Stroke is a leading cause of morbidity and long-term disability in the United States and worldwide. Therefore, a tremendous need exists for the development of new therapies for stroke. Patients with stroke exhibit spontaneous recovery at the behavioral level within the first 3 months after ischemic injury. Studies from experimental stroke demonstrate that endogenous processes of neuronal remodeling such as axonal sprouting and formation of new cortical connections are induced in the area adjacent to the infarct border. Clearly, endogenous mechanisms are not sufficient to restore full neurological function; however, preclinical data in rodents suggest that amplification of endogenous brain repair processes can lead to improved functional outcome after stroke. Investigating the molecular mechanisms underlying neuronal remodeling after ischemic injury can help in identifying new targets for neurorestorative therapy for stroke.

Epigenetic post-translational modifications of histone proteins, such as lysine acetylation and deacetylation, play a major role in the regulation of gene transcription in neurons. Histone deacetylases (HDACs) are a large family of enzymes that regulate histone acetylation levels by catalyzing the removal of acetyl moieties from lysine residues in histone tails. Histone deacetylation consequently leads to compaction of chromatin and gene repression.

HDACs are classified into 4 major classes (I–IV) based on homology to yeast enzymes. Class II HDACs are mammalian histone deacetylase 1–like proteins that include HDACs 4, 5, 6, 7, 9, and 10. On the basis of their structure, we subdivided class II HDACs into 2 subclasses: class IIa (4, 5, 7, and 9) and IIb (6 and 10).

Two isoforms of class IIa HDACs (4 and 5) are abundantly expressed in the brain and have been implicated in the regulation of neurodegeneration, synaptic plasticity, and memory formation. Constitutive HDAC4 knockout mice are not viable and die within 2 weeks of birth because of severe skeletal malformations, whereas conditional loss of HDAC4 in the forebrain leads to impaired motor coordination.

**Background and Purpose**—Histone deacetylases (HDACs) 4 and 5 are abundantly expressed in the brain and have been implicated in the regulation of neurodegeneration. Under physiological conditions, HDACs 4 and 5 are expressed in the cytoplasm of brain cells where they cannot directly access chromatin. In response to external stimuli, they can shuttle to the nucleus and regulate gene expression. However, the effect of stroke on nuclear shuttling of HDACs 4 and 5 remains unknown.

**Methods**—Using a rat model of middle cerebral artery occlusion, we examined the subcellular localization of HDACs 4 and 5 in the peri-infarct cortex during brain repair after stroke.

**Results**—Stroke significantly increased nuclear HDAC4 immunoreactivity in neurons, but not in astrocytes or in oligodendrocytes, of the peri-infarct cortex at 2, 7, and 14 days after middle cerebral artery occlusion. Neurons with nuclear HDAC4 immunoreactivity distributed across all layers of the peri-infarct cortex and were Ctip2+ excitatory and parvalbumin+ inhibitory neurons. These neurons were not TUNEL or BrdU positive. Furthermore, nuclear HDAC4 immunoreactivity was positively and significantly correlated with increased dendritic, axonal, and myelin densities as determined by microtubule-associated protein 2, phosphorylated neurofilament heavy chain, and myelin basic protein, respectively. Unlike HDAC4, stroke did not alter nuclear localization of HDAC5.

**Conclusions**—Our data show that stroke induces nuclear shuttling of HDAC4 in neurons in the peri-infarct cortex, and that increased nuclear HDAC4 is strongly associated with neuronal remodeling but not with neuronal cell death, suggesting a role for nuclear HDAC4 in promoting neuronal recovery after ischemic injury. (Stroke. 2015;46:1909-1915. DOI: 10.1161/STROKEAHA.115.009046.)

**Key Words:** epigenenomics ■ stroke
learning, and memory.26 Interestingly, conditional HDAC5 knockout mice do not display similar dysfunctions in learning and memory,26 indicating that despite their similarity in structure, HDACs 4 and 5 play nonredundant roles in neurons. HDACs 4 and 5 are predominantly expressed in the cytoplasm,27 where they cannot directly access chromatin; however, in response to external stimuli, they can also shuttle to the nucleus.23–25 For example, previously published studies showed that under physiological conditions HDAC4 is localized to the cytoplasm of cerebellar granule neurons (CGNs), but shuttles to the nucleus when CGNs are treated with nondepolarizing media.16,17 To our knowledge, no information is available about whether stroke affects the subcellular localization of class IIa HDACs. We hypothesized that ischemic conditions may stimulate nuclear shuttling of HDACs 4 and 5. Therefore, in the present study, using a rat model of focal cerebral ischemia, we investigated whether stroke induces nuclear shuttling of HDACs 4 and 5 in neurons in the peri-infarct brain. Furthermore, we also examined whether nuclear shuttling of HDACs is associated with apoptosis or promoted remodeling of neurons after ischemia.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal Model

Adult male Wistar rats (270–300 g; n=6 per group) were subjected to permanent middle cerebral artery occlusion (MCAO) by advancing a 4-0 surgical nylon suture with an expanded tip.26 Sham-operated rats were used for control. Rats were injected once daily with bromo-deoxyuridine (BrdU) (Methods in the online-only Data Supplement) harboring at 2, 7, and 14 days after surgery. The brains were fixed by transcardial perfusion with saline followed by 4% paraformaldehyde before being embedded in paraffin.}

Tissue Preparation, Immunohistochemistry, and TUNEL Assay

The brains were fixed by transcardial perfusion with saline followed by 4% paraformaldehyde before being embedded in paraffin. The brains were cut into 4-μm-thick sections, which were deparaffinized and rehydrated followed by antigen unmasking. The sections were incubated with antibodies against HDAC4, MAP-2, and phosphorylated neurofilament heavy chain (p-NFH). In the cortex, HDAC4 immunoreactivity was mainly detected in nuclei of sham-operated rats and contralateral cortex. Immunoreactive cells were counted using National Institutes of Health ImageJ software, and immunoreactive areas were calculated using Micro Computer Imaging Device imaging analysis system.

Results

Stroke Induces Nuclear Shuttling of HDAC4 in Neurons

To examine whether stroke induces nuclear shuttling of HDAC4 in neurons during brain repair, rats were euthanized at 2, 7, and 14 days after MCAO and double immunohistochemistry was performed with antibodies specifically against HDAC4 along with microtubule-associated protein 2 (MAP-2) and phosphorylated neurofilament heavy chain (p-NFH). In the cortex of sham-operated rats, immunoreactivity of HDAC4 was mainly localized to MAP-2+ neuronal cytoplasm and dendrites (Figure 1A), but not to p-NFH+ axons (Figure I in the online-only Data Supplement). However, after MCAO, we found a robust increase in HDAC4 immunoreactivity in nuclei of neurons in the peri-infarct cortex (Figure 1A and 1D). Quantitative analysis of HDAC4/MAP-2 revealed the percentage of nuclear (B) and dendritic (C) HDAC4 immunoreactive neurons in the peri-infarct cortex at 2, 7, and 14 days after MCAO compared with sham. A schematic representation of a brain coronal section (D) shows that images were acquired from 6 layers of the peri-infarct cortex as outlined in the numbered boxes. DAPI indicates 4',6-diamidino-2-phenylindole. Scale bar, 20 μm.

Image Acquisition and Analysis

Laser-scanning confocal microscopy (Zeiss LSM 510 NLO, Carl Zeiss, Germany) was used for 3-dimensional imaging. For quantification, coronal sections were digitized under a ×20 or ×40 objective of an epifluorescence microscope (Axiopt, Carl Zeiss, Inc) via a Micro Computer Imaging Device system (Imaging Research Inc). Six to 8 fields of view were acquired from the peri-infarct cortex (≤500 μm from the lesion border; Figure 1D). For comparison, additional fields of view from homologous areas were acquired from sham-operated rats and contralateral cortex. Immunoreactive cells were counted using National Institutes of Health ImageJ software, and immunoreactive areas were calculated using Micro Computer Imaging Device imaging analysis system.

Statistical Analysis

One-way ANOVA with post hoc Bonferroni test was used for data analysis. Statistical significance was set at P<0.05. Pearson correlation coefficients were calculated among histological measurements and their correlation with nuclear HDAC4. All values are presented as mean±SE for illustration.
analysis of HDAC4/MAP-2 immunoreactive cells at multiple time points after MCAO revealed a temporal dynamic increase in the percentage of nuclear HDAC4+ neurons after stroke when compared with sham (P<0.05; Figure 1B). Concomitantly, stroke-induced nuclear expression of HDAC4 was accompanied by a significant (P<0.05) decrease in HDAC4 immunoreactivity in neuronal dendrites over the same period (Figure 1C). We did not detect a significant increase in nuclear HDAC4 immunoreactivity in MAP-2+ neurons in contralateral homologous areas of the cortex (31±5% at 2 days, 32±5% at 7 days, and 27±2% at 14 days after MCAO versus 27±3% in sham; P<0.05). Together, these data show that stroke induces nuclear shuttling of HDAC4 in neurons in the peri-infarct cortex.

**Stroke Induces HDAC4 Shuttling Across Cortical Layers in Interneurons and Pyramidal Neurons**

The cerebral cortex is composed of 6 layers that are mainly composed of pyramidal neurons (excitatory) and interneurons (inhibitory).27 We used HDAC4/MAP-2 double immunohistochemistry to examine whether nuclear shuttling of HDAC4 after stroke exhibits a specific laminar distribution (Figure 2A; Figure II in the online-only Data Supplement). We found that in the normal brain, HDAC4 immunoreactivity was predominantly in the cytoplasm of neurons in both superficial (L1–4) and deep (L5–6) cortical layers. The number of nuclear HDAC4 immunoreactive neurons in the normal cortex was low and evenly distributed throughout the superficial and deep cortical layers. Starting 2 days after stroke, we found a significant increase in nuclear HDAC4 immunoreactive neurons in deep cortical layers (L5–6) compared with sham control (Figure 2A; Figure II in the online-only Data Supplement). The percentage of nuclear HDAC4 immunoreactive neurons in deep cortical layers continued to increase until 2 weeks after MCAO (Figure 2A; Figure II in the online-only Data Supplement). On the contrary, nuclear HDAC4 immunoreactivity in superficial cortical layers was delayed and significantly increased only at 7 and 14 days after MCAO (Figure 2A; Figure II in the online-only Data Supplement). Together, these data show that stroke-induced nuclear HDAC4 shuttling first starts in the deep cortical layers and then it expands to superficial layers.

To examine whether nuclear HDAC4 shuttling is induced in a specific neuronal population after stroke, we performed double immunohistochemistry for HDAC4 with Ctip2 (pyramidal neuron marker) and parvalbumin (interneuron marker). We found that stroke significantly increased nuclear HDAC4 immunoreactivity in both Ctip2+ pyramidal cells (Figure 2B) and parvalbumin+ interneurons (Figure 2C). These data suggest that stroke induces nuclear HDAC4 shuttling in both excitatory and inhibitory neurons of the peri-infarct cortex.

Taken together, nuclear shuttling of HDAC4 after stroke is induced in different neuronal populations across all cortical layers, suggesting that nuclear shuttling of HDAC4 plays a major role in regulating neuronal response to ischemia.
HDAC4, but Not HDAC5, Shuttles to Neuronal Nuclei After Stroke

It has been reported that both HDACs 4 and 5 possess the ability to translocate between the cytoplasm and the nucleus. Therefore, we investigated whether stroke also induces nuclear shuttling of HDAC5 in neurons. In sham-operated rats, HDAC5/MAP-2 and HDAC5/p-NFH double immunohistochemistry showed that the subcellular localization of HDAC5 in cortical neurons was similar to HDAC4; HDAC5 immunoreactivity was also mainly localized to MAP-2+ cytoplasm and dendrites of neurons (Figure 3A) and not to p-NFH+ axons (Figure I in the online-only Data Supplement). However, in contrast to HDAC4, stroke did not significantly increase nuclear HDAC5 in neurons in the peri-infarct cortex across cortical layers at 2, 7, or 14 days after MCAO (Figure 3A). These data suggest that stroke only induces nuclear shuttling of HDAC4 in neurons in the peri-infarct cortex.

Stroke-Induced Nuclear Shuttling of HDAC4 Is Specific to Neurons

Previously, we found that stroke induces diverse changes in the expression profiles of individual class I and II HDACs in oligodendrocytes. Using double immunohistochemistry for HDAC4 with adenomatous polyposis coli (oligodendrocytes marker) and glial fibrillary acidic protein (astrocytes marker), we also examined whether stroke induces nuclear shuttling of HDAC4 in glial cells. We did not detect nuclear HDAC4 immunoreactivity in adenomatous polyposis coli–positive oligodendrocytes or glial fibrillary acidic protein–positive astrocytes in the peri-infarct cortex (Figure 3B and 3C, 7 days after MCAO). These data indicate that stroke-induced nuclear shuttling of HDAC4 is specific to neurons and does not occur in glial cells of the ischemic brain.

Stroke-Induced HDAC4 Nuclear Shuttling in Neurons Is Not Associated With Apoptosis

Previous studies presented contradictory evidence on whether nuclear HDAC4 shuttling has a positive or deleterious effect on neuronal survival. Thus, we examined whether nuclear HDAC4 immunoreactive neurons in the ischemic brain are primed to die. Using immunohistochemistry for HDAC4 with TUNEL assay for identification of DNA fragmentation, we found that nuclear HDAC4+ cells in the peri-infarct cortex did not exhibit TUNEL staining (Figure 4A, 7 days after MCAO), indicating that nuclear HDAC4 immunoreactive neurons are not apoptotic cells. In addition, we examined whether nuclear HDAC4+ cells re-enter the cell cycle using double immunofluorescent staining with antibodies against HDAC4 and BrdU. We found that nuclear HDAC4 immunoreactive cells were not BrdU+ (Figure 4B, 7 days after MCAO), suggesting that nuclear HDAC4 immunoreactive neurons do not re-enter an abortive cell cycle that could have led to apoptosis. Together, these data show that nuclear HDAC4 shuttling under ischemic conditions in vivo is not associated with neuronal apoptosis or abortive cell cycle re-entry.

Nuclear Shuttling of HDAC4 Is Correlated With Increased Dendritic, Axonal, and Myelin Densities After Stroke

Neuronal remodeling such as rewiring neuronal circuitry and remyelination occurs in peri-infarct region during stroke recovery. Thus, we examined whether increased nuclear HDAC4 immunoreactivity after stroke is associated with increased neuronal remodeling, by performing double immunofluorescent staining for HDAC4/MAP-2, HDAC4/p-NFH, and HDAC4/MBP at 2, 7, and 14 days after MCAO.
Stroke Induces Nuclear Shuttling of HDAC4

Discussion

In the present study, we show for the first time that stroke induces nuclear shuttling of HDAC4 in neurons in the peri-infarct cortex and that nuclear HDAC4 is strongly associated with improved neuronal remodeling and not with neuronal cell death, suggesting a role for nuclear HDAC4 in promoting brain repair after ischemic injury.

Class IIa HDACs are mainly expressed in the cytoplasm in physiological conditions but are also able to shuttle into the nucleus. In the nucleus, HDACs can access histone proteins and act as epigenetic regulators.\(^1\)\(3\)\(2\)\(3\)\(2\)\(5\) Interestingly, in addition to their known cytoplasmic and nuclear localizations, we also found that HDACs 4 and 5 are expressed in dendrites of neurons in the normal and ischemic cortex. This finding suggests a function for HDACs in dendritic outgrowth in addition to gene regulation.

Synaptic activity in hippocampal and cerebellar granular neurons is known to regulate the intracellular shuttling of class IIa HDACs.\(^2\)\(3\)\(2\) Whether ischemic stimuli can similarly affect the subcellular localization of HDACs has not been previously explored. The present study shows that stroke induces nuclear shuttling of HDAC4 in neurons in the peri-infarct sensorimotor cortex. The translocation of HDAC4 from the cytoplasm to nuclei induced by stroke is specific because nuclear translocation of HDAC5 was not detected although both HDACs are closely similar in structure (70% homology).\(^1\) Moreover, along with increased nuclear HDAC4 shuttling after stroke, we detected a significant decrease in dendritic HDAC4 immunoreactivity. Thus, our data strongly suggest that cerebral ischemia affects the subcellular localization of HDAC4 leading to increased trafficking of HDAC4 from dendrites and cytoplasm into the nucleus. Although nuclear HDAC4 continued to gradually increase ≤14 days after MCAO, dendritic HDAC4 decreased from 19% in sham to 6% to 8% at 2 days after MCAO and remained stable afterward. This finding suggests that trafficking of HDAC4 from dendrites to the nucleus is most robust in early stages after stroke. Possibly, a minimal level of dendritic HDAC4 is required to maintain dendritic integrity and function. Later on, at 7 and 14 days after MCAO, the increase in nuclear HDAC4 most likely results from increased shuttling of HDAC4 from the cytoplasm to the nucleus.

We previously reported that stroke induces diverse changes in the expression profiles of class I and II HDACs in oligodendrocytes.\(^2\)\(9\) However, the present study demonstrated that stroke-induced nuclear shuttling of HDAC4 is neuronal specific and is not induced in oligodendrocytes or astrocytes. In addition, examination of the laminar distribution of nuclear HDAC4 shuttling after stroke revealed that nuclear HDAC4 shuttling starts in deep cortical layers and then expands to superficial layers. We also found that nuclear HDAC4 shuttling is not specific to 1 neuronal population and is induced in both excitatory and inhibitory neurons, indicating that nuclear HDAC4 may play a major role in regulating neuronal response to ischemic injury.

The role of nuclear HDAC4 in neuronal cell death and survival remains debated in the literature.\(^3\)\(5\) The controversy largely stems from contradictory reports on the effect of HDAC4 shuttling in CGNs. Bolger and Yao\(^1\) reported that in response to low potassium or excitatory glutamate conditions that induce CGNs death, HDAC4 shuttled from the cytoplasm to the nucleus. Nuclear HDAC4 then repressed the activity of myocyte enhancer factor 2, a known prosurvival transcription factor and promoted neuronal apoptosis. On the contrary,
Majdzadeh et al\textsuperscript{17} reported that forced expression of HDAC4 in CGNs protected them against low potassium induced apoptosis. The protective effect of HDAC4 was shown to occur in the nucleus and mediated by preventing abortive cell cycle re-entry through inhibition of cyclin-dependent kinase 1. These reports may have yielded contradictory results because of heavily relying on in vitro experiments.\textsuperscript{33} Therefore, in the current study, we used an in vivo model for ischemia to examine whether endogenous stroke-induced nuclear HDAC4 shuttling is associated with cell death.

Using double immunohistochemistry, we found lack of colocalization of nuclear HDAC4 immunoreactive neurons with TUNEL assay and BrdU labeling, suggesting that stroke-induced nuclear shuttling of HDAC4 does not trigger apoptotic cell death or abortive cell cycle re-entry. Furthermore, nuclear HDAC4 shuttling persists for at least 2 weeks after stroke and is positively correlated with endogenous processes of neuronal repair after ischemia. Surviving cortical neurons in the peri-infarct cortex experience axonal sprouting, dendritic outgrowth, and remyelination,\textsuperscript{3,4,34} which may contribute to spontaneous functional recovery after stroke. Indeed, our data show that nuclear HDAC4 is strongly and positively correlated with improved indices of neuronal remodeling including dendritic, axonal, and myelin as determined by MAP-2, p-NFH, and MBP densities, respectively. These findings highlight that further studies are warranted to show a causative relationship between nuclear HDAC4 shuttling and improved neurological outcome after stroke.

In summary, our data show that stroke induces nuclear shuttling of HDAC4 in neurons in the peri-infarct cortex, and that nuclear HDAC4 is strongly associated with improved neuronal remodeling and not with neuronal cell death, suggesting a possible positive role for nuclear shuttling of HDAC4 in promoting neuronal recovery after ischemic injury. Additional studies to investigate the effects of specific blocking of neuronal HDAC4 in the ischemic brain would advance our understanding of the mechanisms by which HDAC4 possibly promotes neuronal remodeling after stroke.

Acknowledgments
We thank Qinge Lu and Sutapa Santra for technical assistance.

Sources of Funding
This work was supported by National Instituted of Health (NIH) grants RO1 NS088656 (Dr Chopp) and RO1 NS075156 (Dr Zhang). The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

Disclosures
None.

References
Stroke Induces Nuclear Shuttling of Histone Deacetylase 4
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*Stroke*. 2015;46:1909-1915; originally published online May 12, 2015;
doi: 10.1161/STROKEAHA.115.009046

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/46/7/1909

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/05/12/STROKEAHA.115.009046.DC1

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Bromodeoxyuridine labeling
Bromodeoxyuridine (BrdU), a thymidine analog that is incorporated into cells during DNA synthesis, was used for S-phase labeling. Using a cumulative labeling protocol\(^1\), rats (n=6/group) were intraperitoneally injected once daily with BrdU (50 mg/kg, Sigma-Aldrich, St. Louis, MO) for 2 or 7 consecutive days, starting 24 hours after surgery. Rats receiving a daily BrdU injection in the 2-day and 7-day groups were sacrificed at 2 hours after the last BrdU injection. Rats in the 14-day group were injected daily with BrdU for 7 days starting 24 hours after surgery, and sacrificed 7 days after the last BrdU injection.

Immunohistochemistry
A series of coronal sections (6 µm thick) were obtained at the center of the lesion, corresponding to coronal coordinates for Bregma -1 to +1 mm\(^2\). Three coronal sections were used for each immunohistochemistry experiment. Class IIa HDACs were examined using rabbit anti-HDAC4 (1:50, Santa Cruz Biotechnology) and rabbit anti-HDAC5 (1:50, Santa Cruz Biotechnology) antibodies. The following primary antibodies were used: mouse anti-microtubule-associated protein 2 (neuronal somatodendritic marker, MAP-2; 1:400, Millipore), mouse anti-phosphorylated neurofilament heavy chain (axonal marker, p-NFH; 1:500, Covance), rabbit anti-myelin basic protein (MBP; 1:400, Dako), rat anti-COUP-TF-interacting protein 2 (Ctip2; 1:250, Abcam), goat anti-parvalbumin (PV; 1:5000, Swant), mouse anti-adenomatous polyposis coli (APC; 1:20, GenWay), chicken anti-glial fibrillary acidic protein (GFAP; 1:500, Aves Labs) and mouse anti-BrdU (1:100, Dako).

Brain sections were incubated with the primary antibodies listed above and with Cy3 or FITC (Jackson ImmunoResearch) conjugated secondary antibodies. Control experiments consisted of staining brain coronal tissue sections as outlined above, but omitting the primary antibodies. Counterstaining with DAPI (Vector Laboratories) allowed visualization of cells nuclei and determination of cortical layers.

TUNEL assay
TUNEL assay for the identification of apoptotic cells was performed using the ApopTag® Fluorescein in Situ Apoptosis Detection Kit (Millipore) following the manufacturer’s manual.
Supplemental Figures

Supplemental Figure I: HDACs 4 and 5 in p-NFH+ axons

Supplemental Figure I: Immunoreactivities of HDAC4 (A) and HDAC5 (B) were not detected in p-NFH+ processes of cortical neurons. Bar = 20µm.
Supplemental Figure II: Distribution of HDAC4 positive neurons across cortical layers 1, 4 and 6.

Supplemental Figure II: In superficial (L1,4) and deep (L6) cortical layers of sham-operated animals, HDAC4 immunoreactivity was predominantly detected in the cytoplasm of MAP-2+ neurons (arrowheads). Two days after stroke, nuclear HDAC4 (arrows) was increased in neurons of deep (L6), but not superficial (L1,4) cortical layers. Only 7 and 14 days after MCAO, increased nuclear HDAC4 immunoreactivity was also detected in superficial layers (L1,4). Bar = 20µm.

Supplemental References