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Rapid Morphologic Plasticity of Peri-Infarct Dendritic Spines After Focal Ischemic Stroke

Craig E. Brown, PhD; Charles Wong, BSc; Timothy H. Murphy, PhD

Background and Purpose—Focal stroke is associated with cell death, abnormal synaptic activity, and neurologic impairments. Given that many of these neuropathologic processes can be attributed to events that occur shortly after injury, it is necessary to understand how stroke affects the structure of neurons in surviving peri-infarct regions, particularly at the level of the dendritic spines, which transmit normal and potentially abnormal and injurious synaptic signaling. Recently, we described ischemia-induced changes in the structure of layer 1 dendritic tufts of transgenic mice expressing YFP in layer 5 cortical neurons. However, these in vivo imaging experiments could not address ischemia-related phenomena that occur in deeper cortical structures/layers, other cortical regions, or submicron changes in dendritic spine structure.

Methods—Focal stroke was induced in the forelimb sensorimotor cortex by the photothrombotic method. Two, 6, and 24 hours after stroke, brains were processed for Golgi-Cox staining to permit a detailed analysis of primary apical dendritic spine structure from layer 2/3 and 5 cortical pyramidal neurons.

Results—Photothrombotic stroke caused a rapid deterioration of neurons, as revealed by Golgi-Cox labeling, in the infarct core that could be readily distinguished from surviving peri-infarct regions. Analysis of >15 000 dendritic spines revealed that although many spines were lost in the peri-infarct cortex during the first 24 hours after stroke ($\approx$38% lost), spines that remained were significantly longer ($\approx$25% at 6 hours). Furthermore, these effects were found in both layer 2/3 and 5 neurons and were restricted primarily to peri-infarct regions (<200 μm from the infarct border).

Conclusions—These rapid changes in dendritic spine number and length may reflect an early adaptive response of potentially vulnerable peri-infarct neurons coping with postischemic spreading depression-like depolarizations and the loss of presynaptic contacts. (***Stroke***. 2008;39:1286-1291.)

Key Words: neuronal plasticity ■ penumbra ■ excitotoxicity ■ focal cerebral ischemia ■ mice ■ recovery

The sudden loss of blood flow to the brain (ie, ischemia) causes an immediate loss of cells in the ischemic core that is surrounded by an area of compromised, but potentially salvageable, tissue known as the penumbra, or peri-infarct region.[1] Because of this potential for rejuvenation, investigations have examined the physiologic changes that take place within the peri-infarct region during the first few hours and days after stroke.[2] For example, it is known that ischemia and reperfusion rapidly induce the production of reactive oxygen species, mitochondrial dysfunction, and glutamate release that is followed by repetitive spreading depression-like depolarizations and changes in intra- and extracellular loads of electrolytes (ie, calcium, potassium, zinc).[2–4] These abrupt changes in neuronal excitability and ionic homeostasis during the early stages of stroke could conceivably lead to extensive changes in the structure of peri-infarct neurons that may significantly affect their survival. Indeed, in vitro studies of oxygen/glucose deprivation or in vivo models of global ischemia have described acutely dysmorphic dendritic pro-

cesses, spine loss, and filopodial formation within minutes of ischemia.[5–7] Recent in vivo imaging studies have confirmed and extended these observations, showing that focal stroke causes immediate (within 10 minutes), but potentially reversible, damage to apical dendritic tufts and spines[8,9] that was followed by a protracted period of spine proliferation during the recovery phase.[10]

Despite these advances in our understanding of neuronal plasticity after ischemia, very little is know about the nature, timing, and spatial extent to which focal ischemia triggers changes in dendritic spine structure, particularly those occurring at the submicron level and in deeper cortical regions not amenable to in vivo imaging experiments. Resolving this question may help explain which factors limit the spread of focal ischemic damage or lead to aberrant synaptic transmission in peri-infarct regions,[11,12] given that rapid changes in spine morphology or number could influence electrical conduction of postsynaptic potentials,[13] diffusion of signaling proteins between the spine and parent dendrite,[14] and buffer

against an excessive influx of calcium and other ions associated with excitotoxicity.[15,16] In this study, we used the Golgi-Cox staining method to examine rapid, ischemia-induced changes in primary apical dendritic spine structure of layer 2/3 and 5 pyramidal neurons after photothrombotic stroke of the sensorimotor cortex. Our results show that focal stroke triggers rapid spine loss and elongation of remaining spines in the peri-infarct cortex.

Materials and Methods

Twenty adult male C57BL6 mice (>3 months of age) bred at the University of British Columbia animal facilities were used. All protocols were approved by the animal care committee and were consistent with the Canadian Council for Animal Care guidelines.

Focal stroke was induced by the photothrombotic method originally described by Watson et al.[17] In brief, mice were anesthetized by inhalation of 1.5% isoflurane gas while body temperature was maintained at 37°C. To facilitate photoactivation, a 1.5×1.5-mm region of the skull (with the use of stereotaxic coordinates[18]) was thinned to ≈50% of its original thickness. Previously we had shown that this region directly overlies the forelimb sensorimotor cortex.[10] After Rose Bengal injection (1% solution dissolved in phosphate-buffered saline, 100 mg/kg IP), photothrombosis was initiated by illuminating the thinned skull with a collimated green laser (532-nm wavelength at 17 mW) for 15 minutes. After surgery, breathing and body temperature were carefully monitored until mice regained consciousness.

To investigate rapid ischemia-induced alterations in apical dendritic spine structure, mice were euthanized at 2 (n=4), 6 (n=6), or 24 (n=5) hours after photothrombosis. Control mice (n=5) were treated in an identical manner except with the omission of either Rose Bengal or laser light and were euthanized at 6 or 24 hours after stroke. Unlike our previous experiments of imaging urethane-anesthetized YFP transgenic mice,[7,8] isoflurane-anesthetized mice recovered quickly after surgery and were awake and freely moving for the majority of time after stroke. Brains were then processed for Golgi-Cox staining, which allows one to visualize dendritic spine structure in superficial and deep cortical layer neurons.[19,20] In brief, mice were perfused with 0.9% saline, and extracted brains were stored in Golgi-Cox solution for 7 days in the dark at room temperature and then transferred to 30% sucrose solution. Coronal sections were cut at 200 μm and stained by immersing them in NH$_4$OH followed by Kodak fixative solution (both 30 minutes), dehydrated in ethanols, cleared in xylene, and coverslipped.

To map the areal extent to which focal ischemia affects dendritic structure, we selected pyramidal neurons from cortical layers 2/3 and 5 from the peri-infarct primary motor cortex (M1) and more distant ipsilateral secondary motor cortex (M2). Owing to the fact that the photothrombotic method allows for relatively specific targeting of cortical regions that is highly replicable across animals,[21] neurons analyzed for M1 and M2 groups were taken from a coronal section between 0 and 0.5 mm anterior to the bregma, which corresponded to the approximate center of the infarct (ie, the coronal section where infarct area would be maximal). The designation of M1 and M2 regions was based on coordinates from Paxinos and Watson.[18] These regions were chosen on the basis of previous work, showing that forelimb sensorimotor stroke induces significant structural plasticity in the peri-infarct motor cortex but not in more distant ipsilateral cortical regions.[10] In addition, spines were examined in the contralateral barrel cortex (≈1 to 1.5 mm posterior to the bregma), which served as a within-animal control, given that it possesses little, if any, direct connections to the contralateral forelimb cortex.[22] Examination of spines in this distant region would also help rule out any nonspecific effects associated with the surgical procedure. Neurons were selected on the basis of the classic pyramidal morphology of a triangularly shaped cell body, a vertically oriented spiny apical dendrite, and laterally oriented basilar dendrites. All neurons analyzed in the present study did not display any obvious signs of

degeneration, such as beading of the primary apical dendrite, which would obscure visualization and quantification of the spines.

Images were captured with use of a Zeiss Axiophot light microscope coupled to a 12-bit CCD camera (Retiga, Q-imaging). High-resolution image stacks of each primary apical dendrite were generated by capturing 50 z-sections spaced 0.66 μm apart with a 100X Zeiss Neofluor oil-immersion objective (numerical aperture=1.3). Each section consisted of 1024×1024 pixels (0.1 μm per pixel) covering an area of ≈100 μm^2. Several image stacks were captured and tiled together to cover the 300- to 500-μm-long primary apical dendrites.

Dendritic spines were analyzed from 6 pyramidal cells per animal (1 layer 2/3 and 1 layer 5 neurons for each of the 3 regions) with NIH Image J software (v1.35). Owing to the fact that certain aspects of spine structure, such as neck width and in some cases spine head diameter, fall close to or below the resolution limit for optical microscopy, we restricted our analyses to measurements of spine length and density. Furthermore, because we could not definitively distinguish filopodia from long, thin dendritic spines by optical microscopy,[23] measurements of spine length included all types of dendritic protrusions. Only spines that protruded laterally from the primary apical dendrite were included in the analysis. For measurements of spine length, an observer blinded to experimental condition would scroll through each 3-dimensional image stack, locate the center of the spine, and manually draw a line through the center of the spine (from the tip of the spine head to the shaft of the dendrite) by using the bent-line tool. Spine density (number of spines per 10 μm of dendrite) was calculated by dividing the total number of laterally oriented spines by the length of the dendrite. Because many pyramidal neurons have a spine-free zone close to the soma, spine length and density measurements began ≈30 μm from the soma.

Statistical comparisons of spine length and density were done by ANOVA followed by post hoc t tests with a Bonferroni correction for multiple comparisons. All data are expressed as mean±SEM.

Results

The Golgi-Cox staining method labeled a subset of neurons in all layers of the cerebral cortex. Figures 1A through 1D show the general pattern of neuronal labeling in a control mouse and those euthanized 2, 6, and 24 hours after photothrombotic stroke in the forelimb cortex. Consistent with the notion that photothrombosis causes a severe loss of blood flow and YFP-labeled dendritic structure in the ischemic core,[8,9] apical and basilar dendritic morphology in the core, as revealed by Golgi-Cox staining, rapidly deteriorated during the first 24 hours after stroke (see insets in Figures 1B–1D). The loss of staining in the infarct core was evident 2 hours after stroke and contrasted with the relatively full labeling of neurons in regions surrounding the infarct (dotted lines in Figures 1B–1E), denoted as the peri-infarct cortex. At the infarct border, individual neurons had an asymmetric appearance due to the presence of fully labeled basilar dendrites on the nonischemic side of the soma, opposed by blebbed, fragmented dendrites that projected toward the infarct core (Figure 1E). These results further support the idea that focal stroke is accompanied by a very sharp transition zone between normal and infarcted tissue.[9]

A summary of all results pertaining to apical dendritic spine length and density for cells within each cortical layer, region, and time point after stroke is presented in the Table. Owing to the fact that we were unable to trace most apical dendrites to their layer 1 tuft (most were truncated due to sectioning), only the primary apical dendrite was analyzed. Furthermore, because there were no significant differences between layer 2/3 and 5 neurons in spine length ($F_{1,32}$=0.49,
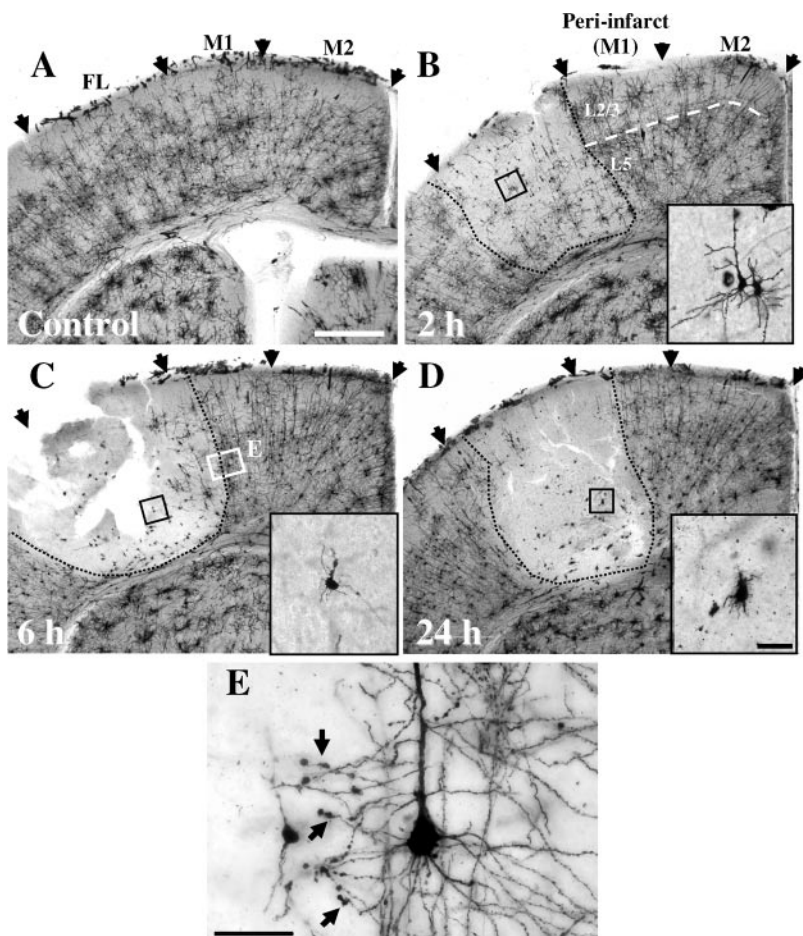
Figure 1. Golgi-Cox staining of cortical neurons in a sham surgery control (A) and mice euthanized 2 (B), 6 (C), and 24 (D) hours after photochemically-induced stroke of the forelimb sensorimotor cortex. In control mice (A), fully labeled neurons were present in superficial and deep cortical layers. Note that after stroke, the infarct border could be easily discerned by more scant neuronal labeling in the infarct core than in adjacent surviving regions (see dashed line representing the border). This segregation between dying and surviving tissue became more striking over time due to the progressive degeneration of neurons in the infarct core (see insets in B–D corresponding to black boxes). In fact, the border was so sharp that beaded apical and basilar dendritic processes (arrows in E) could be found on the more ischemic side of a single neuron, but not the other (E). FL indicates forelimb sensorimotor cortex; M1, primary motor cortex; and M2, secondary motor cortex. Scale bar in A=500 μm; in D and E=50 μm.

P=0.48) or density ($F_{1,32}$=1.29, P=0.26) at any time point after stroke, all measurements from neurons in superficial and deep layers were pooled together.

Recent in vivo imaging studies have shown that layer 1 dendritic spines can undergo dynamic structural modifications after manipulations of sensory experience,[24,25] after synaptic activity,[26] or during long-term recovery from stroke[10] and therefore represent a plausible site of rapid (ie, within hours) morphologic plasticity after focal ischemia. However, those studies were limited in their ability to detect changes in other dendritic compartments or deeper cortical layers. Furthermore, lower imaging resolution in those in vivo experiments (measured at ≈0.65 μm in the *x-y* plane[10] versus 0.26 μm for the present study) hindered assessment of submicron changes in dendritic spine structure.

To determine the temporal and spatial extent to which focal stroke affects dendritic spine length, we measured a total of 15 156 spines along the primary apical dendrite of neurons in the peri-infarct primary motor (174±18 μm from infarct border), secondary motor (738±35 μm from infarct border), and contralateral barrel cortex at 2, 6, and 24 hours after stroke. In accordance with previous estimates of pyramidal neuron spine length assessed by Golgi or virally based labeling methods,[27–30] the average length of dendritic spines in the control primary, secondary motor, and contralateral barrel cortex was 1.29±0.05, 1.16±0.04, and 1.20±0.05 μm, respectively (Figure 2B). Our quantitative analysis revealed

that spine length varied significantly as a function of time after stroke ($F_{3,48}$=3.94, P=0.013) and distance from the site of infarction ($F_{2,48}$=13.14, P<0.0001). Across cortical regions, dendritic spines were significantly longer in the peri-infarct primary motor cortex ($F_{3,16}$=4.53, P=0.017) but not in the secondary motor ($F_{3,16}$=2.01, P=0.15) or contralateral barrel ($F_{3,16}$=0.45, P=0.71) cortex. As shown in Figures 2A and 2B, this increase in dendritic spine length in the peri-infarct cortex was significant at 6 hours (1.61±0.08 μm, 25% increase relative to controls; t_9=3.7, P<0.01) but not at 2 (1.42±0.08 μm, 10% increase; t_7=1.61, P=0.07) or 24 (1.44±0.06 μm, 12% increase; t_8=1.8, P=0.06) hours after stroke. Plotting the cumulative frequency distribution of spine lengths in the peri-infarct cortex (Figure 2D) indicated that the significant increase in spine length was due to an increase in the proportion of medium and long dendritic spines (note the rightward shift of the plots, especially at 6 hours). These findings suggest that focal stroke is associated with a transient increase in dendritic spine length that is specific to peri-infarct regions.

Similar to a report by Ballesteros-Yanez et al,[31] control apical dendritic spine density levels (Figure 2C) for the primary motor (9.61±1.0 spines/10 μm), secondary motor (8.21±0.6 spines/10 μm), and contralateral barrel (9.18±0.6 spines/10 μm) cortex did not differ significantly from one another ($F_{2,12}$=0.98, P=0.40). However, within 24 hours after stroke, spine density levels dropped significantly in the

Table.   Summary of Changes in Dendritic Spine Length and Density

Primary Motor Cortex (Peri-Infarct)	Control (n=5)	2 Hours (n=4)	6 Hours (n=6)	24 Hours (n=5)
Spine length, μm				
Layer 2/3	1.37±0.06	1.47±0.13	1.58±0.05	1.50±0.07
Layer 5	1.22±0.05	1.37±0.06	1.65±0.18	1.38±0.06
Spine density, No. of spines/10 μm				
Layer 2/3	9.69±1.1	7.46±0.5	8.98±0.7	6.06±0.8
Layer 5	9.52±1.7	6.41±0.9	7.44±0.4	5.92±0.4
No. of spines	1588	945	1463	1257
Secondary Motor Cortex (M2)				
Spine length, μm				
Layer 2/3	1.23±0.06	1.40±0.18	1.27±0.08	1.24±0.16
Layer 5	1.09±0.05	1.32±0.04	1.32±0.05	1.40±0.08
Spine density, No. of spines/10 μm				
Layer 2/3	9.81±1.0	6.19±0.5	10.3±1.0	7.43±0.7
Layer 5	6.62±0.9	6.17±0.8	7.95±0.9	7.54±0.9
No. of spines	1585	1067	2050	1897
Barrel Cortex (Contralateral)				
Spine length, μm				
Layer 2/3	1.23±0.04	1.29±0.09	1.21±0.07	1.20±0.05
Layer 5	1.09±0.13	1.13±0.02	1.33±0.15	1.03±0.13
Spine density, No. of spines/10 μm				
Layer 2/3	10.2±0.7	9.53±0.8	9.01±0.6	8.72±0.2
Layer 5	6.19±0.2	7.88±1.0	9.46±0.2	8.28±1.0
No. of spines	732	743	888	943

Values are mean±SEM.

peri-infarct primary motor cortex ($F_{3,16}$=5.68, $P<0.01$), only marginally in the secondary motor cortex ($F_{3,16}$=3.27, $P=0.05$), and not at all in the contralateral barrel cortex ($F_{3,16}$=0.23, $P=0.87$). As shown in Figure 2C, ischemia-induced spine loss was most pronounced in the peri-infarct primary motor cortex, particularly at 2 (6.94±0.5 spines/10 μm, 27.7% reduction; t_7=2.12, $P<0.05$) and 24 (5.98±0.5 spines/10 μm, 37.7% reduction; t_8=3.11, $P<0.01$) hours after stroke. These results indicate that focal ischemia induces a relatively severe (37.7% lost by 24 hours) and local loss of primary apical dendritic spines. The fact that we did not see any changes in the contralateral barrel cortex (Figure 2C) suggests that our data could not be explained by any extraneous variables associated with the surgical procedure (eg, anesthesia) or subtle differences in age of the animals or quality of staining.

Discussion

Dendritic spines are the recipients of most excitatory synapses in the brain, where they participate in the transmission and integration of synaptic signaling.[32] Previous studies have shown that morphologic changes in these tiny structures may play a fundamental role in normal brain processes, as well as in neuropathologic conditions such as Alzheimer disease,

mental retardation, and epilepsy.[27,33,34] Accordingly, our goal was to characterize rapid changes in neocortical apical dendritic spine structure after an in vivo model of focal cerebral stroke. Our results indicate that photochemically induced stroke of the forelimb sensorimotor cortex causes a loss of spines during the first 24 hours after stroke. Quantitative measurements of the spines that remained indicated that they were significantly longer than controls ($\approx$25% at 6 hours). Furthermore, these effects were independent of cell layer and were found selectively within peri-infarct regions ($<$200 μm). These results provide new information regarding rapid, ischemia-induced changes in synaptic structure in vivo, as well as the spatial extent to which these effects can be observed, at least within the first 24 hours after stroke, when tissues are most vulnerable and therapeutic interventions are most likely to occur.

Previous studies with in vitro or in vivo models of global ischemia have reported spine loss within minutes to hours after the insult.[5,6,35] Recent imaging experiments conducted in our laboratory have extended these observations, showing that focal photothrombotic stroke induces spine loss in layer 1 apical dendritic tufts of ischemic YFP-labeled layer 5 neurons.[9,36] It is, however, unknown whether focal ischemia causes acute changes in dendritic spine integrity in deeper
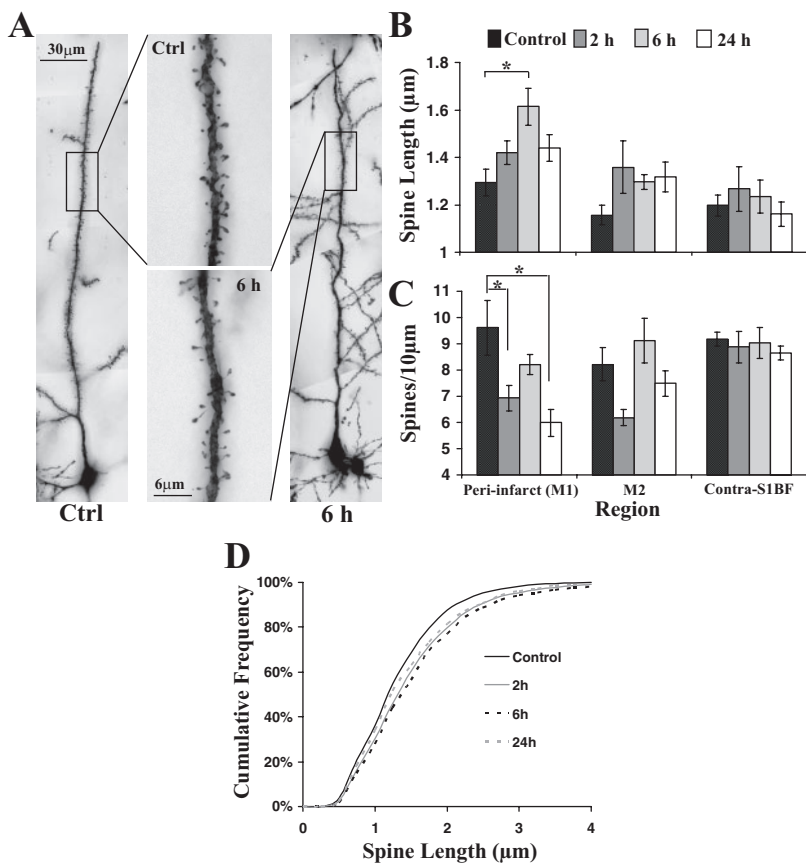
Figure 2. Focal stroke induces spine loss and elongation of existing spines in the peri-infarct cortex. A, Photomicrographs of Golgi-Cox–stained layer 5 pyramidal neurons in control and peri-infarct primary motor cortex, 6 hours after photothrombotic stroke. Similar to those in controls, neurons in the peri-infarct region were labeled in full exquisite detail. As shown in boxed regions, numerous spines could be resolved along the primary apical dendrite. Analysis of apical dendritic spine length (B) and density (C) revealed that focal ischemia induced a transient elongation of dendritic spines in the peri-infarct primary motor cortex ($\approx$174 μm from the infarct border), concomitant with a progressive reduction in spine density. These effects were fairly local, considering that apical dendritic spines in the more distant secondary motor ($\approx$738 μm from the infarct border) and contralateral barrel (S1BF) cortices were largely unaffected. D, Cumulative frequency distribution of spine lengths in the peri-infarct cortex (M1). Note that the cumulative frequency plot for spine lengths was shifted rightward at all times after stroke, indicating a greater proportion of medium to long spines. $*P<0.05$.

cortical layers, other parts of the dendrite, or more distant cortical regions. The present data suggest that, at least initially, the effects of ischemia are limited predominately to neurons close to the infarct border (<200 μm), because we did not find significant changes in spine density in the more distant secondary motor cortex or the contralateral barrel cortex. Furthermore, layer 2/3 and 5 pyramidal neurons seemed to be similarly affected by ischemia, considering that both showed a significant reduction in spine density at 2 and 24 hours after stroke (see the Table). The fact that spine density levels at 6 hours were not significantly different from controls is intriguing and may reflect a brief burst of spine proliferation that has been reported in recovering cultured neurons hours after oxygen-glucose deprivation/glutamate receptor activation[5,6] or in hippocampal dendrites after shorts bouts of global ischemia or chilling/rewarming.[35,37] This increase in spine proliferation at the 6-hour time point may be related to the observed increase in spine length at this time, although our previous work has shown that newly formed spines tend to be slightly shorter than existing ones.[10]

Accumulating data have suggested that changes in dendritic spine length or shape can significantly alter the functional properties of neurons.[38] As such, a number of studies have described correlative changes in dendritic spine morphology during functional recovery from spinal cord injury,[39] experience-dependent plasticity,[40,41] or long-term potentiation/long-term depression–like synaptic plasticity.[42,43] Morphologic plasticity of dendritic spines has also been reported in in vitro models after ischemia, wherein brief oxygen-glucose deprivation or glutamate receptor activation, which does not result in acute cell death, promoted the enlargement of existing spines and filopodial formation within minutes of the insult.[5,6] Here, we have shown for the first time in an in vivo model that focal stroke is associated with a significant increase in dendritic spine length in peri-infarct regions. This change in spine length may be triggered by abrupt, ischemia-induced changes in neuronal excitability or calcium homeostasis, given that similar effects have been observed in culture after stimulating excitatory synaptic activity or intracellular calcium release.[44,45]

Dendritic spine plasticity after stroke may also represent an early adaptive response of vulnerable neurons to damaging events associated with the postischemic brain,[46,47] such as spreading depression-like depolarizations or excessive calcium release.[2,7,11] It is well established that dendritic spines can act as tiny biochemical compartments that restrict the diffusion of calcium[15,16] and potentially other ions implicated in excitotoxicity. Furthermore, longer dendritic spines limit the conduction of electric activity from synapses on the spine head to the soma.[13] Taken together, the net effect of longer spines during ischemia may be to biochemically and electrically isolate excitotoxic events at the spine from spreading to the soma and triggering cell death.

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Disclosures

None.

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