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-l 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

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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 1 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

Received August 2, 2001; final revision received January 7, 2002; accepted January 10, 2002.

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Stroke **is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000013708.54623.DE**

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,[14,15] we have also examined phosphorylation of phosphosite 1 of synapsin-I, against which an antibody has recently been developed.[16] 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 anesthestized 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, P_{CO_2}, P_{O_2}, 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.[12] 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 PF2B) 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,[17] 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 paravertebral 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).

Potentials were amplified and filtered (for unit recordings) by an amplifier (Axoclamp-2A, Axon Instruments). Amplified signals were sampled at 20 Hz, digitized, displayed, and stored in a computer by a data acquisition and analysis system (MacLab/8s, ADInstruments).

Data are presented as means and SEs.

Immunocytochemistry

Rats under pentobarbital anesthesia (n=38) were perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer after 1-hour ischemia followed by 1, 3, 24, and 72 hours of reperfusion. Brains were kept in paraformaldehyde for 48 hours and subsequently embedded in paraffin. Slices 4 μm thick were obtained from 4 mm posterior to the frontal pole, deparaffinized at 56°C overnight, and hydrated in xylol and graded alcohol. Sections were stained with anti–synapsin-I (1:200, courtesy of Dr Andrew Czernic), G-257 anti–phosphosynapsin-I (1:500, courtesy of Dr Andrew Czernic), anti-synaptophysin (1:50, Zymed), anti-synaptotagmin (1:200, courtesy of Dr George Augustine), and anti-phosphotyrosine (1:200, Upstate Biotechnology) antibodies by use of the avidin-biotin method. Diaminobenzidine was used as chromogen. Negative controls were included in every run, and sections from all time points were processed altogether with each antibody to ensure standard staining conditions. Immunostaining of the penumbral cortex, 2.5 to 3 mm lateral to the midline, was compared with the contralateral homologous region on the same coronal section to minimize variability in intensity due to staining procedure.

Additionally, 4-μm-thick slices were deparaffinized and hydrated in xylene and graded alcohol and stained with hematoxylin and eosin (H&E).

Image Analysis

To approximately quantify changes in immunoreactivity, we compared the optical densities of 3 randomly selected areas (0.99 mm^2) in the sensorimotor cortex with homologue regions in the contralateral hemisphere of the same section. A Nikon (Ecclips E600) microscope with a plan achromat ×20 lens was used to take images. Staining intensities were measured as gray-scale values (range, 0 to 255), and correlative histograms were used to integrate the signal intensity. Integration was calculated by summing the gray values of each pixel. The ratio of final integration values was used to compare signal intensities between the 2 hemispheres.

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,[8] 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.[18] 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 RPM 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

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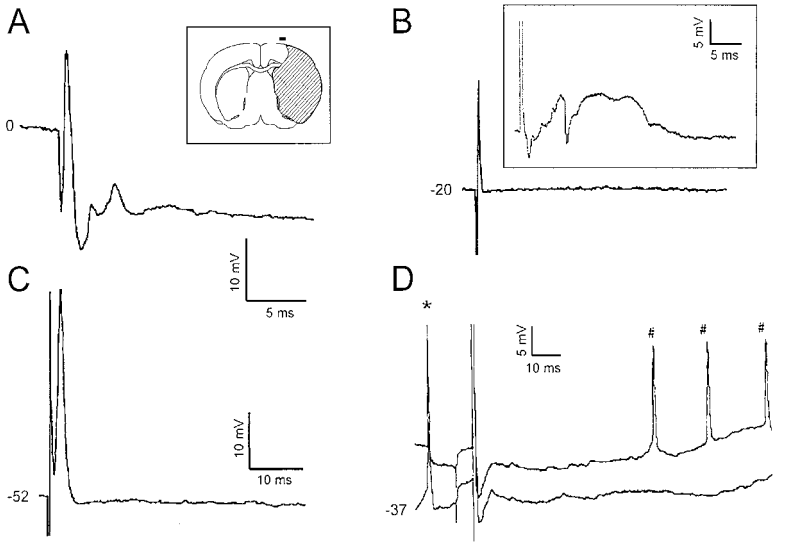

Figure 1. Intracellular recordings from neurons in the ischemic penumbra 2 (A, B, C) and 24 (D) hours after reperfusion after 1-hour MCA occlusion. Inset in A illustrates the infarct area at 72 hours and the border zone of the MCA territory (horizontal bar), where electrophysiological recordings were made. A, An action potential induced by penetration of a depolarized neuron. B, No synaptic potentials could be evoked 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 population spikes and the slow wave reflecting postsynaptic currents). C, A neuron with −52 mV of resting membrane potential generated an antidromically elicited 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.

unsatisfactory) were taken into account. RMPs recorded 1 to 3 hours after recirculation in the ischemia group were significantly depolarized (−26±3 mV; n=46; $P<0.05$ 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). However, no postsynaptic potentials could be recorded from these cells, including those with RMPs <−50 mV and after restoration of the RMP by current injection (Figure 1B to 1D). Presumed glia were identified by lack of action potentials with depolarizing pulses. RMPs of glia were typically more hyperpolarized (<−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 ($P>0.05$ by χ^2 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 considered 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 alterations in phosphorylation with a spatial resolution superior to what could be achieved by using Western blotting or biochemical 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 oriented apical dendrites surrounded by immunostained terminals 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 (Figure 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 reduced in the penumbra (Figures 3 and 4). Unlike synapsin

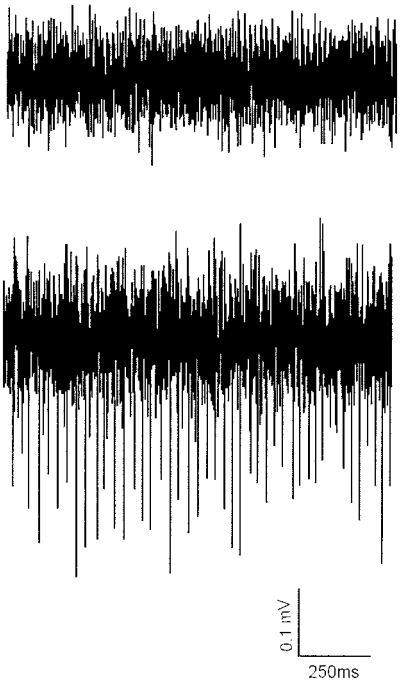

Figure 2. Postsynaptic responsiveness to glutamate was preserved after ischemia. Iontophoresis of glutamate (20 nA, 5 seconds) induced multiple unit discharges in the ischemic sensorimotor cortex 2 hours after reperfusion (bottom tracing). The top tracing was recorded immediately before glutamate application.

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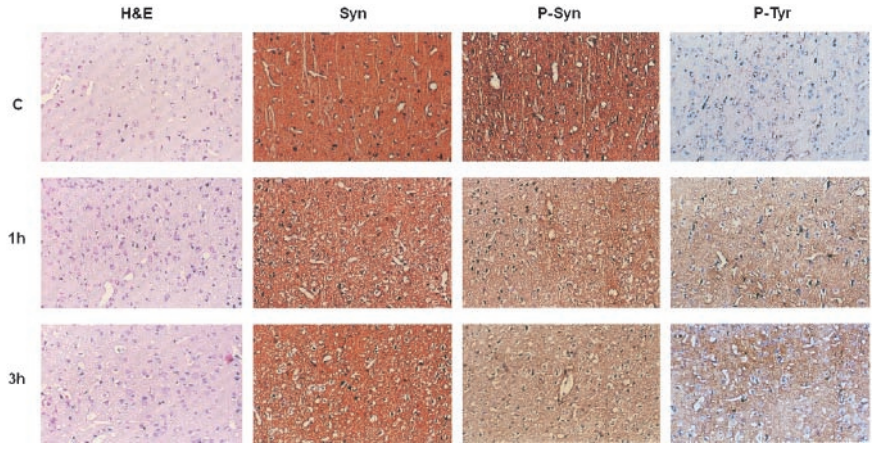

Figure 3. Changes in histology (H&E) and immunostaining for synapsin-I (Syn), phosphosynapsin-I (P-Syn), and phosphotyrosine (P-Tyr) in the frontal penumbral cortex after 1-hour MCA occlusion. Sections (magnification ×200) were obtained after sham operation (top) or 1 hour (middle) and 3 hours (bottom) after reperfusion. Only mild neuropilic sponginess was detected by H&E stainings 1 to 3 hours after reperfusion. Phosphosynapsin-I immunoreactivity was considerably reduced at 1 hour and 3 hours. No change in synapsin-I immunostaining was detected except that the orderly appearance of vertically oriented apical dendrites was distorted, possibly because of swelling of dendrites and astrocytes. Phosphotyrosine immunoreactivity was only robust in microglia in control brains, and the neuropil was faintly stained. After recirculation, phosphotyrosine immunostaining was increased in both microglia and neuropil.

phosphorylation, phosphorylation of tyrosine residues was increased in the neuropil 1 and 3 hours after reperfusion (Figures 3 and 4). In sham-operated rats, phosphotyrosine 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 penumbra 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.

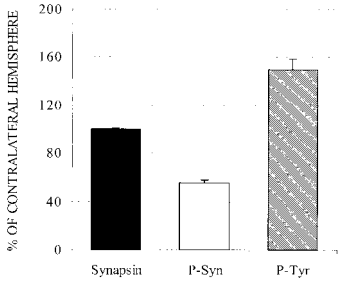

Figure 4. To approximately quantify the changes in synapsin, phosphosynapsin (P-Syn), and phosphotyrosine (P-Tyr) immunoreactivities, we compared the optical densities of 3 selected areas in the sensorimotor cortex with homologue regions in the contralateral hemisphere in rats (n=4) subjected to 1-hour MCA occlusion and 3 hours of reperfusion. Ischemia did not affect synapsin-I immunostaining but caused a decrease in phosphorylation of synapsin-I and an increase in tyrosine phosphorylation. Values are mean±SE.

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 penumbra during 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.

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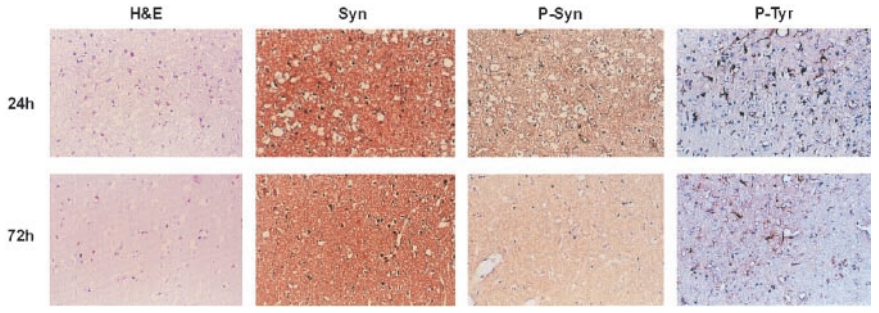

Figure 5. Twenty-four and 72 hours after restitution of blood flow, most of the penumbra was evolving to infarct (H&E-stained sections). Synapsin (Syn) immunoreactivity was preserved, but phosphorylation of synapsin-I (P-Syn) was severely depressed at these time points. Tyrosine phosphorylation (P-Tyr)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

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 restoration of rCBF. This outermost area of the MCA territory was clearly affected during ischemia because neuropil was slightly edematous and phosphotyrosine immunoreactivity was increased. Unfortunately, we could not confirm the transmission failure electrophysiologically 72 hours after reperfusion because of methodological limitations.

We found that most (by 84%) of the neurons that could not generate postsynaptic potentials were partially depolarized. Although it was hard to determine whether cells exposed to ischemia were really depolarized or were less resistant to damage by electrode penetrations, they may have been moderately depolarized because of an unsatisfactory recovery in ATP or because of inhibition of Na^+/K^+-ATPase by unfavorable tissue conditions (eg, by prostaglandins, free radicals).[27] However, postsynaptic depolarization block cannot account for the transmission failure because no postsynaptic potentials could be recorded from cells with RMPs <-50 mV or after restoration of the RMP by current injection. Field potential recordings clearly showed that depression of the synaptic activity was not an artifact caused by electrode penetrations. The transmission failure was likely to be caused by a defect in transmitter release since postsynaptic responsiveness to glutamate was grossly preserved, although a mild postsynaptic dysfunction in glutamatergic synapses may have been undetected by unit recordings.

The mechanisms of a defective transmitter release are probably complex. Lack of energy cannot account for transmission failure because ATP levels are readily restored in the penumbra on reperfusion.[28,29] A study reporting normal intrasynaptosomal Ca^{2+} levels 1 hour after 2 hours of MCA occlusion in the mouse suggests that ATP synthesis resumes also within nerve endings.[30] Since extracellular adenosine accumulation is readily restored on reperfusion, presynaptic inhibition by adenosine of excitatory transmitter release is

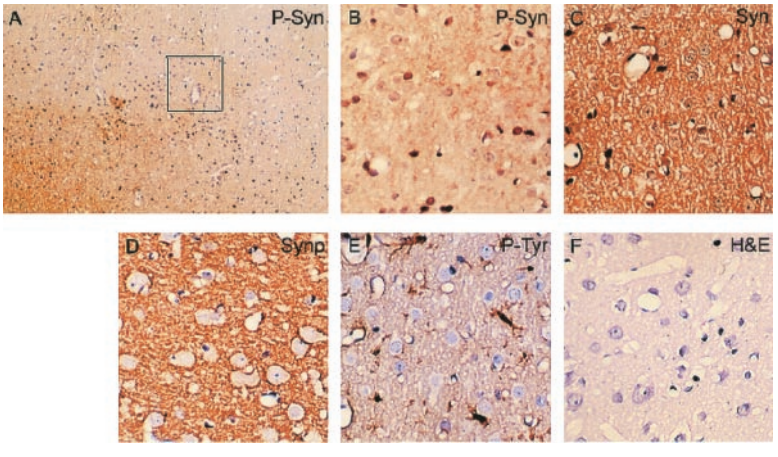

Figure 6. Seventy-two hours after reperfusion following mild ischemia (rCBF >35% of normal during 1-hour MCA occlusion), phosphorylation of synapsin-I (P-Syn) was defective at the peri-infarct area (outer border zone of the penumbra) that survived ischemia (A) (magnification ×100). B (magnification ×400) 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 phosphosynapsin-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 phosphosynapsin-I immunostaining at the border zone of MCA territory at the left lower quarter in (A).

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also unlikely.[28,29] 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.[30,31] Disruption of the optimum geometry between the presynaptic and postsynaptic sides by swollen cellular processes may also reduce the efficiency of synaptic transmission.[8–11] We found no considerable changes in the intensity of synapsin-I, synaptophysin, and synaptotagmin immunostaining, suggesting that the number of presynaptic terminals and vesicles and the amount of these proteins were not noticeably changed in the penumbra during the first 3 hours after reperfusion and, in the peri-infarct area, at 24- and 72-hour time points. Therefore, as the impaired synapsin-I phosphorylation suggests, it is very likely that a selective phosphorylation defect in nerve terminals in the penumbra as well as in the peri-infarct area that survived the ischemic attack may unfavorably affect transmitter release along with some of the aforementioned possible factors. This conclusion is supported by several studies reporting that kinases remain dysfunctional after cerebral ischemia (see below), although suppression of overall synaptic activity after ischemia may also indirectly contribute to decrease in phosphosynapsin-I levels.

Synapsin-I is phosphorylated at serine-9 near the N-terminus (phosphosite-1) by protein kinase A (PKA).[14,32] Calcium/calmodulin kinase-II (CaMK-II) phosphorylates synapsin-I at 2 serine residues near the C-terminus (phosphosites 2 and 3). Synapsin-I is also phosphorylated by mitogen-activated protein kinase 2 (MAPK2), extracellular signal-regulated kinases, and cyclin-dependent kinase at phosphosites 4 to 6 and by CaMK-I at phosphosite-1. The antibody we used (G-257) detects the phospho-form of synapsin-I and synapsin-II (and probably synapsin-III) at phosphosite-1.[16] Therefore, the decreased phosphosynapsin immunoreactivity observed indicates at least a defect in PKA- and/or CaMK-I–catalyzed phosphorylation. This site is constitutively phosphorylated under basal conditions by PKA, and fluctuations in the intraterminal cAMP levels are thought to modulate transmitter release.[16] Synapsin phosphorylation at site I dissociates synapsins from synaptic vesicles[33]; however, phosphorylation of synapsin-I by CaMK-II is the key step for liberation of vesicles from the actin cytoskeleton to move to release sites.[14] Unfortunately, we could not detect any immunoreactivity with RU-19 anti–phosphosynapsin-I antibody, which recognizes CaMK-II–phosphorylated serines, possibly because CaMK-II–mediated phosphorylation is not constitutively present but emerges transiently after stimulation.[16]

Inhibition of PKA after focal cerebral ischemia has been reported.[34] CaMK-II in synaptosomes has been found to remain dysfunctional after global as well as transient focal ischemia.[35–37] Decreased phosphorylation of synapsin-I by PKA and CaMK-II is likely to contribute to failure of transmitter release. Protein kinase C and MAPK2 are also reported to be defective after ischemia.[37] These kinases, like PKA and CaMK-II, phosphorylate synaptic proteins and have other functions regulating synaptic transmission.[15] Interestingly, phosphatases have been reported to be functional after ischemia,[37,38] 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 thrombolytics as well as the functional deficits seen in stroke patients with no lesions in diffusion-weighted MRIs[39] 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,[40] or they may degenerate and induce apoptosis in chronic vascular diseases and therefore contribute to cognitive decline.[41–43]

Acknowledgments

This study was supported by grants from TÜBITAK, Hacettepe University Research Fund, and Brain Research Society (Turkey). Dr Dalkara's work is also supported by the National Academy of Sciences of Turkey (TÜBA). The authors are grateful to Dr Andrew Czernik for kindly providing the anti–phosphosynapsin-I and anti–synapsin-I antibodies, to Dr George Augustine for kindly providing the anti-synaptotagmin antibody and for his comments, to Dr Figen Söylemezoglu for help with neuropathological evaluation and photomicrographs, and to Ünal Seref for help with tissue sections.

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