Persistent Defect in Transmitter Release and Synapsin Phosphorylation in Cerebral Cortex After Transient Moderate Ischemic Injury

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Background and Purpose—Synaptic transmission is highly vulnerable to metabolic perturbations. However, the long-term consequences of transient metabolic perturbations on synapses are not clear. We studied the long-lasting changes in synaptic transmission and phosphorylation of presynaptic proteins in penumbral cortical neurons after transient moderate ischemia.

Methods—Rats were subjected to 1 hour of middle cerebral artery occlusion. After reperfusion, electric activity of neurons in the peri-infarct region was recorded intracellularly and extracellularly in situ. Phosphorylation of synapsin-I and tyrosine residues was studied by immunohistochemistry.

Results—Neurons in the penumbra displayed no postsynaptic potentials 1 to 3 hours after recirculation. However, these cells were able to generate action potentials and were responsive to glutamate, suggesting that postsynaptic excitability was preserved but the synaptic transmission was blocked because of a presynaptic defect. The synaptic transmission was still depressed 24 hours after recirculation in neurons in the peri-infarct area that survived ischemia. The amount of immunoreactive synapsin-I, synaptophysin, and synaptotagmin was not appreciably changed for 72 hours after reperfusion. However, phosphorylation of synapsin-I was significantly decreased, whereas phosphotyrosine immunoreactivity was increased, suggesting a selective defect in synapsin-I phosphorylation.

Conclusions—These data demonstrate that synaptic transmission may be permanently impaired after transient moderate brain injury. Since postsynaptic excitability is preserved, the transmission failure is likely to be caused by presynaptic mechanisms, one of which may be impaired phosphorylation of presynaptic proteins. (Stroke. 2002;33:1369-1375.)

Key Words: cerebral ischemia, focal motor cortex phosphorylation proteins somatosensory cortex synapses rats

It is estimated that 30% of brain metabolic activity is spent to maintain synaptic transmission.\(^1\) Not unexpectedly, synaptic transmission is highly vulnerable to metabolic perturbations.\(^2\)–\(^4\) Synaptic failure is one of the first consequences of hypoxia.\(^5\) However, the long-term effects of transient metabolic disturbances are not well documented. It is likely that synaptic boutons are prone to significant free radical damage due to the high rate of oxidative phosphorylation and Ca\(^{2+}\) shuttling across the mitochondrial membrane.\(^6\) Mitochondria of synaptic origin have been reported to be more sensitive to inhibition of complex I and to depletion of glutathione than are nonsynaptosomal mitochondria.\(^7\) Moreover, repair of damaged macromolecules in nerve terminals is likely to be a complex process because the supply of newly synthesized proteins to synapses depends on remote nuclear control and axonal transport. Dendritic swelling and changes in postsynaptic densities have also been reported to develop after ischemia.\(^8\)–\(^11\) Given the importance of these structures and processes, even brief perturbations are likely to induce long-lasting changes in synaptic function. In support of this view, it was recently demonstrated by evoked potential recordings that brief and mild focal cerebral ischemia could cause a long-lasting synaptic transmission defect in the cortex.\(^12\),\(^13\)

In this study we have searched by intracellular recordings for direct evidence of a persistent transmission block after mild brain injury and investigated the underlying mechanisms. In situ intracellular recordings indicate that synaptic transmission remains severely impaired after 1-hour transient middle cerebral artery (MCA) occlusion in the penumbral cortex, where collateral blood flow prevents full ischemia. We have found that a presynaptic defect is largely responsible...
for the transmission failure. Because phosphorylation of synaptic proteins regulates transmitter release at several stages, including release of vesicles from the reserve pool, priming of vesicles for docking, fusion, and endocytic recycling, we have also examined phosphorylation of phosphosite 1 of synapsin-I, against which an antibody has recently been developed. Phosphorylation of synapsin-I dissociates vesicles from actin skeleton to move to release sites. We have found that phosphorylation of synapsin-I remains impaired despite successful restoration of blood flow after moderate ischemia.

**Materials and Methods**

All experiments were performed in compliance with institutional guidelines for use of animals in research. Wistar rats (weight, 180 to 210 g) were anesthetized with either urethane (1.2 g/kg IP) or chloral hydrate (375 mg/kg IP with atropine 0.01 mg/kg IM). Electrophysiological recordings were always obtained under urethane anesthesia. Chloral hydrate was used during induction of ischemia/reperfusion in experiments with 24- and 72-hour reperfusion times. Rats were kept normothermic (37 ± 0.1°C) with a homeothermic blanket during experiments. The arterial blood pressure, pH, PCO₂, PO₂, hematocrit, and plasma glucose of all rats included in the study were within normal limits.

Proximal occlusion of the right MCA by a nylon filament was performed as described before. Briefly, after a midline incision, the right common and external carotid arteries were ligated by a 5-0 silk suture. A 4-0 nylon filament was inserted into the common carotid artery through a small incision proximal to the bifurcation and advanced to the origin of the MCA. A flexible probe of a laser-Doppler flowmeter (Perimed P2ZB) was placed 3 mm lateral and 1 mm anterior to bregma to monitor the regional cerebral blood flow (rCBF) in the cortex where electrophysiological recordings were to be obtained. If rCBF values were <35% of the control level after MCA occlusion, the contralateral common carotid artery was additionally occluded by snare ligature in some rats. The contralateral common carotid artery was not occluded in experiments with 24- and 72-hour reperfusion times.

**Electrophysiological Recordings**

Borosilicate glass microelectrodes (40 to 100 MΩ) filled with 3 mol/L KCl were implanted 2.5 to 3 mm lateral, 0.5 to 1.5 mm anterior to bregma (depending on localization of the pial vessels) for intracellular recordings. Conventional techniques were used for recording membrane potentials, bridge balancing, and current injection. The dorsomedial frontal cortex, the rat equivalent of premotor area, was stimulated with bipolar tungsten electrodes at 0.7 Hz with rectangular pulses (1 mA, 0.05 ms) to evoke orthodromic and antidromic activity in the sensorimotor cortex. The extracellular field potential was also recorded while the microelectrode was outside the cell. A Ag/AgCl-plated disk placed between left paraventricular neck muscles was used as the ground electrode. Intracellular recordings were obtained for 2 hours starting 1 hour (n = 3 rats) or 24 hours (n = 5 rats) after ischemia and were compared with recordings from the sham-operated group (n = 3 rats). We used KCl electrodes to avoid unfavorable effects of acetate on already jeopardized cellular metabolism. We also minimized current injection and time spent in each cell that intracellular ionic composition would not be perturbed. Multibarreled glass electrodes (10 to 35 MΩ) filled with 1 mol/L glutamate (pH 8.1) and 3 mol/L NaCl were used for microiontophoretic glutamate application and unit recordings at the same coordinates used for intracellular recordings. Unit responses to glutamate injections (10 to 40 nA, 7 to 10 seconds) were recorded 1 to 3 hours after reperfusion (n = 4 rats) and in sham-operated rats (n = 3).

**Results**

**Synaptic Transmission Remains Impaired After Moderate Ischemia**

We recorded intracellularly from neurons in the forelimb area 1 to 3 hours after recirculation. rCBF dropped to 20% to 30% or 35% to 50% of the basal level, depending on whether the contralateral carotid artery was ligated, and resumed to basal levels 10 minutes after recirculation, indicating that recordings were obtained from the penumbral area. As described in detail by Garcia et al., only mild and reversible ischemic alterations such as neuropilic sponginess and occasional neuronal shrinkage and scalloping were found in the recording area when examined at the end of the experiment by H&E staining, confirming that recordings were performed in the penumbra.

In sham-operated rats, the resting membrane potential (RMP) of neurons in the sensorimotor cortex was −57 ± 2 mV (n = 24), conforming with in vivo recordings performed in this region by others. In rats subjected to ischemia, most of the penumbral neurons were partially depolarized after reperfusion. Since it was not possible to differentiate injuriously penetrated neurons from depolarized neurons, we averaged the RMP of all neurons impaled in sham-operated as well as ischemic rats. The mean RMP of sham-operated rats was −44 ± 2 mV (n = 63) when all penetrations (good or
unsatisfactory) were taken into account. RMPs recorded 1 to
3 hours after recirculation in the ischemia group were
significantly depolarized (Δ/H11002/11006 26/3 mV; n/H11005 46;
P/H11021 0.05 by Student’s t test) compared with this value. The magnitude of
depolarization was likely to be overestimated by averaging all
impaled neurons because they all generated action potentials
during penetration or with antidromic stimulation or on
injection of depolarizing pulses (Figure 1A and 1C). How-
ever, no postsynaptic potentials could be recorded from these
cells, including those with RMPs Δ/H11002/11005 50 mV and after
restoration of the RMP by current injection (Figure 1B to
1D). Presumed glia were identified by lack of action poten-
tials with depolarizing pulses. RMPs of glia were typically
more hyperpolarized (Δ/H11002/11005 60 mV) and were more stable than
those of neurons.

To gain insight into the mechanisms of transmission
failure, we determined the postsynaptic sensitivity to glutamate.
To avoid experimental bias, we tested unit responses to
 glutamate with regular short pulses of moderate intensity. The
duration and intensity varied slightly between electrodes, but
the same iontophoretic current pulse was used for a given
electrode, and we did not apply larger pulses if this intensity
failed to generate a response at some recording sites. The
ratio of glutamate responsive units was comparable before
(37%; 18 of 49 U) and after (45%; 33 of 73 U) ischemia
(P/H11022 0.05 by χ² test) (Figure 2).

Phosphorylation of Synapsin-I Remains Defective
After Ischemia
We next searched for a possible presynaptic defect by
examining phosphorylation of synapsin-I. When we consid-
ered the small size of the peri-infarct area and variation in its
coordinates, immunohistochemistry was the only method that
allowed us to correlate morphological changes with alter-
tations in phosphorylation with a spatial resolution superior to
what could be achieved by using Western blotting or bio-
chemical detection methods.

As indicated above, only mild morphological alterations
were detected in the penumbra 1 and 3 hours after ischemia
(Figure 3). There was no appreciable change in synapsin-I
immunoreactivity. The orderly appearance of vertically ori-
eted apical dendrites surrounded by immunostained termi-
nals was distorted, possibly because of swelling of neuronal
and astrocytic processes (Figure 3). The sponginess of the
tissue possibly created an impression of slight decrease in
immunostaining; however, optical density measurements did
not show a significant difference between hemispheres (Fig-
ure 4). Findings similar to those obtained with synapsin-I
staining were obtained with 2 other presynaptic markers,
synaptophysin and synaptotagmin antisera. On the contrary,
phosphosynapsin-I immunoreactivity was considerably re-
duced in the penumbra (Figures 3 and 4). Unlike synapsin

Figure 1. Intracellular recordings from neurons in the
ischemic penumbra 2 (A, B, C) and 24 (D) hours after
reperfusion after 1-hour MCA occlusion. Inset in A
illustrates the infarct area at 72 hours and the border
zone of the MCA territory (horizontal bar), where
electrophysiological recordings were made. A, An
action potential induced by penetration of a depolar-
ized neuron. B, No synaptic potentials could be
elicited in this cell by orthodromic stimulation.
Hyperpolarizing the membrane did not disclose any
synaptic potentials (not shown). No field potentials
could be detected (see initial part of the tracing) in
contrast to recordings from sham-operated rats
(inset; note the antidromic and orthodromic popula-
tion spikes and the slow wave reflecting postsynap-
tic currents). C, A neuron with −52 mV of resting
membrane potential generated an antidromically elici-
ted action potential but no postsynaptic potentials.
D, Twenty-four hours after reperfusion, a neuron in
the peri-infarct area generated action potentials on
depolarizing pulse injection (⁎) or spontaneously (†)
but no postsynaptic potentials. Numbers on the left of
tracings show RMPs.

Figure 2. Postsynaptic responsiveness to glutamate was pre-
served after ischemia. Iontophoresis of glutamate (20 nA, 5 sec-
onds) induced multiple unit discharges in the ischemic sensori-
motor cortex 2 hours after reperfusion (bottom tracing). The top
tracing was recorded immediately before glutamate application.
phosphorylation, phosphorylation of tyrosine residues was increased in the neuropil 1 and 3 hours after reperfusion (Figures 3 and 4). In sham-operated rats, phosphoryrosine immunoreactivity was only robust in microglia and was barely detectable in the neuropil.

**Long-Lasting Effects of Transient Moderate Ischemia on Synaptic Transmission**

Twenty-four hours after restitution of blood flow, the penumbral area had progressed to infarct in rats whose residual rCBF was <35% during ischemia. If the rCBF was 35% to 50% of normal, the outer border zone of penumbral area appeared normal except for mild loosening of the neuropil. Electrophysiological recordings were performed at this mildly injured peri-infarct area. Although some neurons (6 of 38) had normal RMPs and generated postsynaptic potentials and antidromic and orthodromic action potentials, most of the neurons impaled were partially depolarized (−24±4; n=38). This value was significantly higher than the mean RMP of sham-operated rats calculated by averaging measurements from all penetrations (−44±2 mV; n=63; P<0.05 by Student’s t test). The magnitude of depolarization was likely to be overestimated by averaging all penetrations in the 24-hour reperfusion group since these cells were able to generate spontaneous or antidromically evoked action potentials (Figure 1D). However, stimulation of the cortex produced either small (5.5±0.8 mV) or no postsynaptic potentials in these neurons (Figure 1D). Since the amplitude of intracellularly recorded postsynaptic potentials may be affected by recording conditions (eg, chloride leakage, penetration-induced depolarization), we also compared the extracellular field potentials recorded before penetrating cells as a compound measure of postsynaptic currents generated by nearby neurons in the peri-infarct area. The peak amplitude of slow component of the field potential was greatly reduced in rats subjected to 24 hours of reperfusion compared with the potential recorded from the sham-operated rats (2.0±0.1 versus 5.2±0.4 mV; P<0.05 by Student’s t test; Figure 1B). Unfortunately, intense brain swelling in the infarct area led to bulging of the brain through the opened dura and precluded correct identification of coordinates of the peri-infarct tissue during vertical electrode descent; hence, electrophysiological recordings were not performed 72 hours after recirculation.

We studied the synapsin-I phosphorylation 24 as well as 72 hours after reperfusion in rats whose contralateral common carotid artery was not occluded; the rCBF was 35% to 50% of basal levels in the penumbral MCA occlusion. Intensity of the synapsin immunostaining appeared to be slightly reduced in this area, possibly because of edematous changes in tissue 24 and 72 hours after reperfusion, whereas phosphorylation of synapsin-I was severely depressed (Figure 5). Phosphotyrosine immunoreactivity within microglia was further enhanced compared with the immunostaining at 1 and 3 hours after reperfusion, but intense immunoreactivity in the neuropil was reduced to basal levels. Not only the penumbral tissue evolving to infarct but also the peri-infarct tissue that survived the ischemia displayed reduced phosphosynapsin-I immunoreactivity 72 hours after reperfusion despite preserved synapsin-I, synaptophysin, and synaptotagmin immunostaining (Figure 6). The coordinates of this area, which was identified by healthy-looking cells but some loosening in neuropil along with increased phosphotyrosine immunoreactivity (Figure 6), corresponded with those of electrode penetrations at 1- to 3- and 24-hour reperfusion groups.
Discussion

The concept that synaptic transmission is very sensitive to energy deprivation has long been recognized.2,5 Metabolic inhibitors, anoxia, hypoglycemia, and ischemia have been shown to rapidly inhibit synaptic transmission.19–22 Studies on the giant synapse of squid demonstrated that synaptic transmission readily ceased after metabolic inhibition, although presynaptic action potentials continued to fire.19 Evidence from studies on neocortical slices subjected to in vitro ischemia suggests that presynaptic mediator release is very vulnerable to energy perturbations since postsynaptic responses to glutamate and γ-aminobutyric acid iontophoresis were only partially reduced when the synaptic transmission totally failed.23

Although it is known that synaptic transmission is vulnerable to energy deficiency, the long-term consequences of a brief perturbation on synaptic transmission are not well characterized. In the present study we have provided direct evidence by in situ intracellular recordings that the synaptic transmission remains impaired for at least 24 hours after a relatively mild and brief ischemia despite a largely preserved postsynaptic excitability. These findings conform with in vitro studies reporting reduced evoked potential amplitudes in the peri-infarct brain tissue 28 days after ischemia.24–26 In parallel to depression of synaptic transmission, we observed a defect in synapsin-I phosphorylation after recirculation. Defective synapsin-I phosphorylation persisted 72 hours after reperfusion in the peri-infarct tissue salvaged by early reperfusion in the peri-infarct area (outer border zone of the penumbra) that survived ischemia (A) (magnification ×400), illustrating synapsin-I (Syn) (C), synaptophysin (Synp) (D), and phosphotyrosine (P-Tyr) (E) immunostaining. This area appeared histologically normal except for mild loosening of the neuropil in H&E-stained sections (F) but displayed reduced phosphorylsynapsin-I immunoreactivity (B) despite normal synapsin-I (C) and synaptophysin (D) immunostaining. Phosphotyrosine immunoreactivity was still high in microglia and neuropil at the peri-infarct tissue (E). Note dense phosphorylsynapsin-I immunostaining at the border zone of MCA territory at the left lower quarter in (A).

Figure 5. Twenty-four and 72 hours after restitution of blood flow, most of the penumbra was evolving to infarct (H&E-stained sections). Synapsin (Syn) immunoreactivity was preserved, but phosphorylation of synapsin-I (P-Syn) was severely depressed at these time points. Tyrosine phosphorylation (P-Tyr) was further increased in microglia but resumed in neuropil almost back to basal levels. Magnification ×200 for all photomicrographs.

Figure 6. Seventy-two hours after reperfusion following mild ischemia (rCBF >35% of normal during 1-hour MCA occlusion), phosphorylation of synapsin-I (P-Syn) was defective at the peri-infarct area (outer border zone of the penumbra) that survived ischemia (A) (magnification ×100). B (magnification ×400) is the boxed region in A, and C through F are the corresponding regions in adjacent sections (magnification ×400), illustrating synapsin-I (Syn) (C), synaptophysin (Synp) (D), and phosphotyrosine (P-Tyr) (E) immunostaining. This area appeared histologically normal except for mild loosening of the neuropil in H&E-stained sections (F) but displayed reduced phosphorylsynapsin-I immunoreactivity (B) despite normal synapsin-I (C) and synaptophysin (D) immunostaining. Phosphotyrosine immunoreactivity was still high in microglia and neuropil at the peri-infarct tissue (E). Note dense phosphorylsynapsin-I immunostaining at the border zone of MCA territory at the left lower quarter in (A).
also unlikely. However, if nerve terminals are depolarized in a manner similar to that of the postsynaptic side, this may negatively affect evoked transmitter release. Ca\(^{2+}\) influx to terminals may also be adversely affected by decreased phosphorylation of Ca\(^{2+}\) channels. In support of this view, decreases in 30 mmol/L potassium-induced intrasynaptosomal Ca\(^{2+}\) increase 1 hour after 2-hour MCA occlusion and in presynaptic Ca\(^{2+}\) influx during hypoxia-induced synaptic transmission block in hippocampal slices have been reported. Disruption of the optimum geometry between the synaptosome and postsynaptic sides by swollen cellular processes may also reduce the efficiency of synaptic transmission.  

Phosphorylation of synapsin-I by CaMK-II is the key step for liberation of vesicles from the actin cytoskeleton. However, phosphorylation of synapsin-I by CaMK-II is the key step for liberation of vesicles from the actin cytoskeleton. However, phosphorylation of synapsin-I by CaMK-II is likely to contribute to failure of transmitter release. Protein kinase C and MAPK2 are also reported to be defective after ischemia. These kinases, like PKA and CaMK-II, phosphorylate synaptic proteins and have other functions regulating synaptic transmission. Interestingly, phosphatases have been reported to be functional after ischemia, which may shift the balance against phosphoprotein-mediated reactions.

In conclusion, we have provided direct evidence by in situ intracellular recordings that brief, moderate ischemia may cause a long-lasting synaptic transmission defect. These findings suggest that delayed functional recovery after successful and early restoration of flow with thrombolitics as well as the functional deficits seen in stroke patients with no lesions in diffusion-weighted MRIs may be caused by synaptic transmission block. Mechanisms of the long-lasting synaptic dysfunction may be diverse; however, our data point to the importance of phosphorylation defects. It is likely that damaged synapses may be repaired after acute insults, since new synapse formation has been demonstrated after ischemia, or they may degenerate and induce apoptosis in chronic vascular diseases and therefore contribute to cognitive decline.

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**References**


34. Bolay et al. Persistent Synaptic Defect After Mild Ischemia


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