrating extensive degeneration. LA neurons remained intact (arrows). The scale bar represents 25 μ m and applies to all pictures.

Fig. 2.

LA neurons are identified based on morphological and electrophysiological characteristics. (A) An infrared-DIC image of the striatal slice showing one LA neuron (arrow) and one adjacent medium-sized neuron. (B) Photograph of a LA neuron intracellularly stained with neurobiotin showing few spines on the dendrites. (C) Characteristic responses of one LA neuron to hyperpolarizing and depolarizing current pulses under the current-clamp recording. Prominent afterhyperpolarizations after evoked action potentials and the depolarizing sag (arrow) upon hyperpolarizing pulses could be observed. (D) Representative traces of evoked postsynaptic currents (ePSCs), currents recorded after the application of APV+CNQX, APV+CNQX+bicuculline, respectively.

Fig. 3.

Inhibitory synaptic transmission is enhanced in LA neurons while depressed in MS neurons after transient cerebral ischemia. (A) Representative traces of eIPSCs (1T-5T) in LA neurons of the control and at 3 h, 24 h after ischemia. All the traces are averages of 8 consecutive recordings. (B) Grouped data (1T-5T) reveal that the amplitudes of eIPSCs in LA neurons were significantly facilitated at 3 h and 24 h after ischemia. (C) Representative traces of eIPSCs (1T-4T) in MS neurons of the control and at 8 h after ischemia. (D) Grouped data (1T-4T) reveal that the amplitudes of eIPSCs in MS neurons were significantly depressed at 8 h after ischemia. (E) The threshold of stimulus intensities for LA neurons was greatly increased after ischemia. (F) The absolute values of 3T were not necessarily associated with the amplitudes of eIPSCs at 3 h and 24 h after ischemia in LA neurons. The values of the plotting are mean $\pm$ SE. Asterisks indicate the significant changes compared to the control ones if not specified. * $P < 0.05$, ** $P < 0.01$. These definitions of * and ** apply to all subsequent figures.

Fig. 4.

GABAergic terminals on LA neurons are increased after ischemia. (A) Photograph showing doublelabeling immunofluorescence of GAD 65 (green), the synthetic enzyme of GABA in the terminals of MS neurons, and CHAT (red), the synthetic enzyme of acetylcholine found in LA neurons. GAD65-positive terminals were found on the somata and proximal dendrites of LA neurons in the brain slice (50 μ m) of the control rat. (B) Photograph showing doublelabeling of GAD 67 (green), the synthetic enzyme of GABA found in GABAergic interneurons and CHAT (red). A few GAD67-positive terminals were found on the somata and proximal dendrites of the control LA neurons. Photographs showing doublelabeling of GAD 67 and CHAT from the brain slice at 24 h (C) and 72 h (D) after ischemia. More GAD67-positive

terminals were found on the somata and proximal dendrites of LA neurons after ischemia. The scale bar represents 25 μm and applies to all pictures.

Fig. 5. Presynaptic release of GABA is increased after ischemia. (A) Representative traces of paired-pulse tests at 3T in LA neurons. The traces are averages of 10 consecutive recordings. (B) Grouped data show that PPR was significantly decreased at 3 h and 24 h after ischemia. (C) Representative traces of sIPSCs from the control, 3 h and 24 h after ischemia. (D) Cumulative distributions of inter-event intervals from the same neurons shown in C. The curve for cumulative probability of inter-event intervals showed a leftward shift at 3h and 24 h after ischemia (K-S test, $P < 0.05$). Bar graph shows that the frequency of sIPSCs was significantly increased after ischemia. (E) Cumulative distributions of current amplitudes from the same neurons shown in C. The curve for cumulative probability of current amplitudes showed no change after ischemia. Bar graph shows that there was no significant change in the amplitude of sIPSCs.

Fig. 6.

Postsynaptic responses to GABA are depressed in LA neurons at 24 h after ischemia. (A) Representative traces of mIPSCs from the control, 3 h and 24 h after ischemia. (B) Cumulative distributions of inter-event intervals from the same neurons shown in A. The curve for cumulative probability of inter-event intervals showed no change. Bar graph shows that there was no significant change in the frequency of mIPSCs. (C) Cumulative distributions of current amplitudes from the same neurons shown in A. The curve for cumulative probability of current amplitudes showed a leftward shift at 24 h after ischemia (K-S test, $P < 0.05$). Bar graph shows that the amplitude of mIPSCs at 24 h after ischemia was significantly decreased compared to the control and 3 h after ischemia. (D) Representative traces showing LA neuron's response to exogenous GABA application (300 μ M) in the control and after ischemia. (E) Grouped data show that the currents induced by exogenous GABA application at 24 h after ischemia were greatly reduced compared to the control and 3 h after ischemia.

Fig. 7.

Muscimol enhances inhibitory synaptic transmission in LA neurons after ischemia. Representative traces of eIPSCs and PPR before and after muscimol application in the control neurons (A1) and neurons at 24 h after ischemia (A2). (B) Grouped data show that muscimol increased eIPSCs and decreased PPR in LA neurons after ischemia. Muscimol did not significantly change eIPSCs and PPR in the control LA neurons. All columns were normalized to the pre-muscimol application value (dashed lines).

Fig. 8.

Muscimol differentially modulates sIPSCs in control LA neurons and LA neurons after ischemia. (A) Representative traces of sIPSCs before and after muscimol application in control neurons. (B) Cumulative distributions of inter-event intervals and current amplitudes showed a rightward shift after muscimol application from the same neuron shown in A (K-S test, $P < 0.05$). (C) Grouped data show that the frequency of sIPSCs was decreased while the amplitude of sIPSCs was increased in control neurons after muscimol application. All columns were normalized to the pre-muscimol application value (dashed lines). (D) Representative traces of sIPSCs before and after muscimol application after ischemia. (E) Cumulative distributions of inter-event intervals (leftward shift after muscimol application, K-S test, $P < 0.05$) and current amplitudes before and after muscimol application from the same neuron shown in D. (F) Grouped data show that the frequency of sIPSCs was increased and the amplitude of sIPSCs didn't change in LA neurons after ischemia upon muscimol application. All columns were normalized to the pre-muscimol application value (dashed lines).

Fig. 9.

Muscimol's effect on inhibitory synaptic transmission after ischemia is dependent on extracellular calcium and the voltage-gated sodium channels. (A) Representative traces of sIPSCs in the aCSF, Ca^{2+} -free aCSF, after muscimol application (in the Ca^{2+} -free aCSF) and washout in the same LA neuron after ischemia. (B) Bar graph shows that the frequency and amplitude of sIPSCs were greatly reduced in the Ca^{2+} -free aCSF. (C) Cumulative distributions for inter-event intervals and current amplitudes from the same neuron in A before and after muscimol application in the Ca^{2+} -free aCSF. (D) Representative traces of sIPSCs, mIPSCs, mIPSCs after muscimol application and washout in the same LA neuron after ischemia. (E) Cumulative distributions for inter-event intervals and current amplitudes of mIPSCs from the same neuron in D before and after muscimol application. (F) Grouped data show that muscimol did not change the frequency and amplitude of sIPSCs in the Ca^{2+} -free aCSF. All columns were normalized to the pre-muscimol application value (dashed lines). (G) Grouped data show that muscimol did not change the frequency and amplitude of mIPSCs. All columns were normalized to the pre-muscimol application value (dashed lines).