INHIBITORY SYNAPTIC TRANSMISSION IN STRIATAL NEURONS AFTER TRANSIENT CEREBRAL ISCHEMIA

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ABSTRACT

Yan Li

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In the striatum, large aspiny (LA) interneurons survive transient cerebral ischemia while medium spiny (MS) neurons die. Excitotoxicity is believed to be the major cause for neuronal death after ischemia. Since inhibitory tone plays an important role in the control of neuronal excitability, the present study is aimed at examining if there are any changes in inhibitory synaptic transmission in striatal neurons after ischemia and the possible mechanisms.

Transient forebrain ischemia was induced in male Wistar rats using the four-vessel occlusion method. Inhibitory postsynaptic currents (IPSCs) were evoked intrastriatally and whole-cell voltage-clamp recording was performed on striatal slices. The expression of glutamate decarboxylase65 (GAD65) was analyzed using immunohistochemical studies and Western blotting. Muscimol (a specific $GABA_A$ receptor agonist) was injected intraperitoneally to the rats (1 mg/kg) to observe ischemic damage, evaluated by counting the survived cells in the striatum after hematoxylin & eosin (HE) staining.

The amplitudes of evoked IPSCs were significantly increased in LA neurons while depressed in MS neurons after ischemia. This enhancement was due to the increase of presynaptic release. Muscimol (1 μM) presynaptically facilitated inhibitory synaptic transmission in LA neurons at 24 h after ischemia. The optical density of GAD65-positive terminals and the number of GAD65-

positive puncta was significantly increased in the striatum at both 1 day and 3 days after ischemia. Consistently, data from western blotting suggested an increased expression of GAD65 in the striatum after ischemia. For the rats treated with muscimol, the number of survived cells in the striatum was greatly increased compared to the non-treatment group.

The present study demonstrates an enhancement of inhibitory synaptic transmission in LA neurons after ischemia, which is contributed by two mechanisms. One is the increased presynaptic release of GABA mediated by presynaptic $GABA_A$ receptors. The other is the increased expression of GAD. Facilitation of inhibitory synaptic transmission by muscimol protects striatal neurons against ischemia. Therefore, the enhancement of inhibitory synaptic transmission might reduce excitotoxicity and contribute to the selective survival of LA neurons after ischemia.

Zao C. Xu, M.D., Ph.D., Chair

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INTRODUCTION

Stroke

Stroke occurs when a blood clot blocks an artery (ischemic stroke, 80%) or a blood vessel breaks (hemorrhagic stroke, 20%), interrupting blood flow to an area of the brain (definition from National Stroke Association). Ischemic stroke is classified into focal ischemia and global ischemia. Global ischemia is normally seen in patients after heart attack.

As the third leading cause of death and the number one cause of adult disability in the western world, stroke draws lots of devotion from neuroscientists and pharmaceutical companies. Disappointingly, the only approved drug therapy for stroke is tissue plasminogen activator, which acts to restore cerebral blood flow. For the last two decades, neuroprotection agents were the hope for stroke treatment. However, most of the neuroprotection agents including the ones for glutamate antagonism and GABA agonism, failed in clinical trials. The following key factors are blamed for the failure of the translational study. The first is that the time-window to treatment in animal study does not match the one in clinical trials. The second is that animal models do not mimic human stroke due to the difference in age, anatomy and other complications. The third is that the plasma concentration of the agent in clinical trials cannot reach the one in animal studies. All these leave us a big space to reconsider the design of the animal studies and clinical trials (Ginsberg, 2008). Besides the new neuroprotection therapy considered, Lipton (Lipton, 2007) suggested that neuroprotection agents, which are activated by the pathological state that they are intended to inhibit, should be

developed. Other approach, like the stimulation of brain's endogenous repair mechanisms, might bring new hope for the stroke therapy (Garber, 2007).

Excitotoxicity and postischemic neuronal injury

Onley (Olney et al., 1972) first coined the term excitotoxicity as too much glutamate release can be destructive and excite a neuron to death. Excitotoxicity has been widely accepted as one of the major causes for postischemic cell death. During cerebral ischemia, Na^+-K^+-ATPase on the membrane loses its function to transfer Na^+ out of the cell and K^+ into the cell, which results in membrane depolarization and the rundown of ionic gradients (Hansen, 1985; Lipton, 1999). A dramatic increase in extracellular glutamate concentration is found during cerebral ischemia by utilizing microdialysis (Benveniste et al., 1984; Globus et al., 1988; Globus et al., 1991). Excessive glutamate release is initially caused by Ca^{2+}-dependent exocytosis and later by the reversal uptake of glutamate transporters (Katchman and Hershkowitz, 1993; Madl and Burgesser, 1993; Roettger and Lipton, 1996; Rossi et al., 2000).

Through its interaction with ionotropic NMDA (N-methyl-D-aspartate) and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, glutamate triggers excessive calcium and sodium influx into the neurons (Andine et al., 1988; Benveniste et al., 1988). Voltage-gated calcium channels also contribute to an increase of intracellular calcium during ischemia (Paschen, 2000; Zipfel et al., 1999). Intracellular calcium overload activates an array of downstream phospholipases and proteases that will degrade membranes and proteins important for cellular integrity (Choi and Rothman, 1990; Rothman and

Olney, 1986). Additionally, intracellular calcium overload will sequestrate calcium into mitochondria (Dux et al., 1987; Sims and Pulsinelli, 1987) and induce mitochondrial damage through two ways. One is by the activation of enzymes to generate reactive oxygen species (ROS); the other is the formation of mitochondrial pore, which facilitates the release of apoptosis-related proteins (Starkov et al., 2004). The final consequence is oxidative stress, apoptosis and/or necrosis. Therefore, calcium overload is the central theme for excitotoxicity and the final path leading to neuronal death (Figure 1).

In animal studies, application of antagonists to ionotropic glutamate receptors protect cells from ischemic injury (Boast et al., 1988; Buchan et al., 1991; Marcoux et al., 1988; Nellgard and Wieloch, 1992; Noh et al., 2005; Simon et al., 1984). However, failure of NMDA and AMPA receptor antagonists in clinical trials (Albers et al., 1995; Albers et al., 2001; Davis et al., 1997; Diener et al., 2002; Dyker and Lees, 1999; Elting et al., 2002) suggests that other mechanisms might be involved. Some efforts have been focused on glutamate receptor-independent Ca^{2+}-permeable ion channels. Antagonists to voltage-gated calcium channels have been shown neuroprotective after cerebral ischemia in animal studies (Germano et al., 1987; Hara et al., 1990) but cannot be confirmed in clinical trials (Horn and Limburg, 2001). TRPM7 is a member of the transient receptor potential melastatin (TRPM) subfamily. Aarts et al. (Aarts et al., 2003) showed that blockade of TRPM7 currents attenuated anoxic neuronal death by reducing anoxic Ca^{2+} uptake and ROS production. Acid-sensing ion channels (ASICs) are activated by acidosis. Blocking ASICs protects

neurons from ischemic insults in vivo and in vitro (Xiong et al., 2004). Ca^{2+} extruding system on the plasma membrane, like Na^{+}/Ca^{2+} exchanger (NCX), has also been shown to play a role in cerebral ischemia (Bano et al., 2005; Pignataro et al., 2004a; Pignataro et al., 2004b). Besides Ca^{2+}, ionic imbalances caused by other ions, like Zn^{2+} (Sensi and Jeng, 2004) and K^{+} (Yu et al., 2001), has been extensively studied in cerebral ischemia.

Remarks:

Excitotoxicity is believed to be the main mechanism for postischemic neuronal death. Calcium overload is the central theme for excitotoxicity and the final path leading to neuronal death.

Striatum: the microcircuits and function

In the basal ganglia, most of the inputs go to two nuclei, the striatum and the subthalamic nucleus. And most of the outputs are through two nuclei, the internal segment of globus pallidus (GP) and the substantia nigra pars reticulata. Besides these input and output nuclei, there are relay nuclei, the external segment of GP, the substantia nigra pars compacta and the ventral tegmental area, providing and receiving inputs from other nuclei in the basal ganglia (Alexander and Crutcher, 1990). As the largest nuclei in the basal ganglia, striatum is actively involved in the sensory-motor functions, as well as in the cognitive and limbic functions. Striatum has several sources of inputs. It receives excitatory inputs from cerebral cortex, thalamus, and limbic structures like hippocampus and amygdala. It also receives dopaminergic afferents from substantia nigra pars compacta and serotonergic afferents from dorsal raphe

nucleus in the midbrain (Tepper et al., 2007). Studies from Bevan showed that there are GABAergic inputs from GP to the striatum (Bevan, 1998) (Figure 2).

The projection neurons in the striatum are GABAergic medium spiny (MS) neurons, which accounts for about 97.7% of the neuronal populations in the rodents (Rymar et al., 2004). There are two main populations of MS neurons. One population of MS neurons, with highly collateralized axons, expresses Substance P, Dynorphin and mainly D1-type dopamine receptors. They project to the internal segment of GP and substantial nigra, which is called direct pathway. The other population of MS neurons expresses Enkephalin and mainly D2-type dopamine receptors. They project to the external segment of GP and subthalamic nucleus, which is called indirect pathway. The information flowed through the direct and indirect pathway finally goes back to the thalamus and cerebral cortex, where the striatum originally receive the inputs from (Gerfen, 1992; Graybiel, 1990) (Figure 2).

MS neurons receive the excitatory afferents from both the cortex and thalamus. And these afferents mainly innervate the spines of MS neurons (Frotscher et al., 1981; Somogyi et al., 1981). MS neurons receive GABAergic inputs from GABAergic interneurons in the striatum (Koos and Tepper, 1999) and axon collaterals of other MS neurons (Guzman et al., 2003; Tunstall et al., 2002) (Figure 3). Although the axonal collaterals of MS neurons are dense and widespread in the striatum (Oorschot, 1996), in vivo and in vitro studies demonstrated that collateral inhibitions between MS neurons are weak (Jaeger et al., 1994; Stern et al., 1998; Tunstall et al., 2002). On the contrary, as the minor

neuronal populations in the striatum (2%) (Rymar et al., 2004), GABAergic interneurons provide strong inhibitory inputs to MS neurons as demonstrated by large amplitude of inhibitory postsynaptic potentials and low failure rate (Koos and Tepper, 1999; Koos et al., 2004). The GABAergic interneurons, thus, are believed to provide the feedforward inhibition, while the recurrent axon collaterals of MS neurons provide the feedback inhibition in the striatum (Tepper et al., 2004).

Besides MS neurons, other neurons in the striatum are interneurons, which can be categorized into two types, cholinergic large aspiny neurons and GABAergic interneurons based on the neurotransmitter they produce. Therefore, LA neurons are believed to be the only non-GABAergic neurons in the striatum (Wilson, 2007). Different from MS neurons, LA neurons receive substantial excitatory inputs from the thalamus but are almost devoid of corticostriatal afferents, except for light innervations of distal dendrites (Lapper and Bolam, 1992; Meredith and Wouterlood, 1990). LA neurons receive GABAergic inputs from the axon collaterals of MS neurons (Bolam et al., 1986; Martone et al., 1992) (Figure 3).

LA neurons provide strong cholinergic innervations to striatal neurons since acetylcholine (ACh) is tonically released and their axonal arborizations are dense and large. The release of ACh is modulated not only by synaptic inputs but also by neuromodulators and autoreceptors on the cholinergic terminals. For example, activation of D2 dopamine receptors reduces autonomous spiking in LA neurons (DeBoer et al., 1996). Activation of M4 Muscarinic receptors (mACh)

located on the cholinergic terminals reduces ACh release (Calabresi et al., 1998b; Ding et al., 2006).

Muscarinic and nicotinic ACh (nACh) receptors are widely distributed in the striatum. They are either expressed on the presynaptic terminals, modulating presynaptic transmitter release, or on the postsynaptic sites, causing excitation or inhibition. For example, dopaminergic afferents to the striatum express M4 receptors and nACh receptors. The activation of M4 receptors will decrease dopamine release while the activation of nACh receptors will increase dopamine release (Rice and Cragg, 2004). Glutamatergic afferents to the striatum express M2 and M3 receptors (Hersch et al., 1994), the activation of which will inhibit glutamate release as demonstrated in the paired recordings (Pakhotin and Bracci, 2007). Fast spiking (FS) interneurons, a kind of GABAergic interneurons in the striatum, express nACh receptors. The activation of nACh receptors produces powerful excitation in FS interneurons (Koos and Tepper, 2002). Therefore, LA neurons directly or indirectly participate in the modulation of feedforward and feedback inhibition.

It has long been known that LA neurons are actively involved in motivation and reward learning (Graybiel et al., 1994). They respond to salient stimuli during associative learning by displaying a pause, preceded or followed by increased spikes (Aosaki et al., 1994; Apicella, 2002). The understanding of LA neurons' function in movement control largely benefits from the research in movement disorders. In Parkinson's disease (PD), the dopaminergic afferents to the striatum is depleted while the cholinergic signaling is enhanced. In

Huntington's disease, research from Smith et al. suggests that there might be a dysfunctional cholinergic signaling (Smith et al., 2006). LA neurons' function in the striatum is still under active investigation. In fact, we could get some hint from the functional study of cholinergic neurons in the basal forebrain, in which saporin is used to selectively immunolesion the cholinergic neurons. Saporin is coupled to a monoclonal antibody against a certain receptor, which is exclusively expressed by cholinergic neurons in the basal forebrain (Wenk et al., 1994).

Remarks:

The striatum is involved in sensory-motor functions. MS neurons form direct and indirect pathways, which convey the information from the cortex and thalamus, process it and send it back to where it is from. LA neurons, which are the only non-GABAergic neurons in the striatum, actively participate in reward learning and movement control.

Electrophysiological and immunohistochemical characters of striatal neurons

In vivo recording revealed that the most characteristic electrophysiological property of MS neurons is their two-state membrane potential. That is, their membrane potential is maintained either at a depolarized UP state, where the action potentials are elicited from, or a hyperpolarized DOWN state. The UP state is caused by strong and sustained excitatory inputs from the cerebral cortex and thalamus. The DOWN state is dominated by the inwardly rectifying potassium conductances (Wilson and Kawaguchi, 1996). For years, it is believed that the morphological and electrophysiological properties are homogenous

among MS neurons although they are functionally heterogeneous; with some of the MS neurons forming the direct pathway while the others forming the indirect pathway. The development of the transgenic mice, in which different groups of MS neurons could be identified, helps to prove that these two groups of MS neurons are in fact electrophysiologically and morphologically dichotomous (Day et al., 2008; Gertler et al., 2008). There are several immunohistochemical markers for MS neurons. They express calbindin and dopamine- and cAMP-dependent phosphoprotein of 32 kDa (DARPP-32). For the MS neurons forming the direct pathway, they express Substance P, Dynorphin, and D1 receptors. For the MS neurons forming the indirect pathway, they express Enkephalin and D2 receptors. Different from the striatal GABAergic interneurons, which express predominantly glutamate decarboxylase 67 (GAD67), they mainly express GAD65, the key enzyme in the synthesis of GABA.

LA neurons represent 0.3% of the neuronal population in the striatum (Rymar et al., 2004). They have an elongated cell body around 50 μm with the shortest diameter around 20 μm. They are tonically active neurons, firing action potentials as the consequence of the interplay among intrinsic membrane conductances even without synaptic inputs. With broad action potentials, more calcium gets in LA neurons through voltage-gated calcium channels and at the same time activating the calcium-activated potassium channels. As the membrane goes to the hyperpolarizing direction, the calcium-activated potassium channels are inactivated and the hyperpolarization and cyclic adenosine monophosphate dependent cation (HCN) channels are activated. The activation

of HCN channels results in membrane depolarization and if the depolarization reaches the threshold to activate voltage-gated sodium channels, an action potential occurs (Bennett and Wilson, 1999; Wilson, 2005). HCN channels are also responsible for the large-amplitude and long-duration afterhyperpolarizations following the action potentials in LA neurons. If negative currents are injected, an initial hyperpolarization could be observed, followed by sag, which is also caused by the activation of HCN channels (Jiang and North, 1991; Kawaguchi, 1993). LA neurons are strongly stained for the cholinergic markers, choline acetyltransferase (CHAT), acetylcholinesterase, and vesicular Ach transporter.

GABAergic interneurons comprise totally about 2% in the striatal population (Rymar et al., 2004). They could be categorized as three types based on both electrophysiological and neurochemical characters (Kawaguchi et al., 1995). One group of GABAergic interneurons are parvalbumin (PV)-positive. They are also called fast-spiking GABAergic interneurons since they fire short duration action potentials at very high frequency (200-300 Hz) without little frequency adaptation. They also show deep afterhyperpolarizations (Kawaguchi, 1993). Gap junctions exist among these neurons and they provide strong inhibitory inputs to MS neurons (Koos et al., 2004). The second group of GABAergic interneurons contains neuropeptide Y (NPY), somatostatin (SOM), nitric oxide synthase (NOS) and NADPH diaphorase. Electrophysiologically, they show low threshold calcium spikes and are thus named low-threshold spike (LTS) neurons (Kawaguchi, 1993). They also provide strong inhibitory inputs to MS neurons (Tepper and Bolam, 2004). The other group of GABAergic interneurons

contains calretinin and their electrophysiological properties are still unknown. GAD67 is strongly expressed in the GABAergic interneurons, the other isoform of GAD, with the exception of SOM-containing GABAergic interneurons. Normally, GAD67 could not be detected in SOM-containing GABAergic interneurons although GABA immunoreactivity could be detected in their terminals (Kawaguchi et al., 1995).

There are also a very small group of dopaminergic neurons in the striatum. They express tyrosin hydroxylase (TH) and dopamine transporter (DAT). They also express GAD67, a marker for GABAergic neurons (Huot and Parent, 2007). The electrophysiological properties of these neurons are still under investigation.

Remarks:

Striatal neurons could be identified by their electrophysiological and neurochemical characters (for summary, see table 1). Most of the striatal neurons are GABAergic and LA neurons are the only non-GABAergic neurons in the striatum.

Selective cell death in the striatum after ischemia

Transient global ischemia, as a result of cardiac arrest in humans, induces selective neuronal death in vulnerable brain regions. In the hippocampus, CA1 neurons die while CA3 neurons survive (Kirino, 1982; Pulsinelli et al., 1982). In the striatum, MS neurons in the dorsolateral part show visible damage as early as 6 h after transient cerebral ischemia and most of them die in 24 h (Pulsinelli et al., 1982). Most of the interneurons including LA neurons, calretinin-containing and SOM-containing neurons survive ischemia (Chesselet et al., 1990). Whether

PV-positive neurons survive ischemia or not remains controversial. In the global ischemia model of gerbil (Gonzales et al., 1992) and focal ischemia model in mouse (Katchanov et al., 2003), they are spared from ischemic insults. By contrast, studies from Larsson et al. (Larsson et al., 2001; Meade et al., 2000) showed that PV-positive interneurons were dramatically lost after global ischemia in rats.

The mechanisms for such selective neuronal death in the striatum are under intensive investigation. It is found that the intrinsic membrane properties change differentially in MS and LA neurons after ischemia. MS neurons undergo depolarization due to the activation of TTX-insensitive sodium channels while LA neurons undergo hyperpolarization due to the activation of ATP- and Ca^{2+}-dependent potassium channels (Calabresi et al., 1997; Centonze et al., 2001a; Pisani et al., 1999). Studies from Deng et al. demonstrated that LA neurons have decreased excitability after ischemia. Delayed rectifier potassium currents are enhanced while Ih currents through HCN channels are inhibited in LA neurons after ischemia (Deng et al., 2005; Deng et al., 2008). These two types of neurons also respond differentially to excitatory inputs after ischemia. For the activation of ionotropic glutamate receptors, larger depolarizations occur in MS neurons than in LA neurons upon the application of glutamate receptor agonist (Calabresi et al., 1998a). For the activation of metabotropic glutamate receptors, membrane depolarization and calcium accumulation occur in MS neurons but not in LA neurons (Calabresi et al., 1999). Ischemic-long term potentiation (LTP) occurs in MS neurons but not in LA neurons (Calabresi et al., 2002). Studies

from this lab have shown that excitatory synaptic transmission change differentially in these two types of neurons after ischemia. LA neurons have decreased excitatory synaptic transmission (Pang et al., 2002) while MS neurons show increased excitatory synaptic transmission (Zhang et al., 2006).

The massive degeneration of MS neurons (especially the ones in the dorsolateral striatum) and the relative sparing of striatal interneurons after ischemia resemble that in Huntington's disease (Meade et al., 2000). However, is it reasonable to speculate that rats after ischemia will show the movement disorder as seen in the patients with Huntington's disease? Previous studies developed a motor score to test neurological deficits in equilibrium and muscle strength. Ischemic rats show neurological deficits in 24 h but transient cerebral ischemia does not result in long lasting neurological deficits (Combs and D'Alecy, 1987; Gionet et al., 1991). The deficits in spatial learning and swim speed seen in the ischemic rats are also correlated with striatal damage (Block and Schwarz, 1998; Whishaw et al., 1987). Since neuronal death also happens in the hippocampus, especially in the CA1 area, deficits in learning and memory are very obvious in the rats after transient cerebral ischemia (Block, 1999). However, ischemia-induced functional deficits could be better understood if selective excitotoxic damages would be produced selectively in a certain brain area, either in the striatum or in the hippocampus.

Remarks:

LA neurons survive while MS neurons (especially the ones in the dorsolateral striatum) die after transient cerebral ischemia. The mechanism of this selective

cell death after ischemia and its functional meaning is still under investigation. Previous studies showed that these two types of neurons show differential changes in intrinsic membrane properties and excitatory synaptic transmission after ischemia. However, the role of inhibitory synaptic transmission in this selective vulnerability is still unknown.

Inhibitory synaptic transmission and postischemic neuronal injury

GABA is the major inhibitory neurotransmitter in the central nervous system. There are three types of GABA receptors, $GABA_A$, $GABA_B$ and $GABA_C$ (Chebib and Johnston, 1999). For mature neurons, activation of postsynaptic $GABA_A$ receptors causes Cl^- influx that mediates fast synaptic inhibition, which is called phasic inhibition (Cherubini and Conti, 2001; Gaiarsa et al., 2002; Mody et al., 1994). Spillover of GABA and the activation of extrasynaptic $GABA_A$ receptors cause tonic inhibition (Nusser et al., 1998; Semyanov et al., 2004; Wei et al., 2003). Activation of $GABA_B$ receptors triggers G-protein coupled events and mediates a slow inhibitory response via potassium channels (Mott and Lewis, 1994). $GABA_C$ is also a chloride channel mainly expressed in vertebrate retina (Johnston, 1996). Therefore, activation of GABA receptors results in membrane hyperpolarization.

$GABA_A$ and $GABA_C$ receptors are both transmitter-gated ion channels. They are composed of five subunits and each subunit has four transmembrane domains. The second transmembrane domain forms the wall of the channel pore. There are 16 subunits (α1–6, β1–4, γ1–4, δ, ε) encoded for $GABA_A$ receptors. The diversity of the subunits and the existence of different isoforms

for the different subunits yield a huge amount of combinations. But a fully functional $GABA_A$ receptor requires one α, β, and one other subunit type, such as γ, δ or ε (Chebib and Johnston, 1999). $GABA_C$ receptors are homo-oligomerically formed by ρ1 and ρ2 subunits (Johnston, 1996). $GABA_B$ receptors are seven transmembrane metabotropic receptors encoded by two genes- $GABA_BR1$ and $GABA_BR2$. Heterodimerization between $GABA_BR1$ and R2 subunits is required for the formation of fully functional $GABA_B$ receptors (Bettler et al., 2004; Couve et al., 2004).

Excitotoxicity is believed to be the major mechanism for postischemic neuronal death. The membrane depolarization caused by the energy failure is the initiating step to the cascades of excessive glutamate release, over activation of postsynaptic glutamate receptors, calcium overload, and cell death. Since GABAergic synaptic transmission is an important tone in the control of neuronal excitability and GABA functions in opposite to glutamate, GABAergic synaptic transmission might be neuroprotective after ischemia. In fact, ample evidence from animal studies has demonstrated that GABAmimetic drugs are neuroprotective after ischemia. For example, muscimol, a specific $GABA_A$ receptor agonist, is proved to decrease the infarct size and improve the functional recovery after focal ischemia (Lyden and Hedges, 1992; Lyden et al., 2000; Lyden and Lonzo, 1994). Muscimol improves the survival of hippocampal CA1 neurons after global ischemia (Han et al., 2008; Xiao et al., 2007; Xu et al., 2008; Zhang et al., 2007; Zhou et al., 2008). With in vitro ischemia, muscimol helps recover the field potential in the striatum (Costa et al., 2004) and

decreases the apoptosis of CA1 neurons (Han et al., 2008). Besides rats, muscimol is also neuroprotective in rabbits (Lyden and Hedges, 1992) and gerbils (Shuaib et al., 1993). Muscimol shows synergistic effects when given in combination with baclofen (Costa et al., 2004; Xu et al., 2008) and MK-801 (Lyden et al., 2000; Lyden and Lonzo, 1994). More importantly, muscimol is neuroprotective when given before (Han et al., 2008; Xiao et al., 2007; Xu et al., 2008; Zhang et al., 2007; Zhou et al., 2008), during (within 5 min of ischemic onset) (Lyden and Hedges, 1992; Lyden and Lonzo, 1994), and even within 4 h after the ischemic onset (Lyden et al., 2000). It is known that most of the GABAmimetic drugs, like pentobarbitone, diazepam and baclofen are neuroprotective when administered before ischemic insults but not effective when administered after ischemia. The exceptions are muscimol and Clomethiazole (CMZ), which show neuroprotection even after ischemia (Cross et al., 1991). In fact, there is no evidence that neuroprotection for acute ischemic stroke is possible with any agent beyond 6 h. Even within the 6 h, the extent of neuroprotection with any agent is likely to decline with increasing delay to administration (Ginsberg, 2008). Since muscimol enters the blood brain barrier in concentrations that can elicit a persistent GABAmimetic effect (Baraldi et al., 1979), previous studies have shown that both intraventricular (from Lyden's group) or intraperitoneal injection (from Zhang's group) of muscimol work well. The side effect that should be taken into account for all the GABAmimetic drugs is that they sedate the experimental animals and brings down cerebral temperature, which might be neuroprotective after ischemia. Since the

temperature was closely monitored in the studies from Zhang's group (Han et al., 2008; Xiao et al., 2007; Xu et al., 2008; Zhang et al., 2007; Zhou et al., 2008), the neuroprotective effects should come from muscimol's GABAmimetic effects.

One other GABAmimetic drug, CMZ, which is also a $GABA_A$ receptor agonist, was extensively studied by the group headed by Green (Green, 1998). CMZ is found to be neuroprotective in animal models by functional, biochemical and histological measures. It finally went to the clinical trials. The lack of beneficial effect on long-term outcome for all the patients stalls its further clinical studies (Lyden et al., 2001; Wahlgren et al., 1999). The other GABAmimetic drug that was translated from animal studies to clinical trials was Diazepam, a benzodiazepine agonist, which showed no promising effects in stroke patients as CMZ (Lodder et al., 2006).

Some GABAmimetic drugs increase the synaptic concentration of GABA by inhibiting the reuptake of GABA, like Topiramate, or inhibiting GABA-transaminase, like Vigabatrin. Animal studies revealed no significant neuroprotective effects of these drugs in ischemia by functional and histological measures (Madden et al., 2003).

The failure of these translational studies could be explained by many factors as mentioned in the previous paragraph. It could also be explained by several other possibilities considering from different angles. One is that the functions of postsynaptic $GABA_A$ receptors are depressed after ischemia. It is known that $GABA_A$ 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 outcomes induced by ischemia, like calcium overload and the production of arachidonic acids, also depress $GABA_A$ receptor functions (Schwartz et al., 1992). Therefore, there is very little space left for $GABA_A$ receptor agonists to activate these functionally depressed $GABA_A$ receptors after ischemia.

The second is that large accumulation of GABA in the extracellular space has been demonstrated by in vivo and in vitro studies during cerebral ischemia and early after reperfusion (Allen et al., 2004; Globus et al., 1988; Phillis et al., 1994). Further application of the GABAmimetic drugs to increase the extracellular GABA concentration might have little effect to potentiate GABAergic activity.

The third is that after ischemia, the intracellular concentration of chloride might have increased to the extent that further potentiation of GABA activity results in excitatory effects, due to the more depolarized chloride reversal potential relative to the resting membrane potential. Thus, the activation of $GABA_A$ receptors becomes excitotoxic. Studies have shown that chloride concentration is increased in hippocampal neurons after in vitro ischemia. Activation of $GABA_A$ receptors (Inglefield and Schwartz-Bloom, 1998b), 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 increase. All these possibilities make the translational studies more complicated than what we have imagined.

Remarks:

Overall, potentiation of inhibitory synaptic transmission by GABAmimetic drugs is neuroprotective in animal studies after ischemia. However, the failure of the translational studies suggests that more factors should be taken into account before we finally reach the goal.

Presynaptic $GABA_A$ receptors' role in the modulation of neurotransmitter release

Presynaptic ionotropic receptors are very important in the modulation of presynaptic neurotransmitter release. The small changes in membrane potential and input resistance induced by the activation of presynaptic ionotropic receptors have substantial effects on synaptic strength and information processing (Engelman and MacDermott, 2004). Presynaptic $GABA_A$ receptors are of great interest to us since they could be inhibitory or excitatory depending on the intracellular chloride concentration and the shunting effect. The intracellular chloride concentration could be modulated during brain maturation and under disease condition.

Previous studies have found $GABA_A$ receptors on the axons of many cell types, such as hippocampal granule cells (Jang et al., 2006b; Ruiz et al., 2003), cerebellar molecular interneurons (Pouzat and Marty, 1999), cortical pyramidal neurons (Szabadics et al., 2006), calyx of Held (Turecek and Trussell, 2002), horizontal cells in the retina (Kamermans and Werblin, 1992), and dorsal root ganglia neurons (Eccles, 1964; Rudomin and Schmidt, 1999). Presynaptic $GABA_A$ receptors have been reported on the glutamatergic (heterosynaptic)

(Jang et al., 2006b; Ruiz et al., 2003; Stell et al., 2007), glycinergic (heterosynaptic) (Jang et al., 2002), and GABAergic (homosynaptic) terminals (Axmacher and Draguhn, 2004; Xiao et al., 2007). In mature animals, the effects on the activation of these presynaptic $GABA_A$ receptors vary from inhibition (Axmacher and Draguhn, 2004; Eccles, 1964) to excitation (Jang et al., 2001; Jang et al., 2002; Jang et al., 2006b; Stell et al., 2007; Turecek and Trussell, 2002; Xiao et al., 2007) depending on different tissues and preparations.

The main mechanism for the excitatory effects of presynaptic $GABA_A$ receptor activation is the high chloride concentration at the terminals. The chloride concentration in the terminals might be different from that of the soma. In the mammalian central nervous system, the calyx of Held has a higher chloride concentration than that in the soma as demonstrated by perforated-patch recording (Price and Trussell, 2006; Turecek and Trussell, 2001). Thus, following the activation of presynaptic $GABA_A$ receptors, there will be a chloride efflux and depolarization in the presynaptic terminals. If the depolarization exceeds the shunting effects caused by $GABA_A$ receptor activation, voltage-gated sodium and calcium channels will be activated and neurotransmitter release occurs (Jang et al., 2006a; Jang et al., 2001; Leinekugel et al., 1995; Xiao et al., 2007). However, there are also studies showing that $GABA_A$ receptor-induced rises in the intracellular calcium concentration is driven by osmotic tension (Chavas et al., 2004). If the shunting effects could not be exceeded, the excitability of presynaptic terminals will be inhibited (Cattaert and El Manira, 1999; Ruiz et al., 2003). In addition, the depolarization-induced

inactivation of sodium channels will reduce the excitability of the terminals (Graham and Redman, 1994; Zhang and Jackson, 1993).

The other inhibitory neurotransmitter in the nervous system is glycine. Presynaptic glycine receptors have been found on the calyceal synapses in the medial nucleus of trapezoid body, the activation of which depolarizes the terminals, activates calcium channels and increases glutamate release (Turecek and Trussell, 2001). The glycine receptors are also found on the GABAergic terminals synapsed on the dopaminergic neurons in the ventral tegmental area, the activation of which results in hyperpolarization and a decrease in GABA release (Ye et al., 2004).

In the immature neurons, $GABA_A$ and glycine receptors are excitatory (Rivera et al., 1999). Activation of presynaptic $GABA_A$ or glycine receptors results in increased GABA release in the immature dopaminergic neurons of ventral tegmental area in the immature rats (Xiao et al., 2007; Ye et al., 2004). Activation of presynaptic glycine receptors enhances both excitatory and inhibitory synaptic transmission in the immature cerebellar Purkinje neurons (Kawa, 2003). Activation of presynaptic $GABA_A$ receptors results in increased glutamate release on the ventromedial hypothalamic neurons in the immature rats (Jang et al., 2001). The high expression level of NKCC1, an inwardly chloride transport system, are believed to be responsible for the high intracellular chloride concentration in the immature animals (Plotkin et al., 1997).

Besides the immature neurons, neurons under disease conditions, like ischemia and epilepsy, have high intracellular chloride concentration. After in

vitro ischemia, studies have shown that intracellular chloride concentration is increased in hippocampal neurons. Activation of $GABA_A$ receptors (Inglefield and Schwartz-Bloom, 1998b) and NKCC1 (Pond et al., 2006), or downregulation of KCC2 (Galeffi et al., 2004), has been suggested to be involved in the increase. After pilocarpine-induced status epilepticus, dentate granule cells have increased chloride concentration and increased excitability due to the decreased expression of KCC2 (Pathak et al., 2007).

Remarks:

$GABA_A$ receptors are located on central and peripheral terminals. They are actively involved in the modulation of neurotransmitter (including glutamate, glycine and GABA) release. The modulation effects could be varied from inhibitory to excitatory upon the activation of presynaptic $GABA_A$ receptors, depending on the driving force for chloride and shunting effects. Intracellular chloride concentration varies on the maturation states and different disease states, resulting in different modulating effects on the excitability of the presynaptic terminals and neurotransmitter release.

Summary

LA neurons are the only non-GABAergic neurons in the striatum. They receive inhibitory inputs from MS neurons. After transient cerebral ischemia, LA neurons survive while MS neurons die. Previous studies have shown that differential changes in the intrinsic membrane properties and excitatory synaptic transmission play a role in this selective vulnerability. However, the role of inhibitory synaptic transmission in this selective vulnerability is still unknown.

Since inhibitory tone is very important in the control of neuronal excitability, the present study is aimed at examining if there are any changes in inhibitory synaptic transmission in striatal neurons after ischemia and the possible mechanisms. We also examined if facilitation of inhibitory synaptic transmission by muscimol could attenuate ischemic neuronal injury in the striatum after ischemia. Results from this study will improve the understanding of the mechanisms underlying selective neuronal injury after transient cerebral ischemia. We hope this study could contribute to the translational studies for the stroke patients after cardiac arrest.

HYPOTHESIS AND EXPERIMENTAL DESIGN

Hypothesis 1: It is widely accepted that excitotoxicity, triggered by excessive glutamate release, is the major cause for postischemic cell death. Since LA neurons survive ischemic insults, it is reasonable to speculate that they have their own way to defend excitotoxicity. It has been shown that excitatory synaptic transmission is facilitated in MS neurons (Zhang et al., 2005) while it is depressed in LA neurons (Pang et al., 2002) after transient forebrain ischemia. This suggests that differential changes in the excitatory synaptic transmission after ischemia might contribute to the selective neuronal death in the striatum. Inhibitory synaptic transmission is an important source counteracting excitatory synaptic transmission. In addition, GABAergic synaptic transmission provides an important tone in the control of neuronal excitability. Potentiation of inhibitory synaptic transmission might contribute to the postischemic neuroprotection since its contribution to the decrease of excitotoxicity by counterbalancing the excitability. Ample evidence has demonstrated that GABAmimetic drugs are neuroprotective after ischemia as we have discussed in the introduction. Therefore, we hypothesized that inhibitory synaptic transmission is enhanced in ischemia-resistant LA neurons after ischemia. To study the change of inhibitory synaptic transmission in LA neurons after ischemia, evoked IPSCs will be examined.

Hypothesis 2: Studies have shown that postsynaptic $GABA_A$ responses are depressed after ischemia. Cellular mediators that depress $GABA_A$ receptor function, such as arachidonic acid and superoxide radicals, were released after

cerebral ischemia (Patel et al., 1992; Rao et al., 1999). Intracellular calcium overload (Inoue et al., 1986; Stelzer et al., 1988) also contributed to the decreased conductance of $GABA_A$ receptor. $GABA_A$ responses are very sensitive to intracellular ATP levels (Shirasaki et al., 1992) and decreased ATP production after ischemia induces a selective and rapid rundown of the postsynaptic $GABA_A$ responses (Harata et al., 1997). In addition, $GABA_A$ receptors were down regulated in the hippocampus and striatum after cerebral ischemia (Alicke and Schwartz-Bloom, 1995; Mileson et al., 1992; Onodera et al., 1987). Therefore, we hypothesized that presynaptic components contribute to the enhancement of evoked IPSCs in LA neurons after ischemia. To test this hypothesis, paired-pulse test, miniature IPSCs analysis will be performed and exogenous GABA will be applied.

Hypothesis 3: $GABA_A$ receptors could be excitatory or inhibitory depending on two factors. One is the difference between the resting membrane potential and chloride reversal potential, the latter of which is mainly dependent on the intracellular concentration of chloride. The other is the shunting effect. Studies have shown that intracellular chloride concentration is increased after ischemia, which might change the effects of activating $GABA_A$ receptors from inhibitory to excitatory. It is well known that presynaptic $GABA_A$ receptors are actively involved in the modulation or presynaptic neurotransmitter release. We hypothesized that presynaptic $GABA_A$ receptors increase presynaptic GABA release due to their excitatory effects after ischemia. To test this hypothesis,

muscimol's effect on evoked IPSCs, spontaneous and miniature IPSCs will be compared before and after ischemia.

To examine if muscimol could protect striatal neurons from ischemic injury, muscimol will be applied intraperitoneally to the rats at 30 min before ischemia. The reason we administered muscimol at 30 min before ischemia is that most of the agents show neuroprotection when given before ischemic insults. The extent of neuroprotection with any agent is likely to decline with increasing delay to administration (Ginsberg, 2008). Ischemic neuronal injury will be estimated by counting the survived cells in the striatum.

Hypothesis 4: LA neurons receive inhibitory inputs from MS neurons. As early as 6 h after transient cerebral ischemia, MS neurons in the dorsolateral part show visible damage and most of them die in 24 h. Therefore, we hypothesized that the GABAergic innervations to LA neurons is decreased after ischemia due to the massive degeneration of MS neurons. To test this hypothesis, immunoreactivity of GAD65 will be examined in the striatum before and after ischemia. Western blotting will also be used to examine the expression level of GAD65 after ischemia.

Experimental design:

1. To compare the inhibitory synaptic transmission in LA neurons before and after ischemia.

Four-vessel occlusion model will be used to induce transient forebrain ischemia. Using the whole-cell voltage-clamp recording on the brain slices, the evoked IPSCs will be compared in LA neurons and MS neurons before and at

different time intervals after ischemia. Animals are divided into three groups: control, 3 h, and 24 h after ischemia. At 3 h after ischemia, most of the striatal neurons are morphologically intact and functional. By contrast, at 24 h after ischemia, most of the MS neurons in the dorsolateral striatum degenerate (Pulsinelli et al., 1982). Striatal slices in the thickness of 300 µm will be prepared from the control and postischemic rats. LA neurons and MS neurons are visually identified by their soma size under infrared-DIC microscope.

Inhibitory synaptic transmission is pharmacologically separated from excitatory synaptic transmission by using the NMDA receptor antagonist D-APV (50 µM), and the non-NMDA receptor antagonist CNQX (10 µM). $GABA_B$ receptors are also expressed in the striatum (Misgeld et al., 1995). Inclusion of QX-314 in the internal solution, which blocks $GABA_B$ receptors besides sodium currents (Andrade, 1991), assures that we are recording only $GABA_A$ receptor-mediated synaptic transmission in the postsynaptic neuron. IPSC will be evoked intrastriatally with the stimulating electrode being positioned around 150-200 µm close to the recording electrode. LA neurons are held near their resting membrane potential, –60 mV (Bennett et al., 2000). To set up the I-V curve before and after ischemia, LA neurons are held under different holding potentials and evoked IPSCs will be recorded. Recording condition for evoked IPSCs in MS neurons will be the same except that MS neurons are held at -70 mV. The amplitude of evoked IPSCs will be compared before and after ischemia. After successful recordings, some of the neurons will be identified by intracellular staining with neurobiotin.

2. To determine the involvement of pre- and/or postsynaptic mechanisms in the alteration of inhibitory synaptic transmission after ischemia. Paired-pulse test, miniature and spontaneous IPSCs analysis will be performed and exogenous GABA will be applied to LA neurons before and after ischemia to identify the pre- and/or postsynaptic mechanisms.

Paired-pulse stimulation, in which the synapses are activated twice with a short interval, induces two forms of short-term synaptic plasticity: paired-pulse facilitation (PPF) and paired-pulse depression (PPD). Paired-pulse protocol is one of the most widely used approaches to assess the synaptic sites of effects on synaptic transmission. At most synapses, an increase of presynaptic release probability decreases the magnitude of PPF, increases the magnitude of PPD. Conversely, a decrease of presynaptic release probability increases the magnitude of PPF, decreases the magnitude of PPD. Thus, it is widely accepted that a change in PPF and PPD after experimental manipulations is indicative of the involvement of presynaptic mechanisms, especially the presynaptic release probability. In this experiment, two successive stimuli with an interval of 50 ms will be given by bipolar tungsten electrode. Paired-pulse ratio (PPR), which is the ratio of the second current amplitude to the first one, will be compared before and after ischemia.

Miniature IPSCs result from spontaneous, action potential-independent release of GABA from presynaptic GABAergic terminals. Each miniature event is believed to be caused by the release of a single vesicle/quantum. The frequency of miniature IPSCs is determined by two factors. One is the total number of

presynaptic axon terminals that synapse on the postsynaptic neurons; the other is the release probability at each individual synapse. Therefore, it is widely accepted that changes in the frequency reflect presynaptic site of effects (Edwards et al., 1990). The mean amplitude of miniature IPSCs, also called quantal size, is the response of the postsynaptic receptors to the spontaneous release of a single vesicle. For most of the central synapses, transmitters from a single vesicle are enough to saturate postsynaptic receptors. The number and properties of postsynaptic receptors determine quantal size (Nusser et al., 1997). Therefore, changes in the mean amplitude of miniature IPSCs suggest postsynaptic site of effects. For miniature IPSCs recording, TTX (1μM) will be applied to block the action potential-mediated IPSCs. An episode of 5-10 min miniature IPSCs will be collected before and after ischemia. The frequency and amplitude of miniature IPSCs will be compared before and after ischemia.

A change in the function or number of postsynaptic $GABA_A$ receptors might contribute to the alteration of synaptic strength. To test this possibility, exogenous GABA application (300 μM) will be performed to observe the postsynaptic response. The solutions will be applied through a Y tube system by gravity. The tip of the Y tube has a diameter between 100 μm and 150 μm and will be placed close to the recording electrode on the brain slice surface. The duration for GABA application is 10 s.

3. To examine the effects of muscimol on inhibitory synaptic transmission after ischemia. To examine if muscimol treatment could protect striatal neurons from ischemic injury.

Whole-cell patch-clamp recordings are performed on LA neurons on brain slices. Muscimol, at the concentration of 1 μM, is applied via the bath perfusion. Evoked IPSCs, PPR, miniature, and spontaneous IPSCs will be compared in the control LA neurons and LA neurons at 24 h after ischemia, before and after muscimol application. To reveal the role of calcium in muscimol's action, spontaneous IPSCs will be recorded before and after muscimol application in the Ca^{2+}-free solution.

Muscimol (1 mg/kg) will be injected intraperitoneally at 30 min before ischemia. After paraffin embedding, striatal slices in the thickness of 10 μm will be cut. After hematoxylin & eosin (HE) staining, ischemic neuronal injury will be estimated by counting the survived cells in the striatum.

4. To investigate the changes of GAD after ischemia.

GAD Immunoreactivity will be compared in the striatum before and after ischemia by immunohistochemical studies. Western blotting will be used to quantify GAD protein expression before and after ischemia.

MATERIALS AND METHODS

Male Wistar rats of 200-250 g (Charles River Laboratories, Wilmington, MA), with an approximate age of 6-7 weeks (mature synapse function) (Ben-Ari, 2001), 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.

Animal models of transient forebrain ischemia

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 µm in diameter of the tip) filled with 2 M 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 (Figure 4). 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 (Figure 5).

Our ischemia model has the following advantage and disadvantage. The advantage is that this model better mimics stroke patients in the clinics since ischemia is performed in vivo. For in vitro ischemia model, the microenvironment surrounding neurons is artificially controlled by experimenters. Though easier to perform, the pathological process might be different from what observed in the patients. The disadvantage is that we used relatively young rats to yield better quality of the brain slices for recording while most of the stroke patients are old and always have some other cardiovascular diseases.

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% CO_2 and 95% O_2. The composition of the sucrose solution was as follows (in mM): Sucrose 230; $NaHCO_3$ 26; KCl 2.5; NaH_2PO_4 1.25; $CaCl_2$ 0.5; $MgSO_4$ 10; Glucose 10. The rat was then decapitated and the brain was quickly removed from the skull. Coronal striatal slices (300 μm) 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% O_2 and 5% CO_2 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; $CaCl_2$ 2; $MgCl_2$ 2; NaH_2PO_4 1.25; $NaHCO_3$ 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; $CsMeSO_4$ 92; TEA 5; EGTA 2; $MgCl_2$ 1; HEPES 10; QX314 3 and Mg-ATP 4. The internal chloride concentration of 50 mM and the extracellular chloride concentration of 141 mM yield a chloride reversal potential of -28 mV at 30 ˚C by Nernst equation. 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 MΩ 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 GΩ) 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, Sunyvale, 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, Sunyvale, CA). Intrastriatal stimulation was evoked by using the bipolar tungsten electrodes (~5 MΩ, Micro Probe, Potomac, MD). The stimulus electrode was positioned 150-200 μm subjacent to the recording electrode (Figure 6). Isolated stimulator (ISO-Flex, AMPI, Israel) sent out electrical currents with the pulse width of 100 μs every 5 sec (frequency=0.2 Hz) (Master-8, A.M.P.I., Israel). The threshold of stimulus intensity was determined when the amplitude of evoked inhibitory postsynaptic currents just appeared. Since a submerged chamber and the bipolar stimulating electrodes were used, the currents delivered 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 MΩ were included in the analysis. An episode of 5-10 min was collected for miniature IPSCs and spontaneous IPSCs. Tetrodotoxin (TTX, 1 μM) was applied during miniature IPSCs 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 Ca^{2+}-free solution, $CaCl_2$ was removed from the aCSF and 2 mM ethylene glycol tetraacetic acid (EGTA) was added.

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.

Intracellular staining of neurons with neurobiotin

Neurobiotin (1%) was included in the internal solution for intracellular labeling of some recorded neurons before HRP reaction. 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 diaminobenzidine (DAB).

Immunohistochemistry

Under deep anesthesia, the rats were transcardially perfused with heparinized saline followed by 4% paraformaldehyde in 0.15 M NaPBS. The brains were removed and post fixed in the same fixative at 4 ˚C overnight. They were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer (Ph=7.4) for at least 48 h. The brains were frozen in the embedding medium (O.C.T., Sakura Finetek, Torrance, CA) on dry ice for at least half an hour before they were transferred to the cryostat. Coronal sections of 30 μm were cut and kept in the cryoprotective

buffer at -20 ˚C. The cryoprotective buffer was composed of the following (V/V): ethylene glycol (20%), glycerol (30%), 0.01M KPBS (50%).

For immunofluorescence studies, free-floating sections were rinsed in 0.01M KPBS three times and then 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. After that, they were incubated with rabbit anti-GAD65 (1:500; Millipore, AB5082; Bedford, MA), in the blocking serum overnight at room temperature. After three washes in KPBS, they were incubated in the secondary antibody solutions, Fluorescein goat anti-rabbit IgG (1:100; Vector, FI-1000; Burlingame, CA) for two hours. 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.

Some of the sections were mounted on slides for standard HE staining. For the Avidin-Biotin Complex (ABC) reactions, the procedures were the same as those in the doublelabeling immunofluorescence mentioned above except different secondary antibodies were used and the reaction products were revealed with DAB and H_2O_2. Besides rabbit anti-GAD65, other primary antibodies used were: mouse anti-vesicular glutamate transporter 2 (anti-vGLUT2; 1:1000; Millipore, MAB5504) and mouse anti-GAD67 (1:2000; Millipore, MAB5406).

Western blotting

The control rats and rats at 1 day and 3 days after ischemia were sacrificed and the brain slices containing striata were cut at the thickness of 600 μm. Striata were dissected out and stored on the dry ice before they were manually homogenized using ice-cold radioimmunoprecipitation assay buffer (10 mg tissue/100 ml buffer, Boston Bioproducts, Worcester, MA) supplemented with proteinase inhibitors (Roche, Indianapolis, IN). After sonication, the lysates were centrifuged at 14,000 g for 20 min at 4 ˚C. Supernatants were collected for further analysis. Bicinchoninic acid protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA) was used to determine the protein concentration. The final concentration of the protein sample was adjusted to 1 μg/ml in the 2×SDS gel-loading buffer (Invitrogen, Carlsbad, CA). The protein samples were denatured by boiling before being loaded (20 μg) on the 10% SDS-polyacrylamide gel. After separation on the gel, the proteins were transferred to the nitrocellulose membranes (Millipore). The membranes were then blocked with 0.1% bovine serum albumin in Tris-buffered saline containing 0.1% Triton-X for 1 h at room temperature. They were then incubated either in the mouse anti-actin (1:20,000, Sigma) or the rabbit anti-GAD65 (1:6,000, Millipore) antibodies in the blocking buffer at 4 ˚C overnight. After three washes, the membranes were incubated in the secondary antibody: peroxidase labeled anti-mouse (1:30,000, Millipore) IgG or peroxidase labeled anti-rabbit (1:100,000, Millipore) IgG for 1 h at room temperature. The corresponding bands were detected with the enhanced chemiluminescence (Amersham Pharmacia Biotech,

Piscataway, NJ) and visualized by exposing the membrane to autoradiography films (Fuji, Tokyo, Japan). Data analysis was performed by using the software ImageJ from NIH.

Drug administration and sample preparation for paraffin embedding

Muscimol (Enzo Life Sciences International, Plymouth Meeting, PA) was dissolved in saline at 0.5 mg/ml. At 30 min before ischemia, muscimol (1mg/kg) (Costa et al., 2004) was given to the rats intraperitoneally. One day after ischemia, the rats were deeply anesthetized with the Ketaset and then transcardially perfused with 0.01 M NaPBS (pH=7.4) followed by the fixative (10% acetic acid, 10% formaldehyde (37%) and 80% methanol). The brain stayed in situ overnight. The next day, the brain was removed and post fixed in 4% paraformaldehyde for several days before it was transferred to 70% ethanol for 1-2 days. Then they were sent for paraffin embedding by a specialist.

Quantification analysis of the terminals in the striatum

Quantification analysis was performed by an investigator who was blinded to the treatments using the software ImageJ (NIH). Images were captured from dorsolateral and dorsomedial striatum from 3 interaural planes corresponding to 10.6, 8.7, and 8.08 mm.

To analyze the optical density of presynaptic terminals for GAD65, images were captured in black and white 8-bit grayscale under 100× using a CCD camera from DAB stained sections. The density of the terminals was determined in fixed regions measuring 1×1 mm^2 that was kept constant across all of the images. The pixels were converted to square millimeters by employing a

calibration that converts pixels to millimeters. The density in the white matter, such as corpus callosum and striatal fibers, was subtracted as the background. The final numeric value represented the optical density of the terminals in each mm^2. A mean was calculated for each animal and a total mean±SEM was determined for each experimental treatment group.

To obtain the information of terminal number, size and intensity, images were taken from sections after doublelabeling immunofluorescence in black and white 8-bit grayscale under 400× using a CCD camera. Thresholding was performed to distinguish the object terminals from the background. After precise thresholding, the minimum and maximum size of the terminals was set to occlude the objects clearly not of interest. A mean was calculated for each animal and a total mean±SEM was determined for each experimental treatment group.

Quantification analysis of survived cells in the striatum

Quantification analysis was performed by an investigator who was blinded to the treatments. After paraffin embedding, coronal sections of 10 μm were cut and three interaural planes in the striatum, corresponding to 10.6, 8.7, and 8.08 mm, were selected. Four consecutive sections were taken from each plane for HE staining. Dorsolateral and dorsomedial striatum were randomly selected in each hemisphere of a section by light microscopy under 200×. Dead cells showed shrunken nuclei while survived cells remained normal morphology. We assessed ischemic neuronal damage by counting survived cells. 48 microscopic fields (400×300 μm) were used to count survived cells in the dorsal part of the striatum in both hemispheres for each rat.

Data analysis

Numerical data were expressed as mean±SEM. 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 miniature IPSCs and spontaneous IPSCs 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

Selective neuronal death in the striatum

To reveal whether the four-vessel occlusion model used in this project reproduces the selective neuronal death in the striatum, we used HE staining to examine striatal neurons. In control animals, most of the striatal cells were medium-sized, possibly MS neurons and GABAergic interneurons (Figure 7A). Only a few of the striatal cells were large-sized, possibly LA neurons. At 3 h after ischemia, most of the striatal cells showed normal morphology (Figure 7B). But at 24 h and 72 h after ischemia, very few striatal cells remained normal morphology, in which some of them are LA neurons as indicated by the size (arrows, Figure 7C-D). Most of the cells exhibited pyknotic and shrunken nuclei, the identity of which is difficult to tell. They could be MS neurons or GABAergic interneurons with pyknotic and shrunken nuclei, or the activated glial cells. Although we can identify the normal morphology of the survived cell by HE staining, we could not tell whether the cells with abnormal morphology are dead or not. We could not tell whether they are undergoing necrosis or apoptosis either. Previous research from this group have demonstrated that MS neurons undergo both necrosis and apoptosis after transient cerebral ischemia by EM studies (Ruan et al., 2003). The combination of several specific biochemical analyses is needed to identify the type of cell death (Kroemer et al., 2009). Therefore, from the HE staining, we conclude that the large-sized neurons, corresponding to the LA neurons, survive at 24 h after ischemia (Figure 7).

Identification of striatal neurons

Whole-cell voltage-clamp recordings were performed on striatal neurons. LA neurons were identified by their large soma size (25-60 μm in diameter) (Figure 8, 10A). But due to their scarcity, less than five LA neurons could be found on each striatal section. MS neurons were identified by their medium size, 10-15 μm in diameter (Figure 9, 10A). Plenty of MS could be found on each section. After successful recordings, some of the neurons were further confirmed by intracellular staining with neurobiotin. Besides soma size, the main morphological difference is that LA neurons have aspiny dendrites and MS neurons have spiny dendrites (Figure 8, 9). LA neurons also have distinctive electrophysiological characteristics. Since the internal solution used contains cesium, we could not observe the firing pattern of LA neurons. But on the first two minutes after the cell is broken, sometimes the characteristic firing of LA neurons could be recorded. 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 Ih currents (Jiang and North, 1991; Kawaguchi, 1993) (Figure 10B).

Alterations of inhibitory synaptic transmission in LA neurons and MS neurons after ischemia

To examine inhibitory synaptic transmission in LA neurons, evoked inhibitory postsynaptic currents were recorded in LA neurons upon intrastriatal stimulation.

Intrastriatal stimulation was made by a tungsten electrode positioned about 200 µm adjacent to the recording electrode (Figure 6). Threshold intensity was determined when the postsynaptic currents just appeared. Then the intensity was increased to two times (2T), 3T, 4T, and 5T of the threshold intensity. Intrastriatal 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), or the combination of D-APV (50 µM) and CNQX (10 µM), respectively (Figure 10C). D-APV and CNQX were used routinely to pharmacologically isolate GABAergic IPSCs in the subsequent experiments. At a holding potential of -60 mV, inwardly directed evoked IPSCs were elicited.

To examine if the intracellular concentration of chloride is changed after ischemia, we held LA neurons at different potentials (from -100 mV to +20 mV, at an increment of 20 mV) and recorded evoked IPSCs at 3T. The reversal potential derived from the I-V curve equals the chloride reversal potential calculated by the Nernst equation. These data indicate that the reversal potential of chloride is not changed at 24 h after ischemia (Figure 11), suggesting that the intracellular chloride concentration is not changed after ischemia. However, it should be noted that a definitive conclusion cannot be derived using standard whole-cell patch clamp recordings: perforated patch-clamp recordings would be able to better address this issue.

Evoked IPSCs were recorded from LA neurons at 3 h and 24 h after ischemia, respectively. The amplitudes of evoked IPSCs were significantly

enhanced after ischemia across the stimulus intensities. For example, at a stimulus intensity of three times of the threshold stimulus intensity (3T), the amplitude was enhanced from -47±7 pA in the control (n=12) to -136±30 pA at 3 h after ischemia (n=9; $P<0.05$) and -183±30 pA at 24 h after ischemia (n=9; $P<0.05$; Figure 12).

We also recorded evoked IPSCs in MS neurons at 8 h after ischemia. Evoked IPSCs in MS neurons were significantly depressed after ischemia (Figure 13). For example, at 3T, the amplitude was depressed from -91±14 pA of the control level (n=12) to -52±10 pA (n=12; $P<0.05$; Figure 13) at 8 h after ischemia.

We observed a significant increase of the stimulus threshold in LA neurons after ischemia. The stimulus threshold was 0.31±0.02 mA for the control (n=12), 0.86±0.15 mA (n=9; $P<0.01$) at 3 h after ischemia, and 0.82±0.19 mA (n=9; $P<0.01$; Figure 14A) at 24 h after ischemia. The threshold also showed greater variability after ischemia, ranging from 0.35 mA to 2 mA.

The increase of the stimulus threshold at 3 h after ischemia indicated that the terminals might be functionally damaged even though most of the MS neurons were morphologically normal (Figure 7B). The increase of the stimulus threshold means that we need more powerful stimulus to recruit a certain number of GABAergic terminals to evoke IPSCs, which is consistent with the decreased availability of GABAergic terminals as a result of extensive degeneration of MS neurons in the dorsolateral striatum at 24 h after ischemia (Pulsinelli et al., 1982). However, this increased stimulus threshold does not mean decreased GABAergic transmission. As the results shown in the next section (Mechanisms

for the enhancement of evoked IPSCs in LA neurons after ischemia), the frequency of spontaneous IPSCs, recorded without the presence of TTX, was increased at 3 h and 24 h after ischemia, highly suggesting that presynaptic release is actually increased after ischemia.

To examine if this increase of evoked IPSCs was resulted from the increase of the stimulus threshold, we plotted the absolute values of 3T against the amplitudes of evoked IPSCs at 3 h and 24 h after ischemia. No linear correlation was observed in both groups, indicating the increase of evoked IPSCs was not due to the increase of the stimulus threshold (Figure 14B).

Therefore, inhibitory synaptic transmission is significantly enhanced in the ischemia-resistant LA neurons while depressed in the ischemia-vulnerable MS neurons after ischemia.

Mechanisms for the enhancement of evoked IPSCs in LA neurons after ischemia

The enhancement of evoked IPSCs in LA neurons after ischemia could be due to the increased presynaptic GABA release, or the increased postsynaptic response to GABA. To further determine the pre- and/or postsynaptic mechanisms, we performed paired-pulse tests, recorded spontaneous IPSCs, miniature IPSCs, and postsynaptic currents on exogenous GABA application.

LA neurons exhibited paired-pulse depression, indicating a high initial release probability (Figure 15A). 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; Figure 15B). The decreased PPR suggests an increase of presynaptic release probability after ischemia.

Spontaneous IPSCs compose of both the quanta-release events and the action potential-mediated events. The frequency of spontaneous IPSCs 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). The amplitude of spontaneous IPSCs was slightly decreased, although this was not statistically significant. 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; Figure 16A-C).

Miniature IPSCs represent only the random quantal release of neurotransmitters. The frequency of miniature IPSCs showed no change 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 miniature IPSCs 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; Figure 17A-C).

To confirm that postsynaptic responses are depressed after ischemia, exogenous GABA application (300 μM) 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; Figure 18).

An increase of presynaptic release probability could be concluded from the PPR study. However, the frequency of miniature IPSCs did not change after ischemia, which does not support this conclusion. The explanation for the increase in the frequency of spontaneous IPSCs is that the excitability of presynaptic terminals is significantly increased after ischemia, which results in an increase of presynaptic release. Therefore, 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 (Table 2).

Modulation of GABA release by presynaptic $GABA_A$ receptors in the control LA neurons

Morphological studies have shown that LA neurons receive very few inhibitory inputs (Chang and Kita, 1992), which tells why the frequency of miniature and spontaneous IPSCs is very low as observed in this study and previous studies (Bennett and Wilson, 1999).

To explore whether presynaptic $GABA_A$ receptors play a role in the modulation of inhibitory synaptic transmission, we recorded evoked IPSCs in the

control LA neurons before and after muscimol application. It has been known that muscimol could activate $GABA_B$ receptors but at a higher EC50 of 25 μM (compared to the EC50 of 1 μM for $GABA_A$ receptors) (Yamauchi et al., 2000). Therefore, at a concentration of 1 μM used in the present study, muscimol should have only activated $GABA_A$ receptors. Muscimol (1 μM) 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. After muscimol application, the amplitude of evoked IPSCs (at 3T) was depressed by 64±10%. Before muscimol application, the amplitude was 53±13 pA (n=7). After muscimol application, the amplitude was 35±10 pA (n=7; P>0.05; Figure 19A1, B). No significant change of PPR was detected after muscimol application (before: 0.69±0.04; after: 0.74±0.12; 108±17% of the pre-application level, n=7; P>0.05; Figure 19A1, B).

We also examined spontaneous IPSCs before and after muscimol application. The frequency of spontaneous IPSCs was decreased (before: 1.01±0.16 Hz; after: 0.71±0.12 Hz; 64±9% of the pre-application level, n=7; P<0.05) and the amplitude was increased (before: 16.48±1.80 pA; after: 22.21±2.90 pA; 136±8% of the pre-application level, n=7; P<0.01; Figure 20) after muscimol application.

From the data above, muscimol activates both pre- and postsynaptic $GABA_A$ receptors. Presynaptically, muscimol decreases GABA release as inferred from the decrease in the frequency of spontaneous IPSCs. Postsynaptically, muscimol enhances $GABA_A$-mediated currents as inferred from

the increase in the amplitude of spontaneous IPSCs. Since muscimol has opposite effects on pre- and postsynaptic sites, no significant changes could be detected in the amplitude of evoked IPSCs and PPR.

Modulation of GABA release by presynaptic $GABA_A$ receptors in LA neurons at 24 h after ischemia

We also examined whether muscimol has the same effects on inhibitory synaptic transmission in LA neurons at 24 h after ischemia. Muscimol significantly increased the amplitude of evoked IPSCs to 177±15% of the pre-application level. Before muscimol application, it was 95±34 pA. After muscimol application, it was 140±44 pA (n=8; P<0.01; Figure 19A2, B). Paired-pulse tests showed that PPR decreased to 76±5% of the pre-application level. Before muscimol application, it was 0.51±0.04. After muscimol application, it was 0.40±0.03 (n=6; P<0.05; Figure 19A2, B).

The frequency of spontaneous IPSCs was increased (before: 2.02±0.36 Hz; after: 2.91±0.28 Hz; 148±14% of the pre-application level, n=6; P<0.05) and the amplitude showed no change (before: 16.43±2.33 pA; after: 17.21±1.68 pA; 107±5% of the pre-application level, n=6; P>0.05; Figure 21) after muscimol application.

Therefore, muscimol presynaptically increases GABA release after ischemia. Muscimol differentially modulates presynaptic GABA release in the control and ischemic neurons.

To reveal the role of calcium in muscimol's facilitative effects on inhibitory synaptic transmission, spontaneous IPSCs were recorded before and after

muscimol application at 24 h after ischemia in the Ca^{2+}-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 normal aCSF to the Ca^{2+}-free aCSF, the amplitude (before: 16.89±1.03 pA; after: 13.90±0.62 pA; 87±4% of the pre-Ca^{2+} removal, n=8; $P<0.05$) and frequency (before: 1.54±0.29 Hz; after: 0.89±0.19 Hz; 61±10% of the pre-Ca^{2+} removal, n=8; $P<0.05$; Figure 22A, B) of spontaneous IPSCs 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±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±7% of the pre-application level, n=7; P=0.25; Figure 22A, C, D) of spontaneous IPSCs after ischemia. Therefore, muscimol's facilitation on presynaptic GABA release after ischemia is sensitive to the extracellular calcium.

Unlike spontaneous IPSCs, miniature IPSCs showed no significant changes in the frequency (before: 1.35±0.50 Hz; after: 1.27±0.56 Hz; 95±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±4% of the pre-application level, n=6; $P>0.05$; Figure 23) 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.

The above data indicates that activation of presynaptic $GABA_A$ receptors enhances presynaptic GABA release after ischemia, which is sensitive to both extracellular calcium and voltage-gated sodium channels. Therefore, activation of presynaptic $GABA_A$ receptors on the GABAergic terminals results in differential effects in the postsynaptic LA neurons of control and after ischemia (Table 3). We make the following explanation. In the control condition, intracellular chloride concentration is very low and the reversal potential for chloride is more negative than the resting membrane potential. Therefore, following the activation of presynaptic $GABA_A$ receptors, there will be a chloride influx and membrane hyperpolarization in the presynaptic terminals. After ischemia, intracellular chloride concentration is increased and the reversal potential for chloride is more positive than the resting membrane potential. Following the activation of presynaptic $GABA_A$ receptors, there will be a chloride efflux and membrane depolarization in the presynaptic terminals, which will activate voltage-gated sodium channels and voltage-gated calcium channels. Thus, presynaptic transmitter release is increased and a bigger postsynaptic response occurs in the postsynaptic LA neurons (Figure 24).

GAD expression in the striatum before and after ischemia

MS neurons provide inhibitory inputs to LA neurons (Bolam et al., 1986; Martone et al., 1992). With the massive degeneration of MS neurons (especially in the dorsolateral striatum) after ischemia, how could the inhibitory synaptic transmission be enhanced in LA neurons? To answer this question, we studied GAD in the striatum before and after ischemia.

There are two isoforms of GAD in the striatum, GAD65 and GAD67. GAD65 is abundant in the terminals of MS neurons whereas GAD67 is most abundant in the cell bodies of GABAergic interneurons (Mercugliano et al., 1992). Since LA neurons receive inhibitory inputs from MS neurons, we examined the immunoreactivity of GAD65 before and after ischemia. GAD65-immunopositive terminals were observed in striatal sections after Avidin-Biotin Complex (ABC) reactions (Figure 25A). The optical density of GAD65-positive terminals in the dorsolateral striatum showed a significant increase from 25.31±5.94 in control (n=6) to 69.01±2.79 in 1 day (n=6; $P<0.05$) and 69.36±14.21 in 3 days (n=6; $P<0.05$; Figure 25B) after ischemia. There were no significant differences between 1 day and 3 days.

We also did immunofluorescence labeling of GAD65 in the striatal sections. The number, size, and optical density of GAD65-positive puncta were estimated in the dorsolateral region of the striatum by using the image analysis software, Image J (NIH). After ischemic insult, the number of GAD65-positive puncta significantly increased (Figure 26A). For the control group, the number was 554.27±109.26/field (200x150 μm; n=6). 1 day after ischemia, the number was 2077.17±341.5/field (n=6; $P<0.05$). 3 days after ischemia, the number was 2494.94±294.81/field (n=6; $P<0.05$; Figure 26B). There were no significant differences between 1 day and 3 days. The average size of the GAD65-positive puncta showed no significant change (data not shown). The optical density of the GAD65-positive puncta was significantly increased at 3 days after ischemia.

It was 60.41±1.75 in the control (n=6), 64.02±0.33 in one day (n=6; $P>0.05$), and 69.97±3.74 in 3 days after ischemia (n=6; $P<0.05$; Figure 26C).

The increased number of GAD65-positive puncta observed after ischemia could be an artifact due to the easy detection of high-density puncta after ischemia. Therefore, the number of inhibitory synapses in the control is actually more than that after ischemia. But the low optical density makes them not detectable in the control striatum. Data from the miniature IPSCs recordings does not support the increase in the number of inhibitory synapses since the frequency didn’t change after ischemia. The massive degeneration of MS neurons, the major GABAergic neuronal population in the striatum, does not support the increase in the number of inhibitory synapses after ischemia. In addition, the increase in the threshold of the stimulus intensity indicates that there is a decreased availability of inhibitory terminals after ischemia. The increase in the optical density of GAD65-positive puncta after ischemia indicates that there is an increase in the number of synapses that highly express GAD65.

Because the striatum is mainly composed of MS neurons (Rymar et al., 2004), ischemic injury results in a vast decrease of the striatal cellular population. This dramatic loss in density may suggest that the components remaining in the striatum are more readily accessible to antibodies. The ischemic injury itself may also facilitate the penetration of the antibodies to the cells. Therefore, the increased immunoreactivity of GAD65 might not result from postischemic molecular or intracellular changes but rather due to easier access to the antibodies. To address this issue, we examined the immunoreactivity of vGLUT2

and GAD67 after ischemia. If the increased immunoreactivity of GAD65 is due to increased access to antibodies, the immunoreactivity of vGLUT2 and GAD67 might also increase after ischemia. VGLUT2 is specially located on the glutamatergic terminals from the thalamus that form synapses on LA neurons in the striatum (Fremeau et al., 2001; Herzog et al., 2001; Lapper and Bolam, 1992). Our data suggests that there is no significant increase in the optical density of vGLUT2 immunoreactivity after ischemia (Figure 27A and B). In fact, at three days after ischemia, there is decreased immunoreactivity of GAD67 in the dentate gyrus of the hippocampus compared to the control (Figure 28). Our data suggests the increased penetration of antibodies does not contribute to the increased immunoreactivity of GAD65.

To test if the increased immunoreactivity of GAD65 reflects the increased expression level of GAD65 after ischemia, we performed western blotting. Striatal sections were dissected out from the control rats, rats at 1 day and 3 days after ischemia. Equal amounts (20 μg) of protein were analyzed for GAD65 expression level. The relative density of the bands for GAD65 increased significantly after ischemia. For the control group, it was 100±16.93% (n=4). 1 day after ischemia, it was 289.86±37.85% (n=4; $P<0.05$). 3 days after ischemia, it was 284.86±61.70% (n=4; $P<0.05$; Figure 29). There were no significant differences between 1 day and 3 days.

Therefore, GAD65 expression is enhanced in the striatum confirmed by both immunohistochemical and western blotting analysis. The increased expression of GAD65 highly suggests that presynaptic GABA synthesis is

enhanced after ischemia, which might contribute to the enhanced inhibitory synaptic transmission in LA neurons after ischemia.

Muscimol's effect on ischemic neuronal injury in the striatum

We observed an enhancement of inhibitory synaptic transmission in ischemia-resistant LA neurons and a depression of inhibitory synaptic transmission in ischemia-vulnerable MS neurons. Muscimol enhanced inhibitory synaptic transmission in LA neurons by presynaptically increasing GABA release after ischemia. To answer the question whether facilitation of inhibitory synaptic transmission could protect striatal neurons against ischemia, we treated rats with muscimol 30 min before ischemia and examined the ischemic neuronal injury by counting survived cells in the striatum. Previous studies have shown that muscimol has neuroprotective effects against ischemia, which has been detailed in the introduction part (inhibitory synaptic transmission and postischemic neuronal injury). However, most of the studies were performed in the hippocampus and very few in the striatum.

Compared to the ischemic rats without muscimol treatment, the number of survived cells (defined as cells with normal morphology) in the striatum (both dorsolateral and dorsomedial parts) was significantly increased (Figure 30). For the ischemia group, the number of survived cells was 85.12±14.67/field (400x300 μm; n=6). For the muscimol treatment group, the number was 171.44±29.54/field (n=9, $P<0.05$). This was mainly contributed by the increase in the survived cells in the dorsomedial striatum (ischemia group: 101.18±15.30/field, n=6; muscimol treatment group: 224.22±36.78/field, n=9;

$P<0.05$). The number of survived cells in the dorsolateral striatum showed no significant changes with muscimol treatment (ischemia group: 69.07±14.59/field, n=6; muscimol treatment group: 118.67±25.40/field, n=9; $P=0.16$; Figure 31). Although the difference is not statistically significant, the data suggests that there might be an increase. Additional studies are needed to determine definitively whether or not there is a protective effect in the dorsolateral striatum.

The significant increase in the survived cells was found in the dorsomedial striatum, suggesting that cells in the dorsomedial part are not severely damaged and are savable from ischemic injury. This is consistent with previous studies, showing that ischemic damage is more severe in the dorsolateral striatum than that in the dorsomedial striatum (Pulsinelli et al., 1982). We don't know the identities of the survived cells after muscimol treatment by HE staining. Besides LA neurons as indicated by the large size, we assume that most of them are MS neurons as indicated by their medium size. Whether progenitor cells migrated from the SVZ contribute to the pool of the survived cells after muscimol treatment remains unclear.

In all, our study demonstrated that inhibitory synaptic transmission was enhanced in ischemia-resistant LA neurons after transient cerebral ischemia. There are two mechanisms contributing to this enhancement. One is that the activation of presynaptic $GABA_A$ receptors increased presynaptic GABA release after ischemia. The other is the increased expression of GAD. Facilitation of inhibitory synaptic transmission by muscimol significantly increased the number of survived cells in the striatum, especially in the dorsomedial striatum after

ischemia. Therefore, the enhancement in inhibitory synaptic transmission might reduce excitotoxicity and contribute to the selective survival of LA neurons after ischemia.

DISCUSSION

Mechanisms for the alterations of inhibitory synaptic transmission in LA neurons after ischemia

The present study observed an increase of evoked IPSCs in LA neurons after ischemia. Both pre- and postsynaptic mechanisms are involved in the alterations of inhibitory synaptic transmission based on the following findings. 1. PPR is depressed while the frequency of spontaneous IPSCs is increased after ischemia. 2. The mean amplitude of miniature IPSCs and the currents induced by exogenous GABA application are depressed after ischemia. Therefore, presynaptically, the GABA release is increased. Postsynaptically, the $GABA_A$ receptors show depressed responses at 24 h after ischemia (Table 2).

We found that the activation of presynaptic $GABA_A$ receptors by muscimol decreased presynaptic GABA release onto the control LA neurons while facilitated GABA release onto LA neurons after ischemia based on the following observations. 1. The frequency of spontaneous IPSCs was decreased while the amplitude was increased in the control LA neurons after muscimol application. The opposite effects on the frequency and amplitude also helped explain why there was no significant change in the amplitude of evoked IPSCs and PPR. 2. The amplitude of evoked IPSCs was increased and PPR was depressed in LA neurons after muscimol application at 24 h after ischemia. 3. The frequency of spontaneous IPSCs was increased in LA neurons after muscimol application at 24 h after ischemia. Muscimol's facilitative effect after ischemia was dependent on extracellular calcium and voltage-gated sodium channels (Table 3).

Upon the vesicle release, the size of postsynaptic currents is determined by quantal size (q), the readily releasable pool (RRP) of vesicles (N), and the probability of each vesicle being released (pr) (Johnson and Wernig, 1971; Quastel, 1997; Zucker, 1973). The quantal size is decreased since the mean amplitude of miniature IPSCs was depressed after ischemia. There are three ways to determine the size of RRP. One is by delivering a train of action potentials (Rosenmund and Stevens, 1996). The second is by step-like depolarization (Schneggenburger et al., 2002). The third is by using hypertonic sucrose solution, which is only applicable to cultured neurons (Rosenmund and Stevens, 1996). However, most of the studies used hippocampal slices, cell cultures, or calyx of Held to determine the size of RRP. Whether these methods are applicable to striatal slices or not is still unknown. As to the presynaptic release probability, data from paired-pulse stimulation highly suggests the increase of presynaptic release probability after ischemia. However, no change was found in the frequency of miniature IPSCs confounding this situation (Table 1). There are two possibilities for this. One is that the presynaptic release probability is really increased but the postsynaptic receptors failed to detect them due to the decrease of the quantal size. The other is that presynaptic release is increased, which is action-potential dependent, due to the increase in the frequency of spontaneous IPSCs. The latter possibility seems more probable since the data from muscimol application also supports this. A possible explanation for this is that the excitability of the functional terminals might have increased 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.

Previous studies 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 number and the function of postsynaptic receptors are the main determinants of quantal size. Normally, in the central nervous system, the presynaptic vesicles could saturate postsynaptic receptors, the number of postsynaptic receptors is very important in determining the quantal size. However, if the presynaptic vesicles could not saturate postsynaptic receptors, the presynaptic release kinetics and the neurotransmitter amount in the vesicles are important determinants of quantal size. Therefore, presynaptic factors could also participate in the modulation of quantal size (Atwood and Karunanithi, 2002; Liu, 2003).

Decreased number and/or depressed functions of postsynaptic $GABA_A$ receptors might contribute to the depressed postsynaptic responses as observed from the exogenous GABA application and miniature IPSCs recording. Whether the number of postsynaptic $GABA_A$ receptors is changed after ischemia remains unclear. The functions of postsynaptic $GABA_A$ receptors might have 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 function (Schwartz et al., 1992).

The main $GABA_A$ receptor subunits in the rat striatum are α1, β2/3, and γ2. β2/3 subunits were commonly found on MS neurons and GABAergic interneurons (Fujiyama et al., 2000). In MS neurons, the expression of α5 and β3 subunits is responsible for the tonic conductances (Ade et al., 2008; Janssen et al., 2009). Studies from Yan et al. observed that nearly all LA neurons co express α2, α4, β1, and γ2 mRNAs (Yan and Surmeier, 1997). Whether ischemia could modulate these subunits in the striatum is still unknown.

As to muscimol's facilitative effect after ischemia, we speculated that the chloride concentration in the presynaptic terminals was increased after ischemia, which induced a depolarized reversal potential of chloride. Excitatory effects occurred if the depolarization overcomes the shunting effects while at the same time, the inactivation of sodium and calcium channels not yet happen. Since we speculated that the intraterminal chloride concentration was increased after ischemia, it is very natural to ask the question: is the chloride concentration in LA neurons also increased after ischemia? We could not answer this question since we used whole-cell patch-clamp recording in this study. However, even if chloride concentration is increased in LA neurons after ischemia, LA neuron cell bodies have lower susceptibility to the disruption of ionic imbalance than MS

neurons since their smaller surface-to-volume ratio. What that means is that LA neurons might have a slower and smaller shift in the reversal potential of chloride compared to that of the MS neurons. And they are less likely to convert from inhibitory to excitatory upon the $GABA_A$ receptor activation. One example from previous studies is that in CA3 region, the activity-driven shift in the chloride reversal potential is much faster and larger in the interneurons than in the pyramidal cells. Besides the different surface-to-volume ratio, they also mentioned other factors involved. These two types of neurons might have different expressions of chloride transportation systems or be in different phosphorylation cascades that control the transporters. The transportation systems in pyramidal neurons might be more efficient in extruding the chloride out of the cell. They might have different carbonic anhydrase activity and different availability to $HCO3^-$. The interneurons might have more access to $HCO3^-$ and the $GABA_A$ receptors on interneurons have high permeability to $HCO3^-$ (Lamsa and Taira, 2003).

Inhibitory synaptic transmission and postischemic neuronal injury

Large accumulation of GABA in the extracellular space has been demonstrated by in vivo and in vitro studies during ischemia and early after reperfusion (Allen et al., 2004; Globus et al., 1988). But as to the alterations of GABAergic synaptic transmission, different neurons show different changes. Inhibitory synaptic transmission is depressed or disappeared in striatal MS neurons during ischemia and early after reperfusion in vivo (Gajendiran et al., 2001; Xu, 1995). The frequency of spontaneous IPSCs is increased in neocortical layer 5 neurons and

hippocampal CA1 neurons during simulated ischemia in vitro (Allen and Attwell, 2004; Fleidervish et al., 2001). In the present study, we observed an enhancement of inhibitory synaptic transmission in the ischemia-resistant LA neurons at 3 h and 24 h after transient cerebral ischemia while a depression in inhibitory synaptic transmission in ischemia-vulnerable MS neurons at 8 h after transient cerebral ischemia.

The enhancement of inhibitory synaptic transmission in the ischemia-resistant LA neurons 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 would limit calcium influx and stop the cascades to postischemic neuronal death. The differential changes of the synaptic transmission in LA neurons and MS neurons might contribute to their differential vulnerability to ischemia. In the hippocampus, CA1 pyramidal neurons survive (Pulsinelli et al., 1982) transient cerebral ischemia while CA1 interneurons die (Schlander et al., 1988). They also show differential changes in inhibitory synaptic transmission after transient cerebral ischemia. CA1 pyramidal neurons exhibit depressed responses to GABA while CA1 interneurons not (Zhan et al., 2006).

In the present study, the application of muscimol at 30 min before the ischemic insults significantly increased the number of survived cells in the striatum. This is consistent with previous studies showing that muscimol is neuroprotective against ischemia, as detailed in the introduction part (inhibitory synaptic transmission and postischemic injury). However, considering the neuronal swelling as the consequence of chloride influx during ischemia (Inglefield and Schwartz-Bloom, 1998a, b), the neuroprotective effects by facilitating inhibitory synaptic transmission is not that convincing. In fact, there are studies demonstrating that chloride influx either through $GABA_A$ receptors or other chloride channels are not neuroprotective against excitotoxic injury. Van Damme et al. observed that AMPA application to motoneurons results in chloride influx through the activation of chloride channels, which exacerbates excitotoxic death by increasing AMPA receptor conductance and enhancing the driving force of calcium through repolarization. Co-application of GABA and AMPA enhances chloride influx and excitotoxic cell death (Van Damme et al., 2003). Application of GABA or activation of GABA receptors does not show neuroprotection in cultured neurons, while blockade of $GABA_A$ receptors alleviates cell death (Erdo et al., 1991; Erdo and Michler, 1990; Koh and Choi, 1987). Different experimental preparations might explain the different effects of $GABA_A$ receptor activation and chloride influx. One other possible explanation is that during or after excitotoxic insults, the intracellular chloride concentration might have increased to the extent that further potentiation of GABA activity results in excitatory effects, due to the more depolarized chloride reversal

potential relative to the resting membrane potential. Under this condition, the activation of $GABA_A$ receptors may not be neuroprotective. Therefore, the extent of $GABA_A$ receptor activation should be delicately controlled to obtain neuroprotection.

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 (Costa et al., 2004; Dave et al., 2005; Jackson-Friedman et al., 1997; Kulinskii and Mikhel'son, 2000; Kuramoto et al., 2007). Studies from Kuramoto et al. showed that $GABA_B$ receptors are phosphorylated by 5'AMP-dependent protein kinase (AMPK), which is activated under ischemia, and results in neuronal survival (Kuramoto et al., 2007). Considering the activation of presynaptic $GABA_B$ autoreceptors, the release of glutamate, GABA, and a group of other neurotransmitters will be decreased. From one point of view, this is neuroprotective under ischemic injury since synaptic activity will be decreased, energy will be conserved, and neuronal exposure to excitotoxicity will be limited. From the other point of view, varying effects might occur if the release of a group of neurotransmitters is decreased, which makes the situation more complicated to explain.

Functional significance and limitations of the present study

We demonstrated an enhancement of inhibitory synaptic transmission in ischemia-resistant LA neurons after transient cerebral ischemia. Muscimol application at 30 min before the ischemic insults significantly improves the

number of survived cells in the striatum compared to the non-treatment group. Our data strongly suggests that facilitation of inhibitory synaptic transmission protects striatal neurons against cerebral ischemia. This is corroborated by the previous studies from Zhang' s group, which showed that muscimol application protects hippocampal CA1 pyramidal neurons against transient cerebral ischemia (Han et al., 2008; Xiao et al., 2007; Xu et al., 2008; Zhang et al., 2007; Zhou et al., 2008).

One of the mechanisms for the enhancement of inhibitory synaptic transmission in LA neurons after ischemia is that presynaptic $GABA_A$ receptors help increase GABA release. Large accumulation of GABA in the extracellular space has been demonstrated by in vivo and in vitro studies during ischemia and early after reperfusion (Allen et al., 2004; Globus et al., 1988). It is known that GABA release is initially by exocytosis and later by reversed uptake through GABA transporters (Allen et al., 2004). The initial exocytosis is due to the depolarization by the rundown of Na^+-K^+-ATPase. The activation of presynaptic $GABA_A$ receptors increases the chloride concentration in the presynaptic terminals. GABA release happens when chloride reversal potential is positive than the resting membrane potential. Based on this study, the large accumulation of GABA might be, at least in part, due to the activation of presynaptic $GABA_A$ receptors located on the GABAergic terminals. In addition, $GABA_B$ receptors are activated under ischemic injury (Kuramoto et al., 2007), which results in depressed synaptic activity. Whether GABA release will be facilitated or depressed is determined by the interplay among the extent of

energy rundown, presynaptic $GABA_A$ receptors (inhibitory or excitatory), and presynaptic $GABA_B$ receptors. This also explains why GABA and glutamate accumulation only occurs during ischemia and early after reperfusion but not long lasting. Thus, the present study provides a new way to explain the relationship among GABA, $GABA_A$ or $GABA_B$ receptors, and GABAergic synaptic transmission after ischemia.

The present study could not directly demonstrate that the facilitation of inhibitory synaptic transmission will reduce excitotoxicity and thus protect LA neurons, considering excitotoxicity is the major mechanism for postischemic neuronal death. Since LA neurons survive ischemia, they must have undergone little or no excitotoxicity. Previous studies from this group showed that LA neurons have depressed excitatory synaptic transmission (Pang et al., 2002) and depressed excitability (Deng et al., 2008) after ischemia. But we could not equal the depressed excitability to the depressed excitotoxicity. Whether facilitation of inhibitory synaptic transmission in LA neurons is directly linked to depressed excitotoxicity or by some other mechanisms still needs further investigation.

We demonstrated that the facilitation of inhibitory synaptic transmission by muscimol protects striatal neurons against ischemic insults. However, we could not prove the causal link between facilitation of inhibitory synaptic transmission and the selective survival of LA neurons after ischemia. The causal link is confirmative if LA neurons degenerate when the enhancement of inhibitory synaptic transmission in LA neurons is selectively blocked. However, it seems not feasible to only block synaptic transmission in LA neurons. Systemic or local

drug application of the $GABA_A$ receptor antagonist, like bicuculline, will affect a group of neurons and predispose them to epileptic activities, which will complicate the ischemia model.

Although we did not perform behavioral tests in this study, we observed that most of the rats displayed hunched back, hypomobility, twitches and tremors in 24 h after transient cerebral ischemia. Some of the rats showed body rotation. Since rats were sacrificed within 72 h, we could not examine long lasting neurological deficits (unpublished observations). In fact, muscimol's neuroprotective effects could be further corroborated by functional studies. Previous studies showed that sensorimotor dysfunctions occur mainly in 24-48 h after transient cerebral ischemia but no longer after that (Combs and D'Alecy, 1987; Gionet et al., 1991). If muscimol could improve the sensorimotor functions after ischemia, we would expect to see the ischemic rats with muscimol treatment show improvement in the tests of equilibrium and muscle strength, which are severely damaged in the rats in 24 h after global ischemia (Combs and D'Alecy, 1987; Gionet et al., 1991).

We used spontaneous IPSCs recordings to reveal the mechanisms for the enhancement of evoked IPSCs in LA neurons after ischemia. By doing so, we assumed that the vesicles fused spontaneously are from the same pool as the vesicles fused during stimulation. We also assumed that the release dynamics are the same for spontaneous release and stimulation-induced release. The last assumption we made was that spontaneous and stimulation-induced release activate the same postsynaptic structures. However, these assumptions are

greatly challenged by the recent progress made in neurotransmitter release. It is controversial whether the spontaneous release and evoked release are from the same pool. Research from Kavalali's group demonstrated that spontaneously endocytosed vesicles populate a reluctant/reserve pool that has little crosstalk with the activity-dependent pool in hippocampal synapses (Sara et al., 2005). Studies from other groups demonstrated that these two kinds of release are from the same pool (Groemer and Klingauf, 2007; Prange and Murphy, 1999). If these two kinds of release are from different pools, it is highly possible that the release dynamics are also different. Moreover, spontaneous release and evoked release activate different protein synthesis in the dendrites (Sutton et al., 2007; Sutton et al., 2004) thus differently affecting postsynaptic functions. It is reported that spontaneous and evoked glutamate release activates two populations of NMDA receptors with limited overlap (Atasoy et al., 2008). If these differences also existed in the GABAergic synaptic transmission in the striatum, it would be useless to explain the mechanisms for the enhancement of inhibitory synaptic transmission in LA neurons after ischemia by using the data from spontaneous and miniature IPSCs recording. Therefore, we should be cautious when explaining the data.

We observed an increased expression of GAD65 in the striatum by both immunohistochemical and western blotting analysis. We concluded that there is an increase in the number of synapses that highly express GAD65. As to the origin of this increased expression of GAD65, there are several possibilities. One is that it is originated from the terminals of the survived MS neurons or the MS

neurons that are not severely damaged (especially the ones in the dorsomedial striatum). Studies from Pulsinelli et al. systematically estimated the effect of duration of ischemia on the severity of ischemic neuronal damage. They found that twenty-minute of transient forebrain ischemia will induce neuronal damage in 50% of the striatal populations after 72 h of survival (Pulsinelli et al., 1982). The remaining 50% of the striatal neurons are still functional. Considering the majority of striatal neurons are MS neurons (97.7%) in the striatum (Rymar et al., 2004), most of the functional neurons after ischemia should be MS neurons (especially the ones in the dorsomedial striatum) (Pulsinelli et al., 1982), which might contribute to the increased expression of GAD65. The other possibility is that the survived GABAergic interneurons (GAD67 positive) might express GAD65 after ischemia and this should be confirmed by further investigations.

Since GAD65 is the key enzyme for the production of GABA, we speculated that presynaptic synthesis of GABA was increased and contributed to the increase of presynaptic GABA release. GAD67 is also a key enzyme for the synthesis of GABA. However, we focus on the expression of GAD65 due to the following difference between GAD65 and GAD67. GABA synthesized by GAD65 is mainly found in phasically active neurons and more likely to be involved in synaptic transmission. GABA synthesized by GAD67 is mainly found in the tonically active neurons and mostly provides a pool of GABA for general metabolic activity (Martin and Rimvall, 1993). However, the increased expression of GAD65 does not mean increased function. Or the increased expression is just to compensate for an actually depressed function of GAD65.

We don't know whether this increased expression of GAD65 in the striatum is transient or permanent since we only examined GAD65 expression in the rats at 1 day and 3 days after ischemia.

Future studies

During this study, we encountered many interesting questions. If possible, I would pursue the following projects.

1. The inhibitory inputs from GABAergic interneurons to LA neurons. Are there any inhibitory inputs from GABAergic interneurons to LA neurons? This has long been doubted though not substantiated yet (Bennett and Wilson, 1998; Koos and Tepper, 1999). GABAergic interneurons provide strong inhibitory inputs to MS neurons, which is demonstrated by both morphological (Bennett and Bolam, 1994; Kita et al., 1990) and electrophysiological studies (Koos and Tepper, 1999; Koos et al., 2004). However, there are almost no corresponding reports showing that GABAergic interneurons provide inhibitory inputs to LA neurons. 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). The synaptic connections between calretinin-containing GABAergic interneurons and LA

neurons are not reported. Therefore, neuropeptide Y-containing GABAergic interneurons are the candidates providing inhibitory inputs to LA neurons. The scarcity of LA neurons and GABAergic interneurons (2-3% of striatal population) makes it extremely difficult to catch their electrical connections. 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).

The present study highly suggests that GABAergic interneurons provide inhibitory inputs to LA neurons. Inhibitory synaptic transmission in LA neurons is enhanced at 24 h after transient cerebral ischemia, a time point at which MS neurons are severely damaged while GABAergic interneurons have survived. We speculated that the inhibitory inputs to LA neurons come from the survived GABAergic interneurons after ischemia. Considering the small inhibitory inputs to LA neurons under control condition (Bennett and Wilson, 1999; Chang and Kita, 1992), there are two possibilities. One is 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 of the molecular mechanisms implicated 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 postischemic LTP. The other possibility is that GABAergic interneurons establish inhibitory inputs onto LA neurons after ischemia as a result of synaptic reorganization.

To answer these questions, paired recordings of GABAergic interneurons and LA neurons are necessary. EM studies are also necessary to provide morphological evidence.

2. Presynaptic $GABA_A$ receptors in the striatum. The present study demonstrated that presynaptic $GABA_A$ receptors increase presynaptic GABA release after ischemia, which is sensitive to the extracellular calcium and voltage-gated sodium channels. This is concluded from electrophysiological data, for example, the PPR and the frequency of spontaneous IPSCs. Whether these receptors are located on the presynaptic terminals where GABA is released or not is unclear. It is known that synaptic activities in the dendrite and soma could control the generation of action potentials and the synaptic transmission that these action potentials initiate (Shu et al., 2006). Therefore, we speculated that the exact location of the presynaptic $GABA_A$ receptors could be the presynaptic terminal, soma, or even the dendrites. However, morphological studies, like post-embedding techniques, could not unequivocally localize the $GABA_A$ receptors to the pre- or postsynaptic structures due to the distortion between the membrane from the whole thickness of the section and the most superficial layer of the membrane available for the antibody (Fujiyama et al., 2000).

We found that muscimol's effect on neurotransmitter release is inhibitory in control but facilitative after ischemia. It is speculated that this is due to the increase of intraterminal chloride concentration after ischemia, which results in a more depolarized reversal potential of chloride. To confirm this speculation, it is necessary to measure chloride concentration in the presynaptic terminals. One way is to perform perforated-patch recording, in which intracellular chloride concentration could be derived from its reversal potential. The other way is to use chloride indicator to get an estimate of chloride concentration from the optical signals. However, both methods could only estimate the chloride concentration in cell bodies due to the fine structures of axons (except the calyx of Held). The chloride concentration in the terminals might be different from that of the soma. In the mammalian central nervous system, the calyx of Held has a higher chloride concentration than that in the soma as demonstrated by perforated-patch recording (Price and Trussell, 2006; Turecek and Trussell, 2001). Therefore, it would be a great challenge to confirm both the location of presynaptic $GABA_A$ receptors and the chloride concentration in presynaptic terminals.

3. The electrophysiological properties of GABAergic interneurons after ischemia. GABAergic interneurons provide feedforward inhibition in the striatum via their strong inhibitory inputs onto MS neurons. The excitability of GABAergic interneurons greatly affects not only the inhibitory outputs from the striatum but also the inhibitory microcircuits in the striatum. Studies from striatal slices show that fast-spiking GABAergic interneurons have relatively negative resting

membrane potential (-80 mV) and low input resistance (less than 140 MΩ). They could fire action potentials only upon strong and constant depolarization. But they could be synchronized to fire action potentials since they are connected to each other by gap junctions. LTS GABAergic interneurons have depolarized resting membrane potential (-50 to -60 mV) close to action potential threshold and very high input resistance (greater than 500 MΩ) (Kawaguchi, 1993). However, whether their electrophysiological characters would change after ischemia remains unknown.

The present study found that muscimol, by bath application, activates presynaptic $GABA_A$ receptors and increases GABA release after ischemia, which is dependent on the extracellular calcium and voltage-gated sodium channels. However, we could not exclude the possibility that inhibitory network properties in the striatum are changed or they are modified by muscimol after ischemia, leading to the phenomena we observed. Since most of the GABAergic interneurons survive ischemic insults (Chesselet et al., 1990; Pulsinelli et al., 1982), any changes in the excitability of these neurons, would significantly change the inhibitory network properties in the striatum after ischemia. Other studies found that chronic dopamine deprivation could change firing properties in a subgroup of striatal GABAergic interneurons, thus change the IPSCs in MS neurons (Dehorter et al., 2009). The main obstacle to study GABAergic interneurons after ischemia is the scarcity of these neurons in the striatum. The identification of these neurons could be benefited from neurobiotin staining after successful recordings.

Overall summary

This study demonstrated that inhibitory synaptic transmission was enhanced in ischemia-resistant LA neurons at 3 h and 24 h after ischemia while depressed in ischemia-vulnerable MS neurons at 8 h after ischemia. The enhancement observed in LA neurons was due to the increased presynaptic GABA release. There are two mechanisms contributing to the increased GABA release. One is that the activation of presynaptic $GABA_A$ receptors increased presynaptic GABA release after ischemia. The other is the enhanced expression of GAD. Facilitation of inhibitory synaptic transmission by muscimol protected striatal neurons against ischemic insults. Therefore, the enhancement in inhibitory synaptic transmission might reduce excitotoxicity and contribute to the selective survival of LA neurons after ischemia.

TABLES

	Percentage	Immunohistochemical characters	Firing properties
MS neurons (GABAergic)	97.7%	GAD65, Calbindin, DARPP-32, Substance P, Dynorphin, Enkephalin	Up and down states
GABAergic interneurons	2%	1.PV, GAD67 2.NPY, SOM, NOS 3.Calretinin, GAD67	1.Fast-spiking 2.Low-threshold spike 3.Unknown
LA (cholinergic) interneurons	0.3%	CHAT, Acetylcholinesterase	Broad action potentials, large-amplitude and long-duration afterhyperpolarizations
Dopaminergic neurons	Very few	TH, DAT	Unknown

Table 1 A table summarizes the percentage, immunohistochemical characters, and firing properties for each type of the striatal neurons.

PPR	↓	Presynaptic release probability ↑
Spontaneous IPSCs	Frequency ↑	Presynaptic release ↑
Miniature IPSCs	Mean Amplitude ↓	Postsynaptic response ↓
Currents induced by exogenous GABA	↓	Postsynaptic response ↓

Table 2 Both pre- and postsynaptic mechanisms are involved in the alteration of inhibitory synaptic transmission in LA neurons after ischemia. Presynaptically, GABA release is increased as indicated from PPR and spontaneous IPSCs. Postsynaptically, $GABA_A$ receptors show depressed responses at 24 h after ischemia as indicated from miniature IPSCs and currents induced by exogenous GABA application. But in all, the presynaptic effects far exceed the postsynaptic effects, which results in the facilitation of inhibitory synaptic transmission after ischemia.

	Control	Ischemia
evoked IPSCs	No change	↑
PPR	No change	↓
Spontaneous IPSCs	Frequency ↓ Amplitude ↑	Frequency ↑ Ca^{2+} & TTX-sensitive
Conclusion	Presynaptic release ↓	Presynaptic release ↑

Table 3 Muscimol application results in differential effects in control LA neurons and LA neurons after ischemia. It decreases presynaptic GABA release in control while facilitates presynaptic GABA release after ischemia.

FIGURES

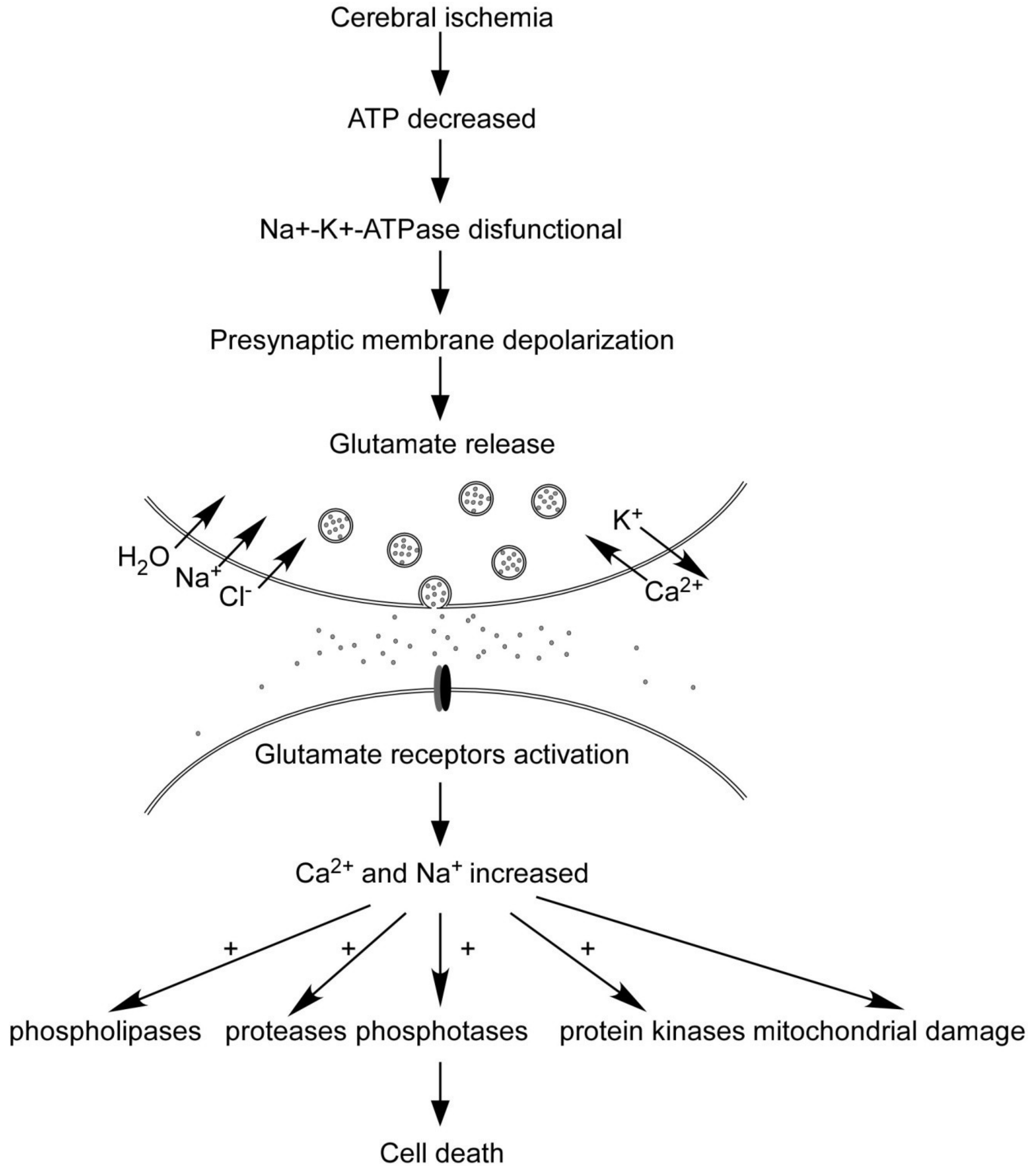

Figure 1 Postischemic neuronal death and excitotoxicity. Excessive glutamate release during ischemia could excite a neuron to death.

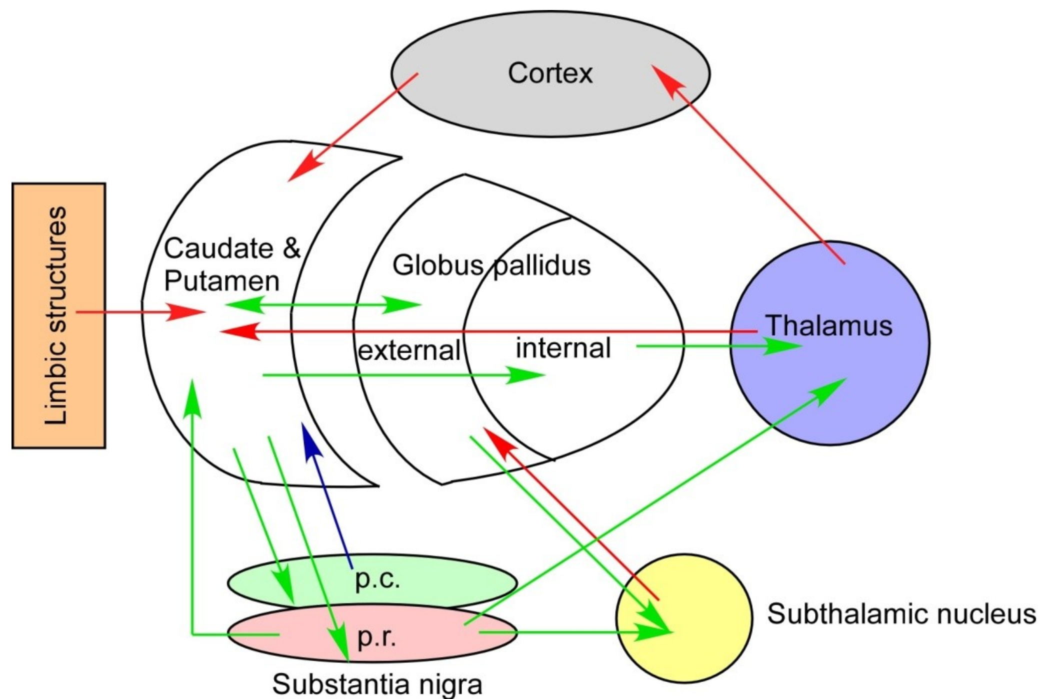

Figure 2 Functional connections of the basal ganglia with the cortex and other brain structures. In the basal ganglia, striatum is the major input nucleus. The internal segment of globus pallidus and the substantia nigra pars reticulate (p.r.) are the two major output nuclei. The information flowed through the striatum finally goes back to the thalamus and cerebral cortex, where the striatum originally receive the inputs from. P.c. represents substantia nigra pars compacta. Red arrows indicate glutamatergic terminals; green arrows indicate inhibitory terminals; blue arrows indicate dopaminergic terminals.

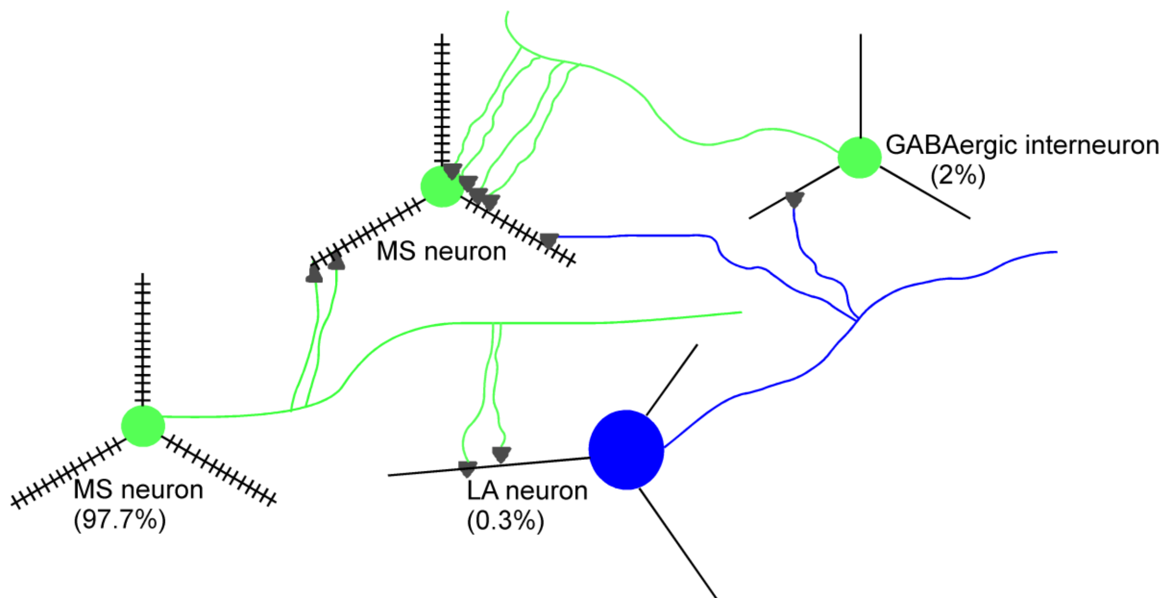

Figure 3 The schematic drawing of the microcircuits in the striatum. MS neurons provide inhibitory inputs to LA neurons and other MS neurons. GABAergic interneurons provide strong inhibitory inputs to MS neurons. LA neurons provide cholinergic inputs to MS neurons and GABAergic interneurons. The percentage for each type of neuron in the striatal population is represented in the brackets. Crossed straight lines represent spiny dendrites; straight lines represent aspiny dendrites; curved lines represent axons. Green represents GABAergic soma or axons; blue represents cholinergic soma or axons.

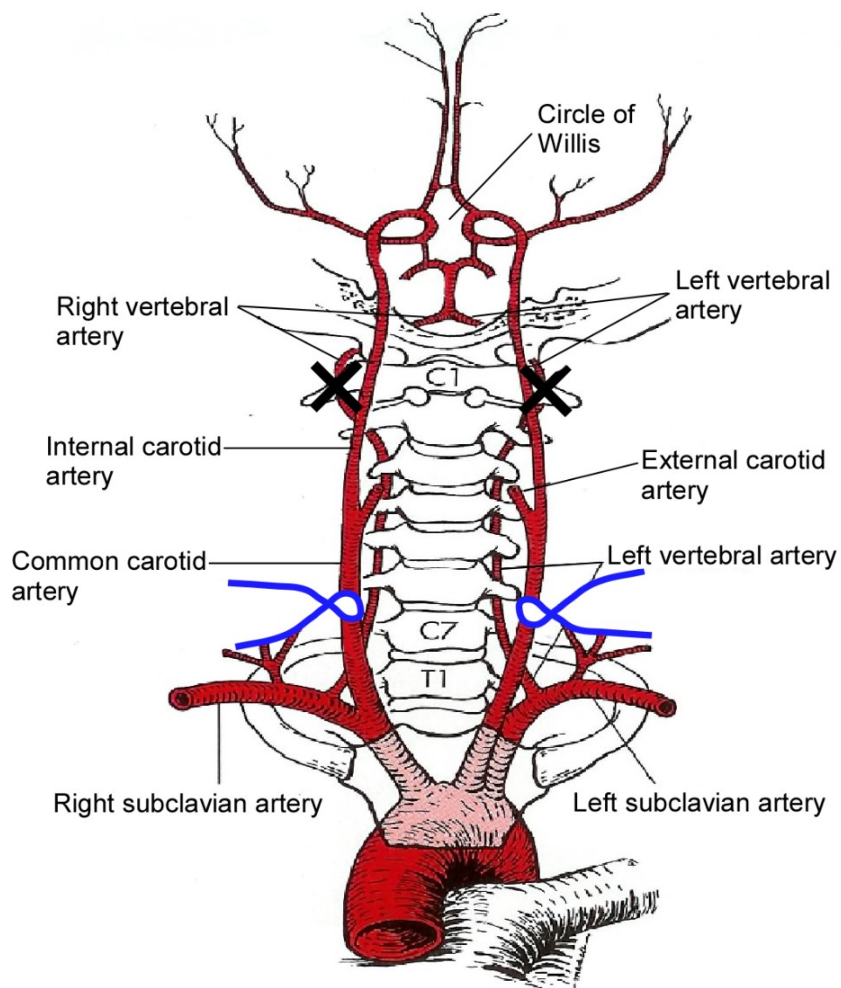

Figure 4 The schematic drawing (modified from Neuroanatomy, fifth edition, by Haines DE) of four-vessel occlusion model used in this project. Two vertebral arteries were electrocauterized (represented by the black crosses) at the C1 level and the two common carotid arteries were occluded (represented by the blue lasso) with the tubing.

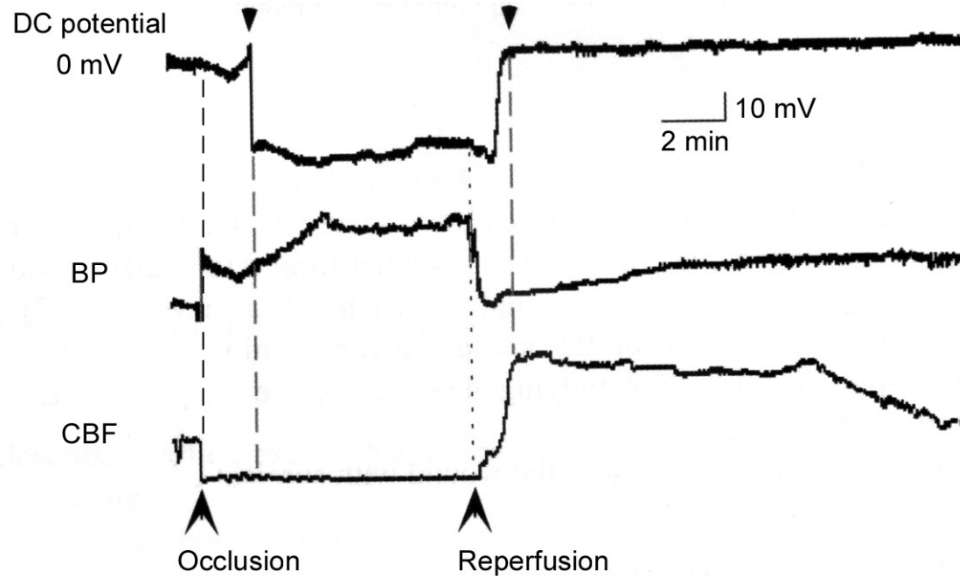

Figure 5 Sample traces of DC potential, blood pressure (BP), and cerebral blood flow (CBF) recording during the occlusion of carotid arteries and reperfusion. DC potential was zeroed before occlusion. After occlusion, it took about 2 min for the DC potential to reach -20 mV. BP immediately increased while CBF decreased after occlusion. The occlusion lasted 20 min, after which DC potential and BP returned to the pre-occlusion value in around 2 min.

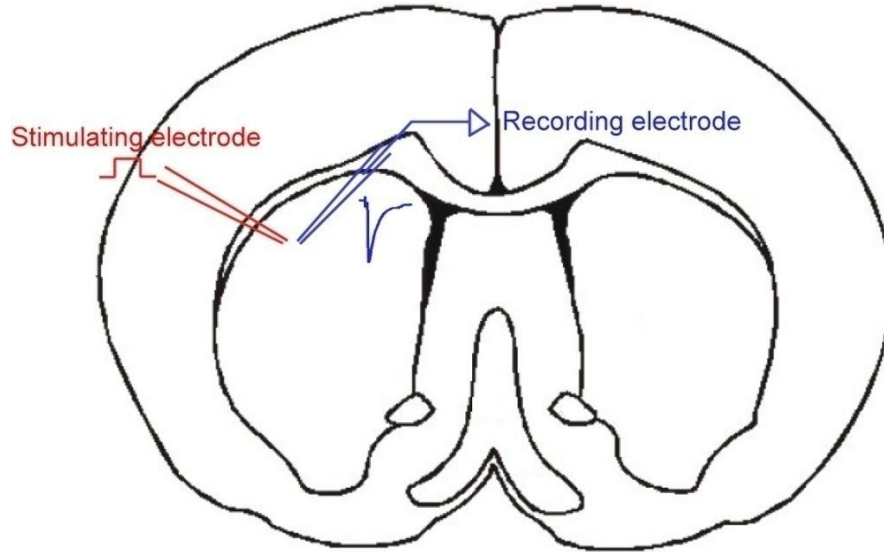

Figure 6 Representative image showing the intrastriatal stimulation used in this study. Stimulating electrode was placed 150-200 μm subjacent to the recording electrode.

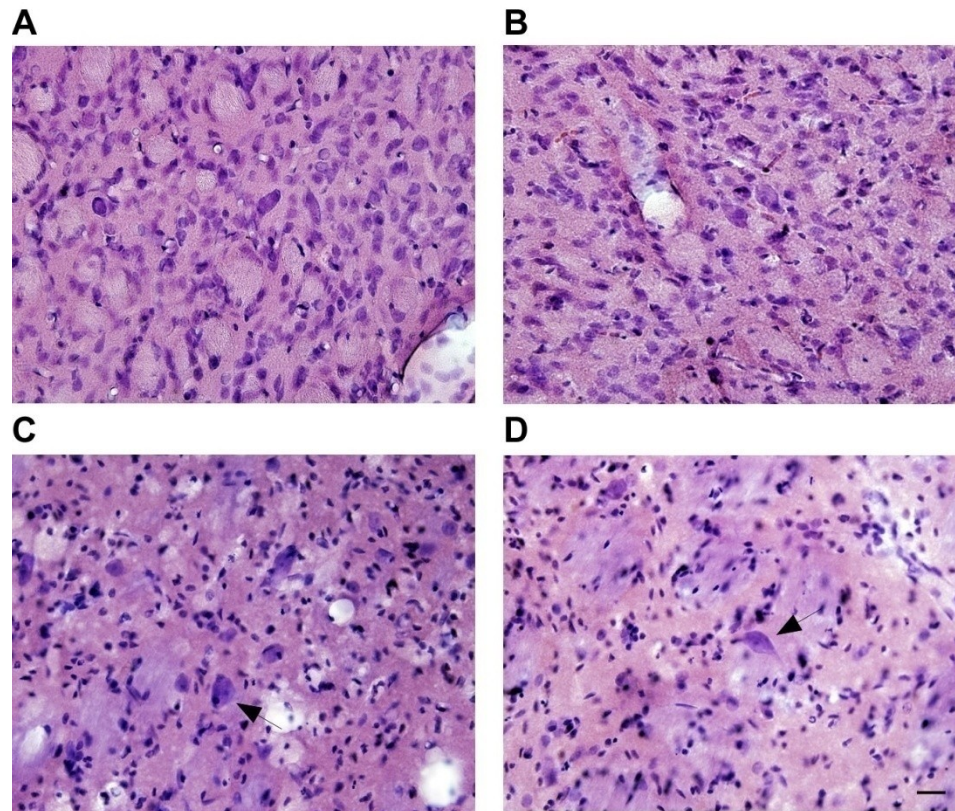

Figure 7 Striatal cells show morphological changes after transient cerebral ischemia. (A) HE staining of the striatal slice (50 μm) from the control rats. LA neurons were recognized by the large soma size among the other medium-sized neurons. (B) HE staining of the striatal slice from the rats at 3 h after ischemia. Most of the neurons remained normal morphology. HE staining of striatal slices from the rats at 24 h (C) and 72 h (D) after ischemia. Most of the striatal cells exhibited pyknotic, shrunken nuclei while LA neurons remained morphologically normal (arrows). Scale bar, 25 μm.

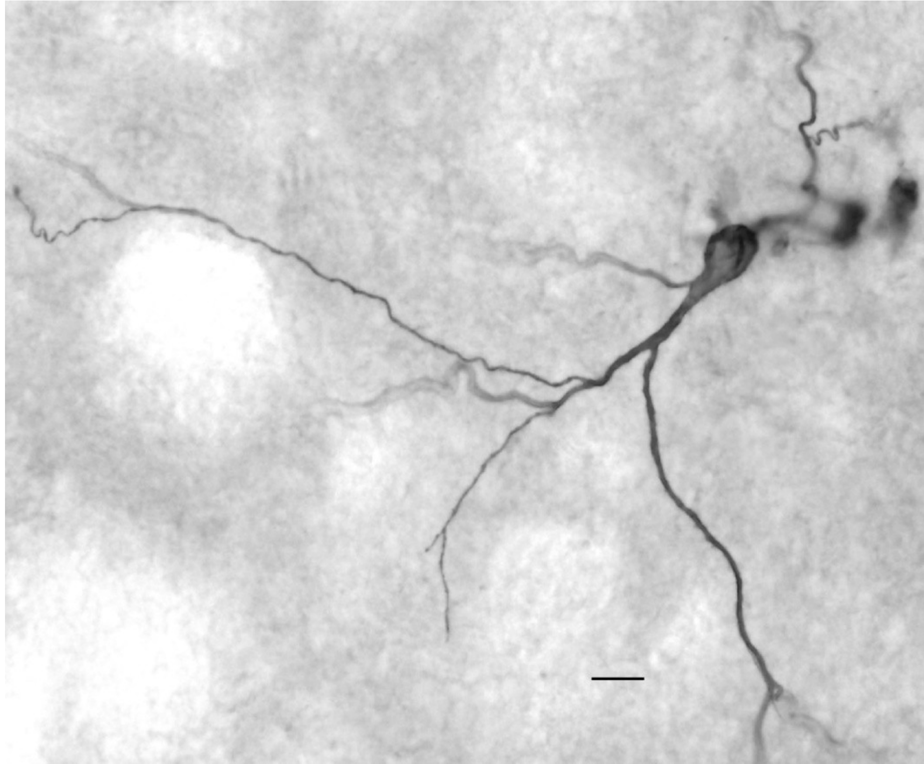

Figure 8 Photograph of one LA neuron intracellularly stained with neurobiotin showing few spines on the dendrites. The longest diameter is around 50 μm for this neuron. Scale bar represents 40 μm.

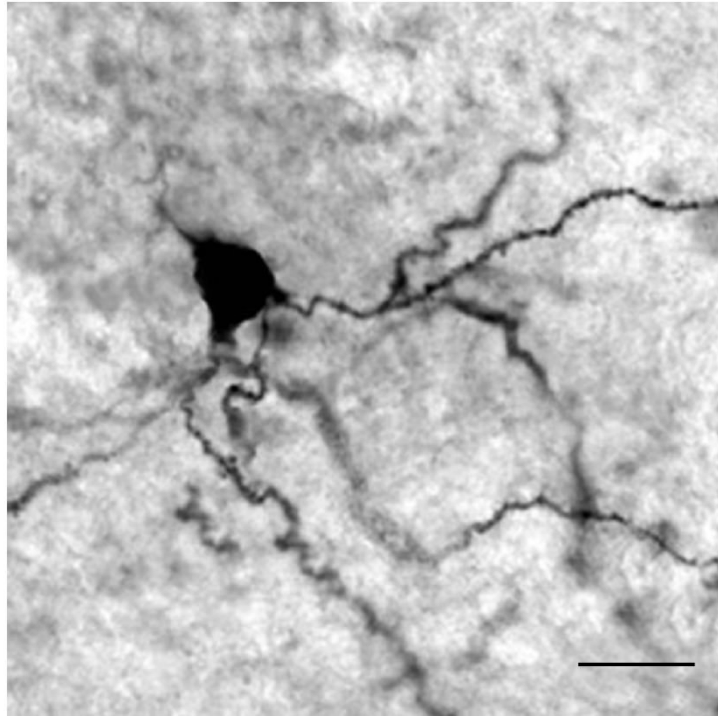

Figure 9 Photograph of one MS neuron intracellularly stained with neurobiotin showing spines on the dendrites. The diameter is around 15 μm for this neuron. Scale bar represents 20 μm.

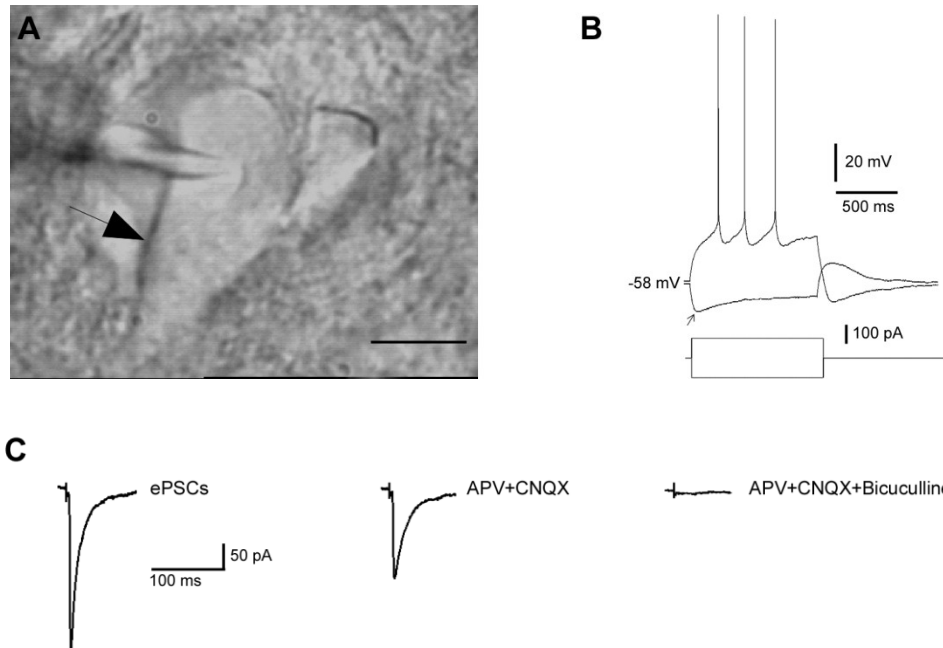

Figure 10 Identification of LA neurons. (A) An infrared-DIC image of the striatal slice showing one LA neuron (arrow) and one adjacent medium-sized neuron. Scale bar represents 25 μm. (B) Characteristic responses of one LA neuron to hyperpolarizing and depolarizing current pulses under the current-clamp recording. Prominent afterhyperpolarizations after evoked action potentials and the depolarizing sag (arrow) upon hyperpolarizing pulses could be observed. (C) Representative traces of evoked postsynaptic currents (ePSCs), currents recorded after the application of APV+CNQX, APV+CNQX+bicuculline, respectively.

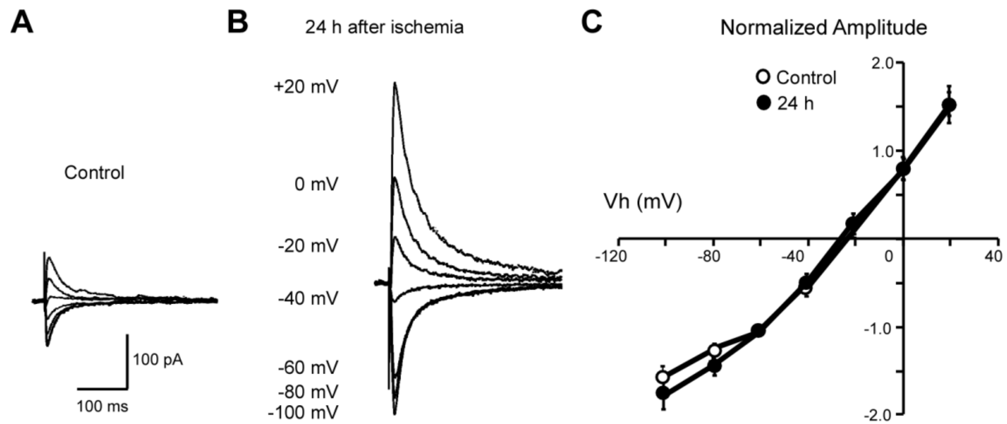

Figure 11 The I-V curves for LA neurons before and after ischemia. The I-V curves showed that the reversal potential of evoked IPSCs was not changed at 24 h after ischemia, indicating that the intracellular chloride concentration in LA neurons was not significantly changed at 24 h after ischemia.

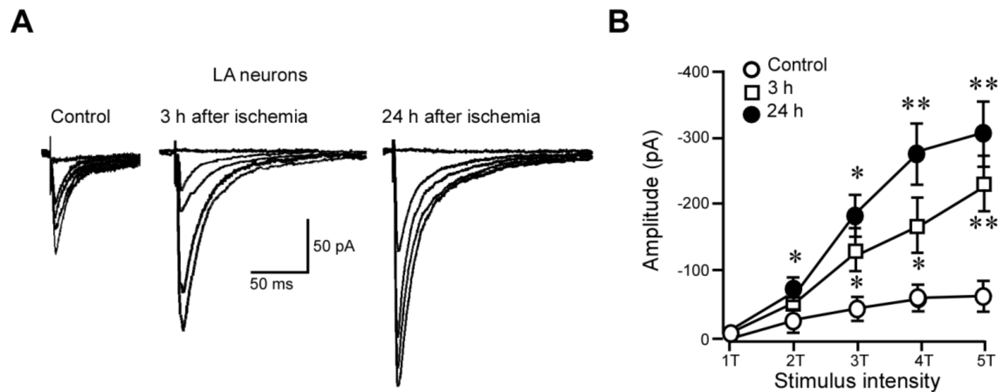

Figure 12 Inhibitory synaptic transmission is enhanced in LA neurons after transient cerebral ischemia. (A) Representative traces of evoked IPSCs (1T-5T) in LA neurons of the control and at 3 h, 24 h after ischemia. All the traces are averages of 8 consecutive recordings. (B) Grouped data (1T-5T) reveal that the amplitude of evoked IPSCs in LA neurons was significantly facilitated at 3 h and 24 h after ischemia. The values of the plotting are mean±SEM. 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.

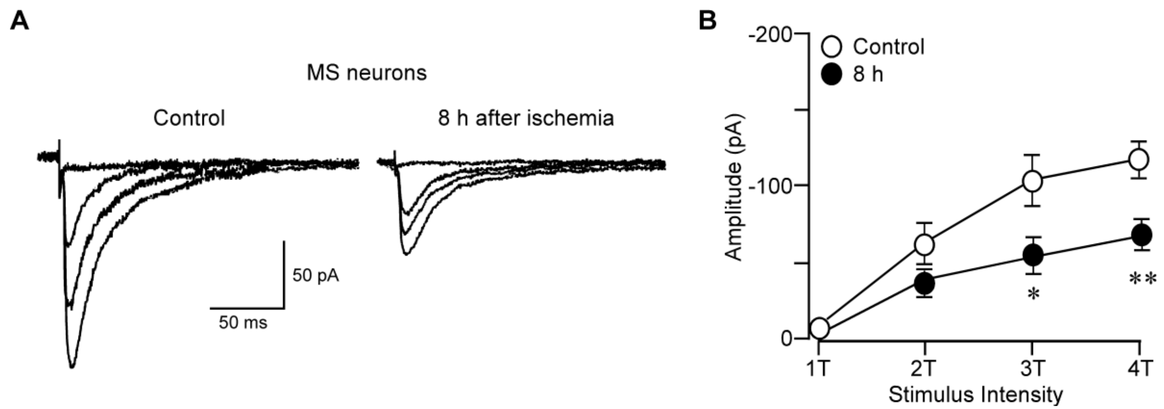

Figure 13 Inhibitory synaptic transmission is depressed in MS neurons after transient cerebral ischemia. (A) Representative traces of evoked IPSCs (1T-4T) in MS neurons of the control and at 8 h after ischemia. (B) Grouped data (1T-4T) reveal that the amplitude of evoked IPSCs in MS neurons was significantly depressed at 8 h after ischemia.

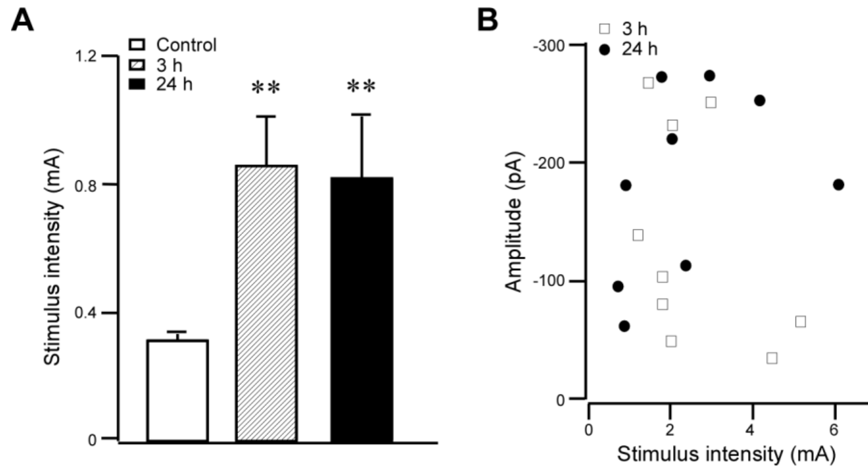

Figure 14 The threshold of stimulus intensity for LA neurons is increased after ischemia but not associated with the increased amplitudes of evoked IPSCs. (A) Bar graph shows the threshold of stimulus intensities before and after ischemia. (B) Scattered plot shows that the absolute values of 3T were not necessarily associated with the amplitudes of evoked IPSCs at 3 h and 24 h after ischemia.

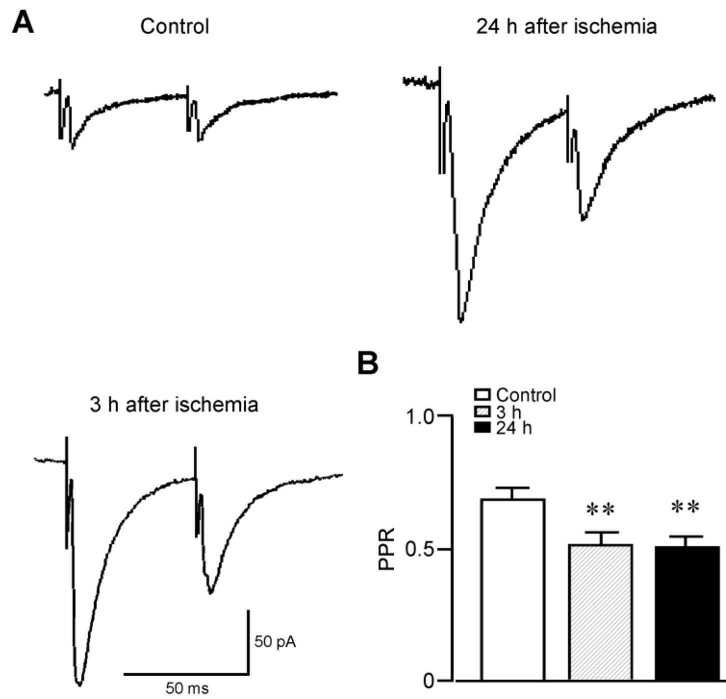

Figure 15 Presynaptic release of GABA is increased after ischemia. (A) Representative traces of paired-pulse tests at 3T in LA neurons. The traces are averages of 10 consecutive recordings. (B) Grouped data show that PPR was significantly decreased at 3 h and 24 h after ischemia.

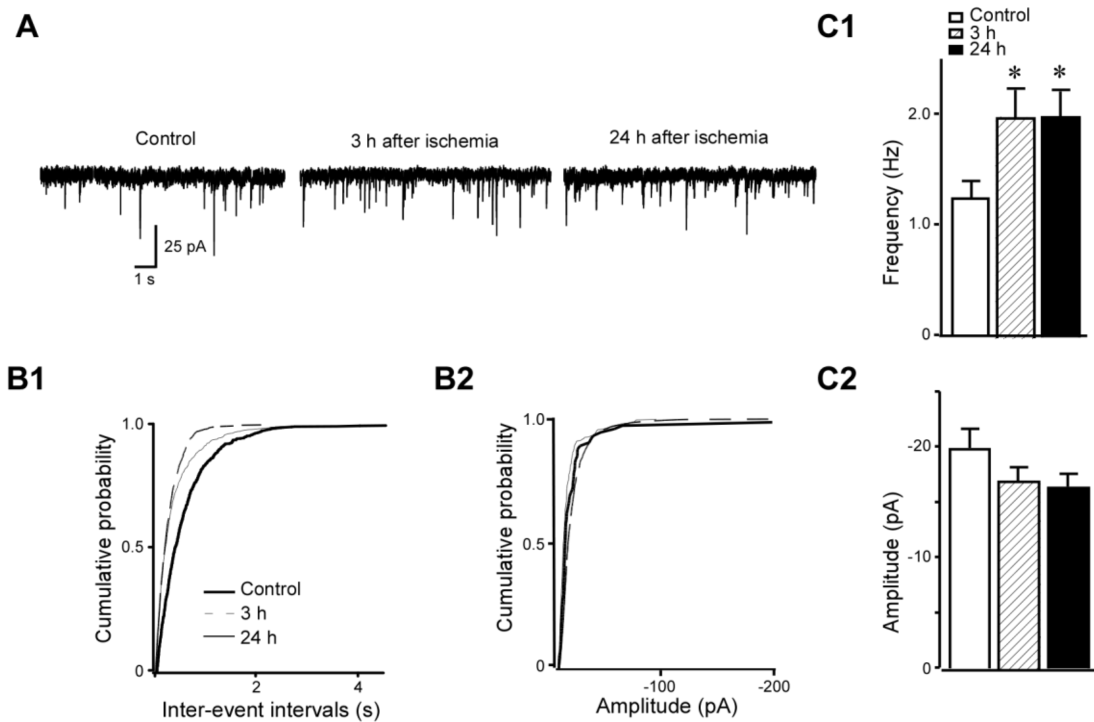

Figure 16 The frequency of spontaneous IPSCs is increased after ischemia. (A) Representative traces of spontaneous IPSCs from the control, 3 h, and 24 h after ischemia. (B1) Cumulative distributions of inter-event intervals from the same neurons shown in A. The curve for cumulative probability of inter-event intervals showed a leftward shift at 3h and 24 h after ischemia (K-S test, P<0.05). (B2) Cumulative distributions of current amplitudes from the same neurons shown in A. The curve for cumulative probability of current amplitudes showed no change after ischemia. (C1) Bar graph shows that the frequency of spontaneous IPSCs was significantly increased after ischemia. (C2) Bar graph shows that there was no significant change in the amplitude of spontaneous IPSCs.

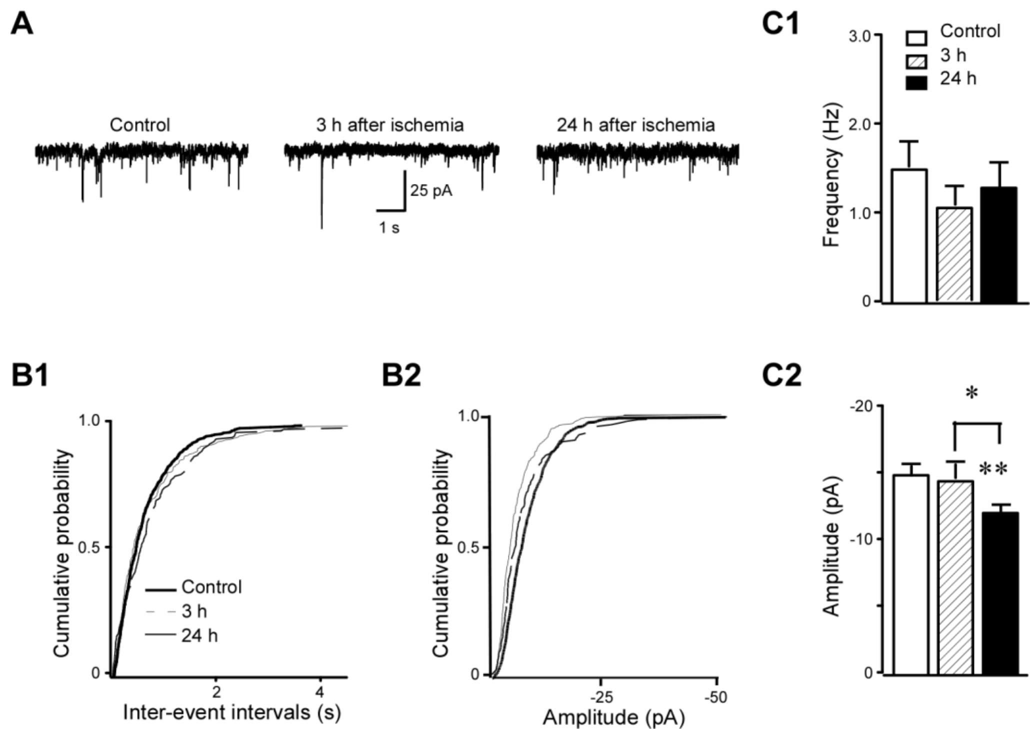

Figure 17 The mean amplitude of miniature IPSCs is decreased at 24 h after ischemia. (A) Representative traces of miniature IPSCs from the control, 3 h, and 24 h after ischemia. (B) Cumulative distributions of inter-event intervals from the same neurons shown in A. The curve for cumulative probability of inter-event intervals showed no change. (B2) 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, $P<0.05$). (C1) Bar graph shows that there was no significant change in the frequency of miniature IPSCs. (C2) Bar graph shows that the amplitude of

miniature IPSCs at 24 h after ischemia was significantly decreased compared to the control and 3 h after ischemia.

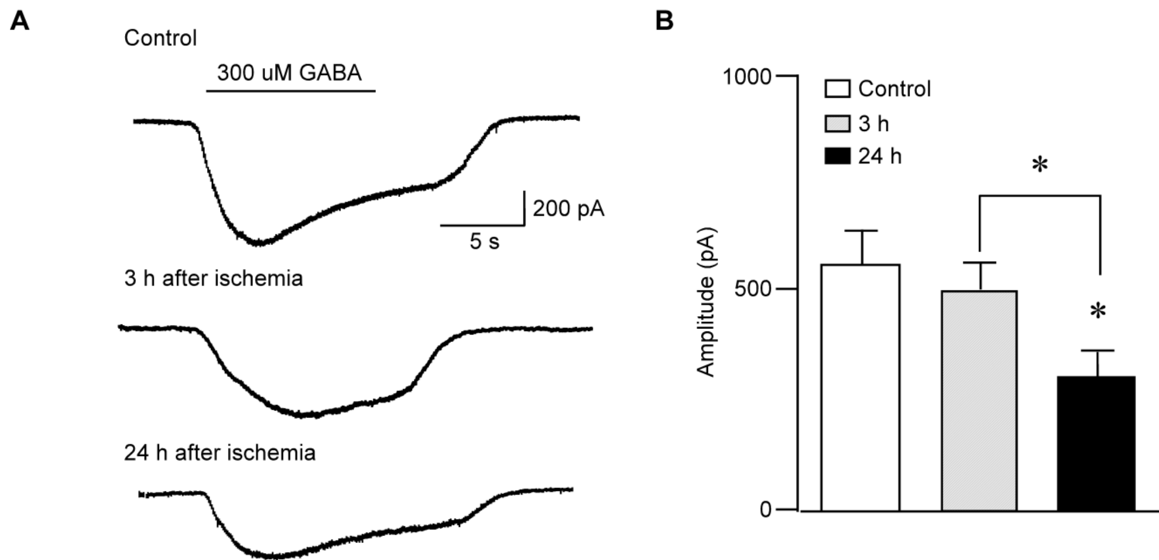

Figure 18 Postsynaptic responses to GABA are depressed at 24 h after ischemia. (A) Representative traces showing LA neuron's response to exogenous GABA application (300 μM) in the control and after ischemia. (B) Grouped data show that the currents induced by exogenous GABA application at 24 h after ischemia were greatly reduced compared to the control and 3 h after ischemia.

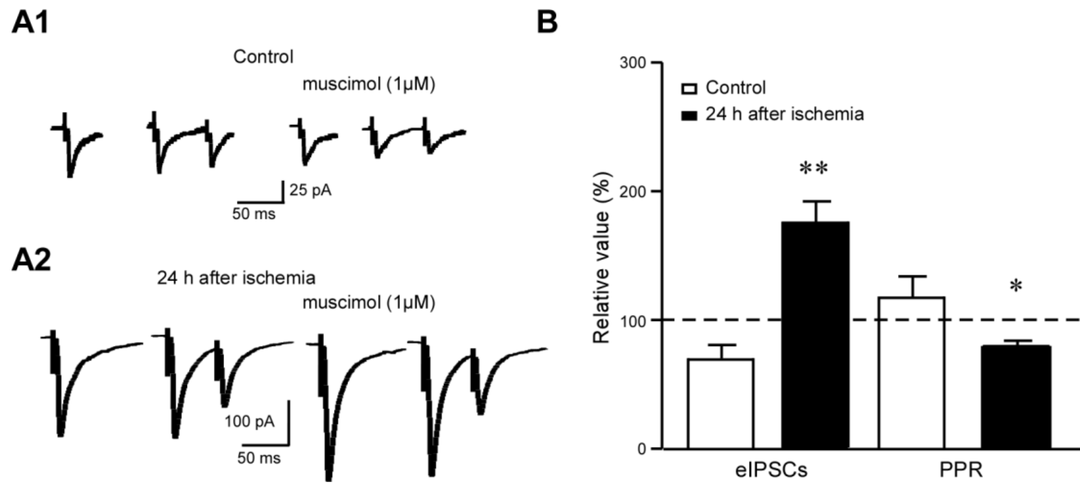

Figure 19 Muscimol enhances inhibitory synaptic transmission in LA neurons after ischemia. Representative traces of evoked IPSCs and PPR before and after muscimol application in the control neurons (A1) and neurons at 24 h after ischemia (A2). (B) Grouped data show that muscimol increased evoked IPSCs and decreased PPR in LA neurons after ischemia. Muscimol did not significantly change evoked IPSCs and PPR in the control LA neurons. All columns were normalized to the pre-muscimol application value and are thus called relative value (dashed lines).

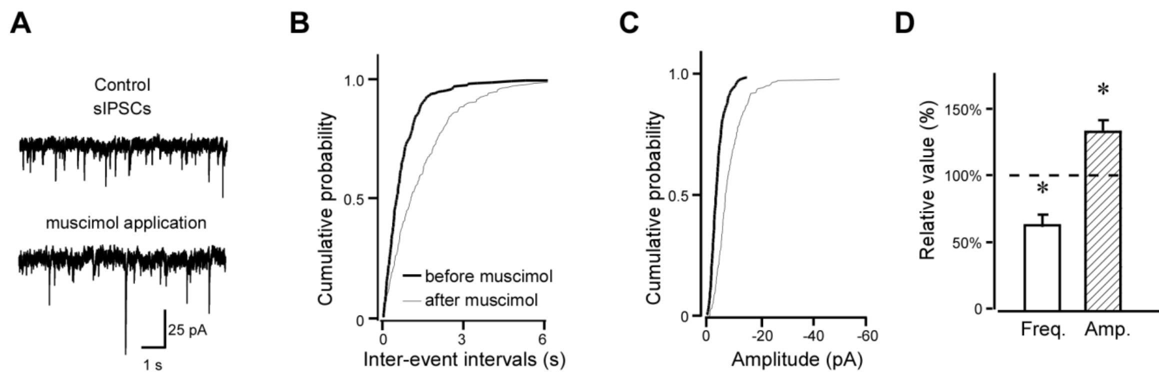

Figure 20 Muscimol decreases presynaptic GABA release in the control LA neurons. (A) Representative traces of spontaneous IPSCs before and after muscimol application in the control LA neurons. (B) Cumulative distributions of inter-event intervals and current amplitudes showed a rightward shift after muscimol application from the same neuron shown in A (K-S test, P<0.05). (C) Cumulative distributions of current amplitudes showed a rightward shift after muscimol application from the same neuron shown in A (K-S test, P<0.05). (D) Grouped data show that the frequency of spontaneous IPSCs was decreased while the amplitude of spontaneous IPSCs was increased in the control LA neurons after muscimol application. All columns were normalized to the pre-muscimol application value (dashed lines).

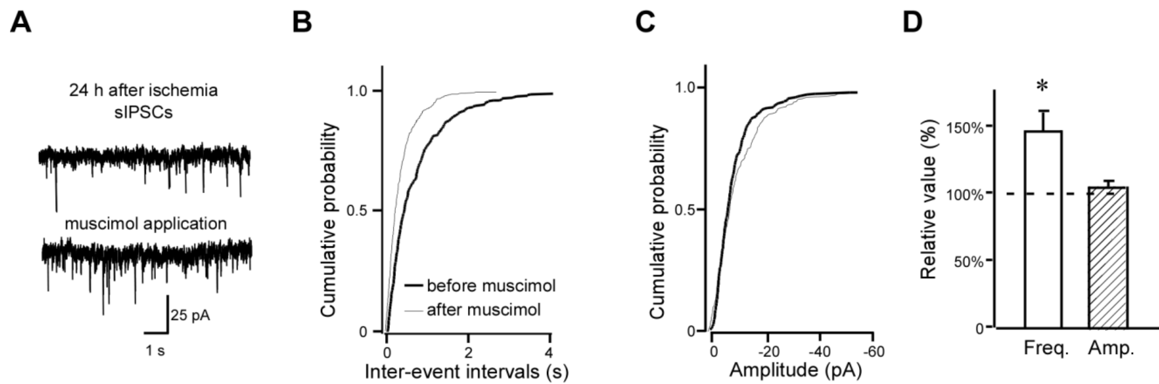

Figure 21 Muscimol increases presynaptic GABA release in LA neurons after ischemia. (A) Representative traces of spontaneous IPSCs before and after muscimol application after ischemia. (B) Cumulative distributions of inter-event intervals showed a leftward shift after muscimol application (K-S test, P<0.05) before and after muscimol application from the same neuron shown in A. (C) Cumulative distributions of current amplitudes showed no change after muscimol application from the same neuron shown in A. (D) Grouped data show that the frequency of spontaneous IPSCs was increased and the amplitude of spontaneous IPSCs didn't change in LA neurons after ischemia upon muscimol application. All columns were normalized to the pre-muscimol application value (dashed lines).

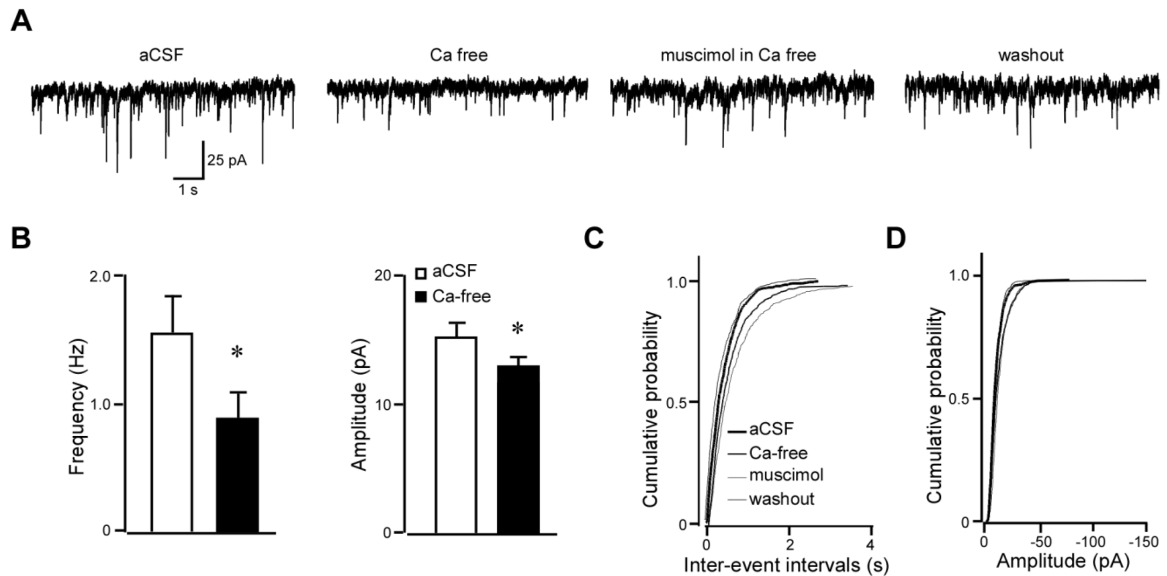

Figure 22 Muscimol's effect on inhibitory synaptic transmission after ischemia is dependent on extracellular calcium. (A) Representative traces of spontaneous IPSCs in the aCSF, Ca^{2+}-free aCSF, after muscimol application (in the Ca^{2+}-free aCSF) and washout in the same LA neuron after ischemia. (B) Bar graph shows that the frequency and amplitude of spontaneous IPSCs were greatly reduced in the Ca^{2+}-free aCSF. (C) Cumulative distributions for inter-event intervals from the same neuron in A before and after muscimol application in the Ca^{2+}-free aCSF. (D) Cumulative distributions for current amplitudes from the same neuron in A before and after muscimol application in the Ca^{2+}-free aCSF.

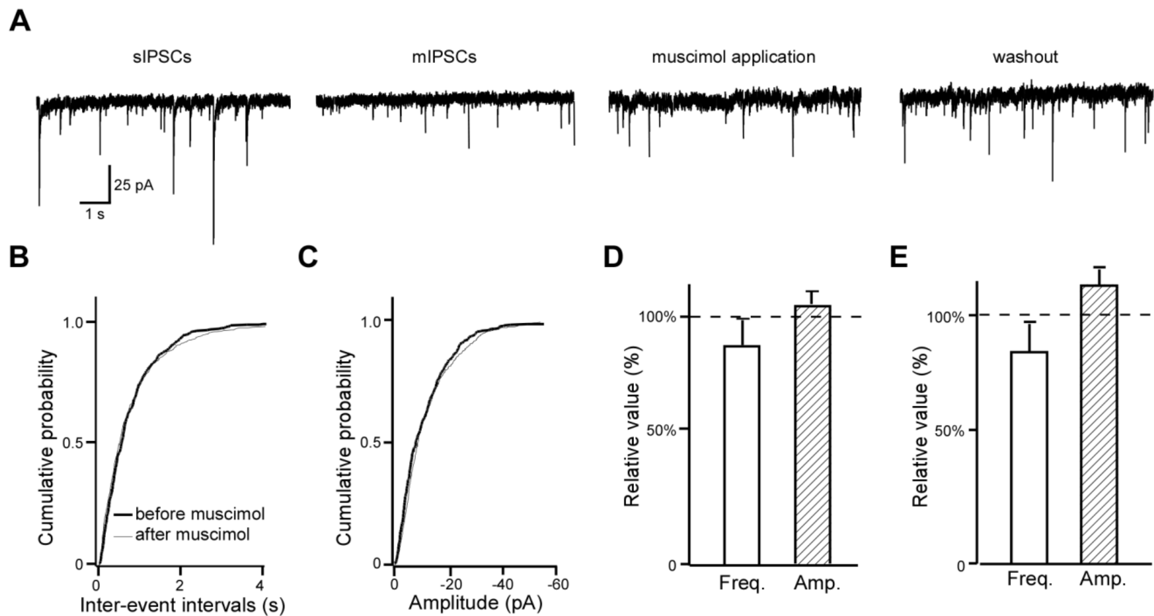

Figure 23 Muscimol's effect on inhibitory synaptic transmission after ischemia is dependent on the voltage-gated sodium channels. (A) Representative traces of spontaneous IPSCs, miniature IPSCs, miniature IPSCs after muscimol application and washout in the same LA neuron after ischemia. (B) Cumulative distributions for inter-event intervals of miniature IPSCs from the same neuron in A before and after muscimol application. (C) Cumulative distributions for current amplitudes of miniature IPSCs from the same neuron in A before and after muscimol application. (D) Grouped data show that muscimol did not change the frequency and amplitude of spontaneous IPSCs in the Ca^{2+}-free aCSF. All columns were normalized to the pre-muscimol application value (dashed lines). (E) Grouped data show that muscimol did not change the frequency and

amplitude of miniature IPSCs. All columns were normalized to the pre-muscimol application value (dashed lines).

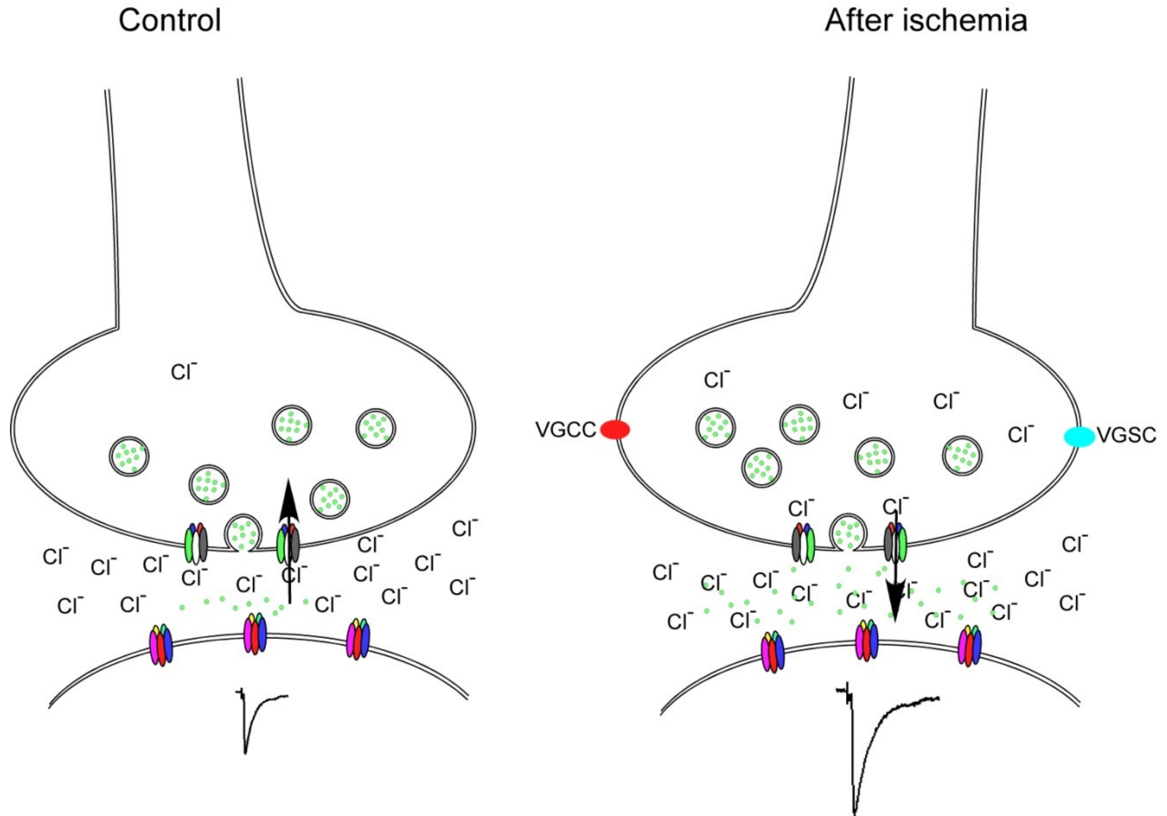

Figure 24 A cartoon shows the presynaptic $GABA_A$ receptors' role in the modulation of inhibitory synaptic transmission in control and after ischemia. In the control condition, intracellular chloride concentration is low and the reversal potential for chloride is more negative than the resting membrane potential. Following the activation of presynaptic $GABA_A$ receptors, there will be a chloride influx and membrane hyperpolarization. After ischemia, intracellular chloride concentration is increased and the reversal potential for chloride is more positive than the resting membrane potential. Following the activation of presynaptic $GABA_A$ receptors, there will be a chloride efflux and membrane depolarization,

which will activate voltage-gated sodium (VGSC) and calcium channels (VGCC). Thus, presynaptic transmitter release is increased and a bigger postsynaptic response occurs.

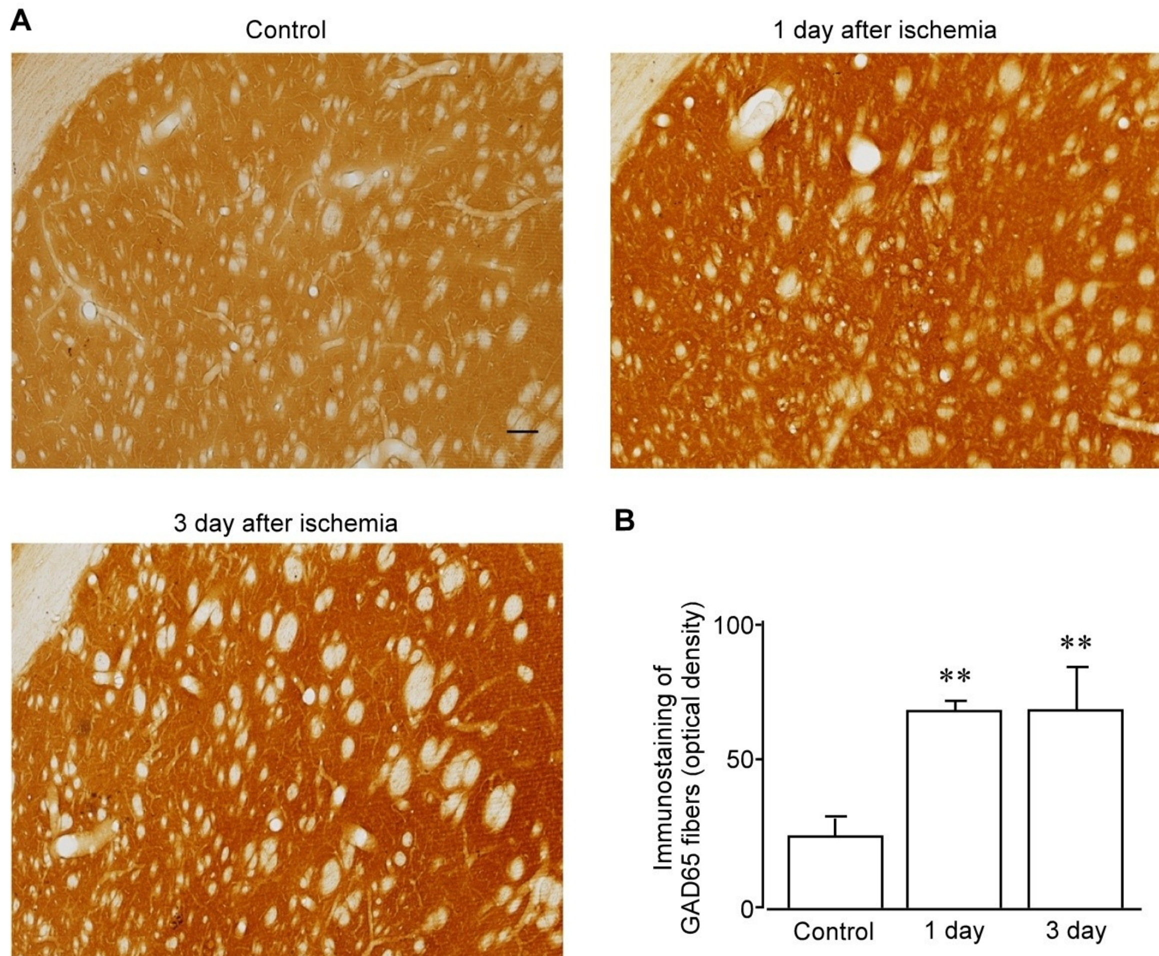

Figure 25 The immunoreactivity of GAD65 is increased in the striatum after transient cerebral ischemia. (A) Representative images of GAD65 immunostaining from the dorsolateral striata of control, 1 day, and 3 days after ischemia. Images were taken under 100×. Scale bar, 100 μm. (B) The bar graph shows a significant increase in the optical density of GAD65 immunoreactivity after ischemia.

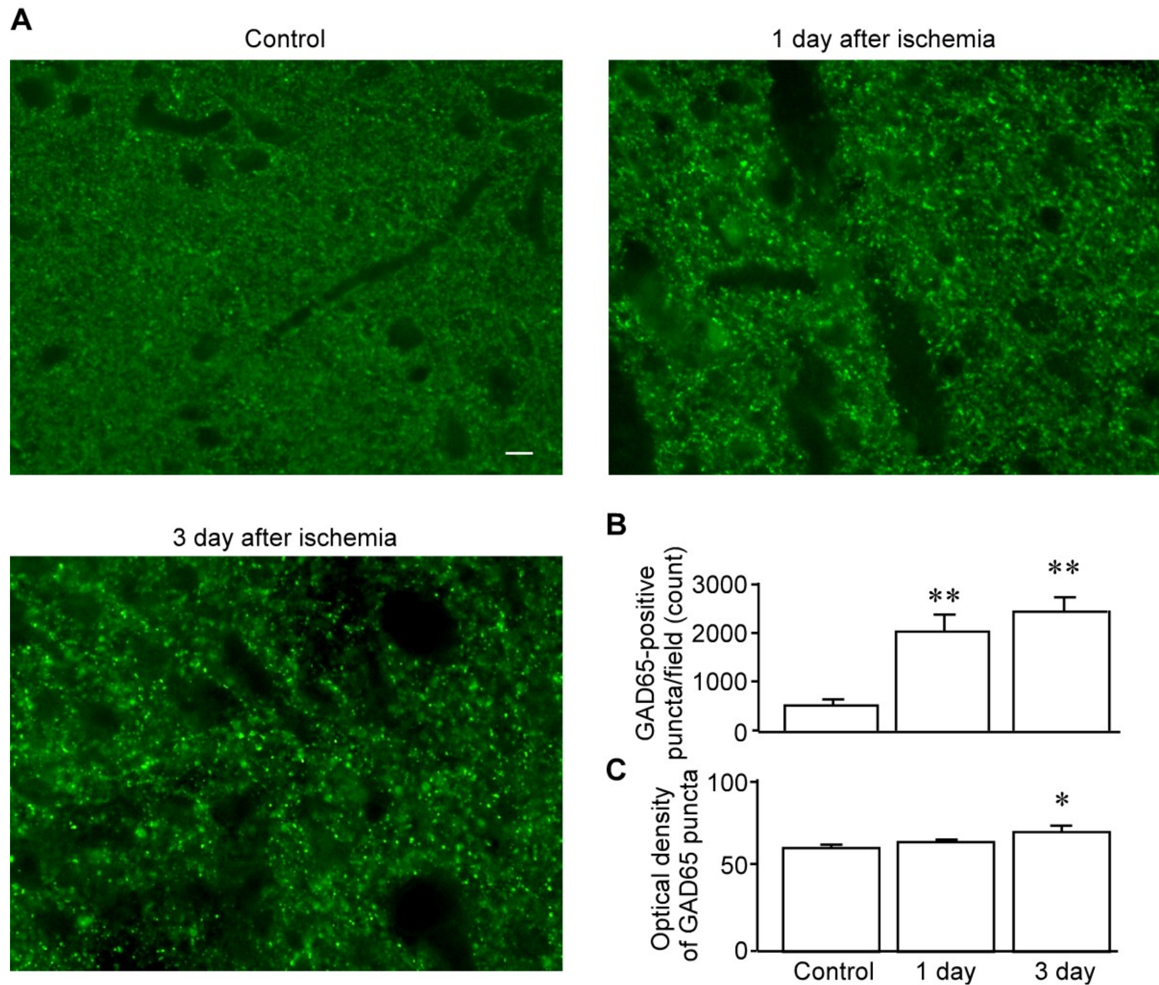

Figure 26 The immunoreactivity of GAD65 is increased in the striatum after transient cerebral ischemia. (A) Representative images of GAD65 (green) after immunofluorescence staining from the dorsolateral striata of control, 1 day, and 3 days after ischemia. Images were taken under 400×. Scale bar, 10 μm. (B) The bar graph shows a significant increase in the number of GAD65-positive puncta after ischemia. (C) The bar graph shows a significant increase in the optical density of the GAD65-positive puncta at 3 days after ischemia.

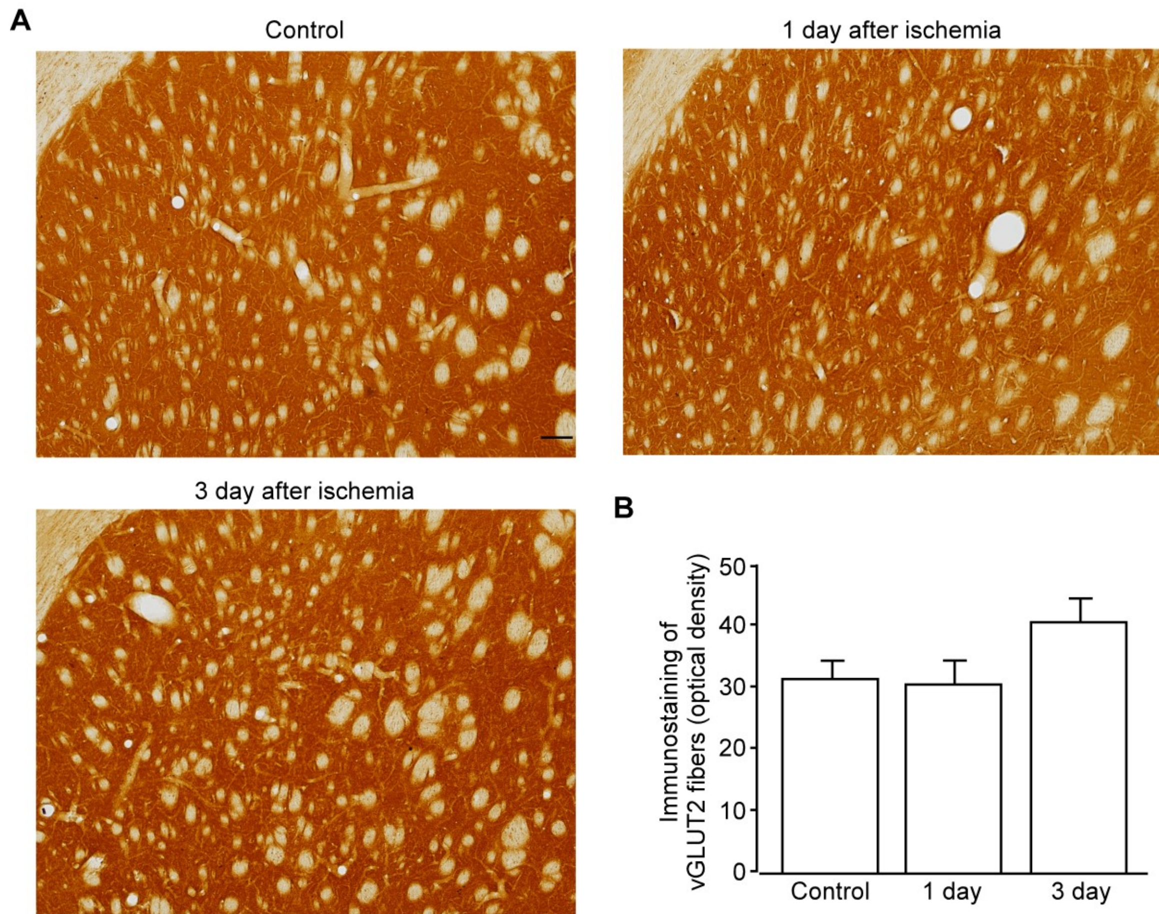

Figure 27 The increase in the immunostaining of GAD65 is not due to the ischemic injury. (A) Representative images of vGLUT2 immunostaining from the striata of control, 1 day, and 3 days after ischemia. Images were taken under 100×. Scale bar, 100 μm. (B) Bar graph shows that there was no increase in the optical density of vGLUT2 immunoreactivity after ischemia.

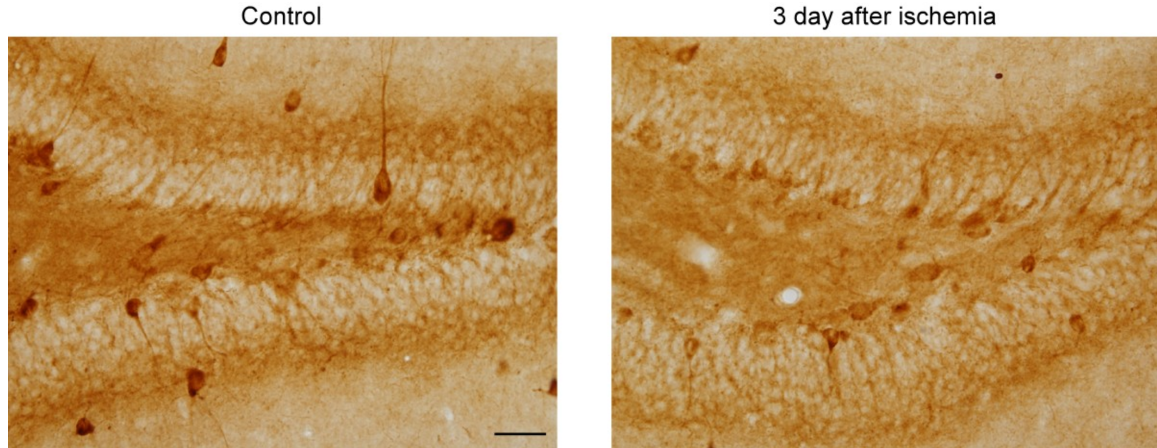

Figure 28 The increase in the immunostaining of GAD65 is not due to the ischemic injury. Representative images of GAD67 immunostaining from the dentate gyrus of hippocampus show a decrease of GAD67 immunoreactivity at 3 days after ischemia compared to that of the control. Images were taken under 400×. Scale bar, 40 μm.

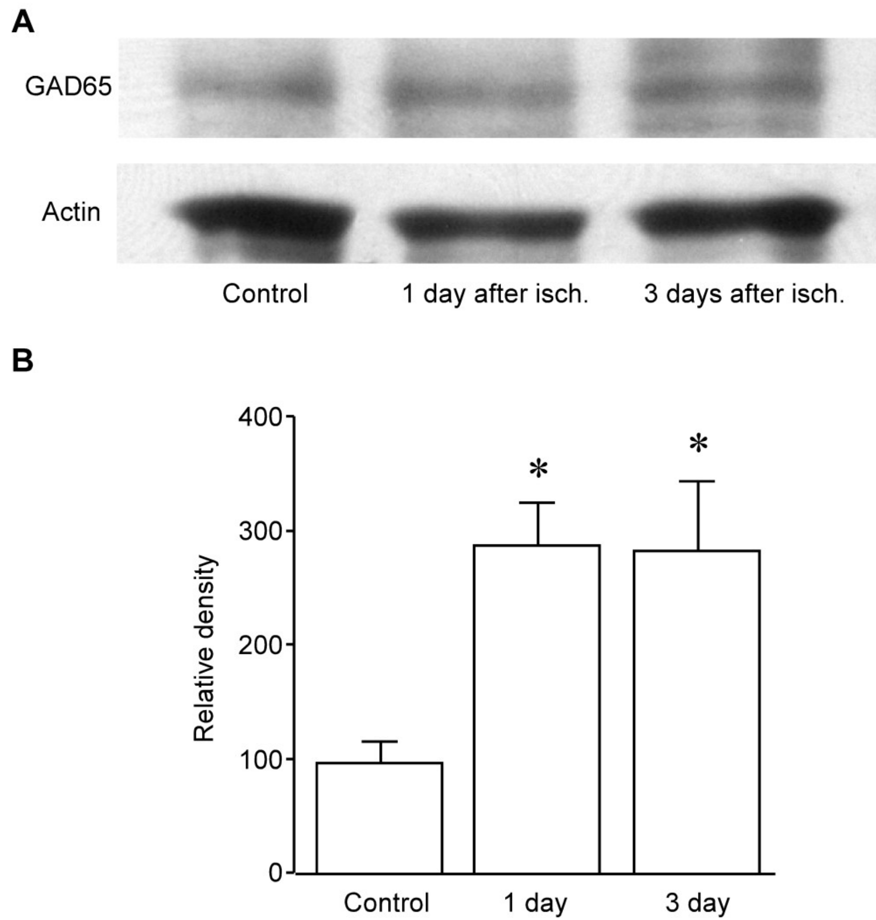

Figure 29 GAD65 expression is increased after ischemia. (A) Representative blots from three independent experiments. (B) Quantification analysis of the relative density of GAD65 in control and after ischemia. Data are represented as mean±SEM.

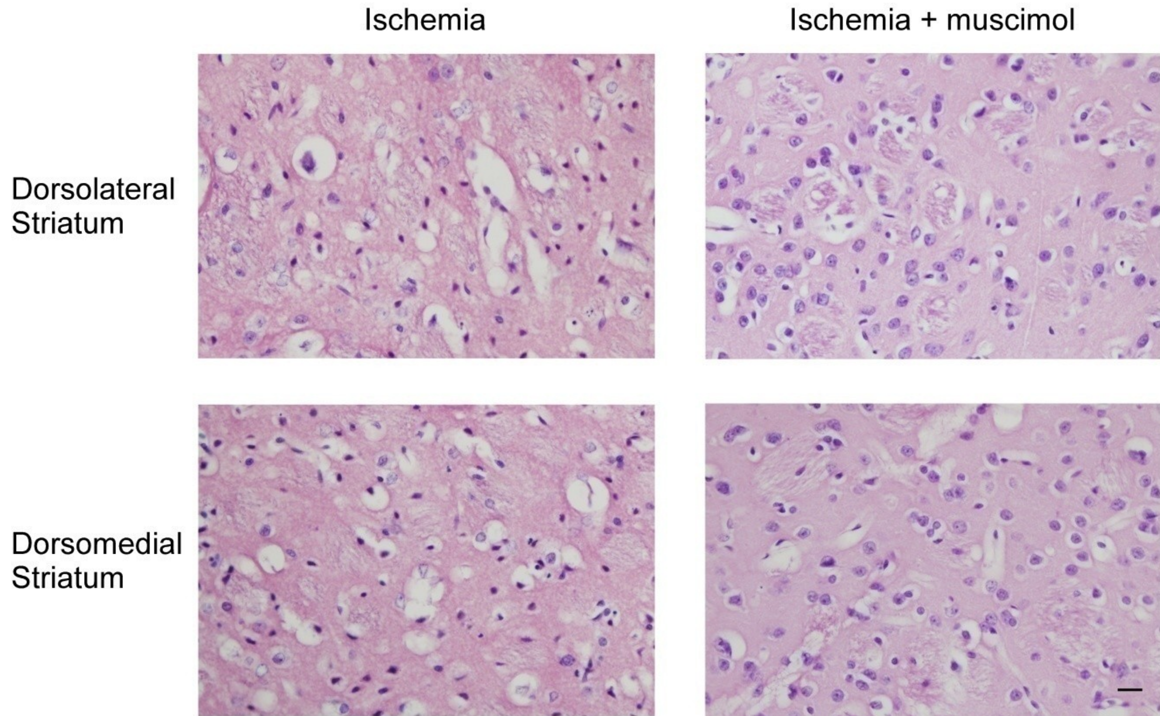

Figure 30 Muscimol application increases the number of survived cells (cells with normal morphology) in the striatum after ischemia. HE staining was performed on striatal slices (10 μm) after paraffin embedding. Rats were subjected to either transient cerebral ischemia alone or ischemia plus muscimol intraperitoneal injections 30 min before the ischemic insults. Images were taken from dorsolateral and dorsomedial striatum under 200×. Scale bar, 30 μm.

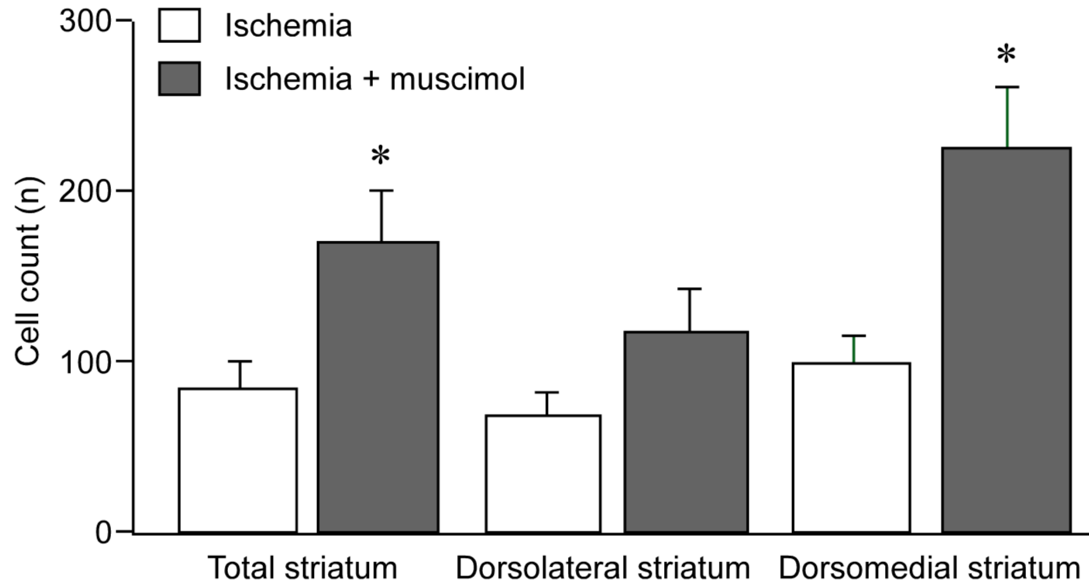

Figure 31 Bar graph shows the number of survived cells in the striatum (dorsolateral + dorsomedial) was increased in the muscimol treatment group. The number of survived cells in the dorsolateral striatum did not show significant increase while in dorsomedial striatum there was a significant increase in the muscimol treatment group.

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