Mitigation of Murine Focal Cerebral Ischemia by the Hypocretin/Orexin System Is Associated With Reduced Inflammation

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Background and Purpose—Brain ischemia causes immediate and delayed cell death that is exacerbated by inflammation. Recent studies show that hypocretin-1/orexin-A (Hcrt-1) reduces ischemic brain injury, and Hcrt-positive neurons modulate infection-induced inflammation. Here, we tested the hypothesis that Hcrt plays a protective role against ischemia by modulating inflammation.

Methods—Orexin/atxixin-3 (AT) mice, a transgenic strain in which Hcrt-producing neurons degenerate in early adulthood, and wild-type mice were subjected to transient middle cerebral artery occlusion (MCAO). Infarct volume, neurological score, and spontaneous home cage activity were assessed. Inflammation was measured using immunohistochemistry, ELISA, and assessment of cytokine mRNA levels.

Results—Infarct volumes 24 and 48 hours after MCAO were significantly larger, neurological score was worse, and spontaneous activity decreased in AT compared with wild-type mice. Macrophage/microglial infiltration and myeloperoxidase-positive cells were higher in AT compared with wild-type mice. Pre-MCAO intracerebroventricular injection of Hcrt-1 significantly reduced infarct volume and macrophage/microglial infiltration in both genotypes and improved neurological score in AT mice. Post-MCAO treatment decreased infarct size in both wild-type and AT mice, but had no effect on neurological score in either genotype. Microglia express the Hcrt-1 receptor after MCAO. Tumor necrosis factor-α production by lipopolysaccharide-stimulated microglial BV2 cells was significantly reduced by Hcrt-1 pretreatment. Sham AT mice exhibit increased brain tumor necrosis factor-α and interleukin-6 mRNA, suggesting chronic inflammation.

Conclusions—Loss of Hcrt neurons in AT mice resulted in worsened stroke outcomes, which were reversed by administration of exogenous Hcrt-1. The mechanism underlying Hcrt-mediated neuroprotection includes attenuation of inflammatory responses after ischemic insult. (Stroke. 2013;44:XXX-XXX.)

Key Words: brain ischemia ■ hypocretin ■ inflammation ■ neurobehavior ■ orexin

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Original Contributions
study, we tested whether endogenous Hcrt-producing neurons promote neuroprotection after MCAO, and whether Hcrt-mediated protection is associated with modulation of the inflammatory response. Using the orexin/ataxin-3 mice (AT) in which the hypocretin/orexin neurons degenerate during early adulthood, we performed transient focal ischemia on wild-type (WT) and AT mice and found that AT mice had larger infarcts, greater behavioral deficits, and increased microglial activation compared with WT mice. mRNA analysis revealed higher levels of both tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in AT mice, suggesting a chronic inflammatory state in this genotype. Importantly, administration to the brain of Hcrt-1 pre- or post-MCAO decreased infarct size in both WT and AT mice, suggesting effects in AT mice are likely a direct result of loss of Hcrt neuropeptides. In vitro experiments support an anti-inflammatory effect of Hcrt that may contribute to its neuroprotection.

Materials and Methods

Animals

Adult male (WT C57/BC6 and orexin/ataxin-3 mice, 3–5 months old, 25–35 g) were used. Although AT mice are normal during early development, the strain has Hcrt-specific expression of ataxin-3, a disease protein that results in gradual degeneration of Hcrt-expressing neurons that is completed by 3 months of age. Details of strain production and animal care can be found in the Materials and Methods in the online-only Data Supplement.

Power analysis for a 30% change in infarct size showed that 11 animals/group were needed for P<0.05, power 80%. Mice that died before perfusion, exhibited brain hemorrhage, or were surgical failures indicated by a fully normal neuroscore of 0 were excluded from analysis. Mice were randomized by being chosen by a blinded experimenter. Numbers of mice excluded and exclusion reasons for each experiment are indicated in Table III in the online-only Data Supplement.

Focal Cerebral Ischemia

Anesthesia was induced with 4% isoflurane and maintained by 1.5% to 2% isoflurane in 70% air and balanced oxygen by a facemask. Rectal temperature was maintained at 37±0.5°C with a heating pad (Harvard Apparatus, Hollister, MA). Transient focal ischemia was induced by MCAO for 60 minutes, which generates infarction in both cortex and striatum, as previously described. Details of surgery protocol can be found in Materials and Methods in the online-only Data Supplement. The surgeon was blinded to genotype and experimental treatment.

Behavioral Testing

Neurological score was evaluated 24 and 48 hours after MCAO according to a neurological grading score, from 0 (no observable neurological deficit) to 4 (unable to walk spontaneously and a depressed level of consciousness). The evaluator was blinded to genotypes and experimental treatment. The SmartCage system (AfaSci, Inc., Redwood City, CA) was used for automated analysis of spontaneous activity as described previously. The home cage activity variables (locomotion, travel distance, velocity, and rear-ups) were determined by photo-beam breaks and automatically analyzed using CageScore software (AfaSci, Inc.). Mice were assessed continuously for 30 minutes during the light phase, 24, and 48 hours after reperfusion.

Measurement of Cerebral Infarction Area

Twenty-four or 48 hours after MCAO and immediately after neurocore assessment, mice were anesthetized with isoflurane and decapitated. Brains were removed and sectioned coronally with a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). Sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St Louis, MO), and infarction core volume as defined by an absence of TTC staining (percent of hemisphere volume) was determined by a blinded observer using 4 sections per brain and corrected for edema using the NIH ImageJ program (Image J 1.37v, Wayne Rasband, NIH) as described previously.

Immunofluorescence

Ischemic or sham-operated mice were euthanized with an overdose of isoflurane and perfused with ice-cold PBS (pH, 7.4) 48 hours after MCAO, followed by 4% paraformaldehyde in PBS as previously described. Brains were removed and postfixed for 72 hours in 4% paraformaldehyde in PBS and cut into 50-μm coronal sections. Details of the immunofluorescence protocol, including antibodies used and cell counting protocol, can be found in Materials and Methods in the online-only Data Supplement.

Reverse Transcription Quantitative Real-time Polymerase Chain Reaction for mRNA Quantitation

Total RNA was isolated with TRIzol (Invitrogen) from the ischemic hemisphere (from +0.8 to −1.2 mm relative to bregma) of WT or AT mice 4 hours after MCAO. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit according to manufacturer’s instructions (Applied Biosystems). Predesigned primer/probes (Applied Biosystems) for mRNAs and GAPDH were also from Applied Biosystems. The expression of mRNAs was normalized using GAPDH as the internal control. Measurements were normalized to GAPDH (ΔCt), and the comparison was calculated as the inverse log of ΔΔCt to give relative fold change value.

Treatment With Recombinant Hcrt-1 In Vivo

Hcrt-1 was injected intracerebroventricularly as previously described. Two microliters of either vehicle (0.1% bovine serum albumin in 0.9% PBS) or containing 2 nmol of Hcrt-1 dissolved in the vehicle was infused over 10 minutes into the left lateral ventricle 30 minutes before or after MCAO. After 48 hours of reperfusion, neurological score was determined, animals were euthanized, and brains removed for TTC staining, as described above.

Measurement of TNF-α Production by BV2 Cells

BV2 microglial cells were treated with 10 ng/mL LPS (Sigma) for 24 hours and fixed with 4% paraformaldehyde. Details of immunocytochemistry protocol, including quantification, can be found in Materials and Methods in the online-only Data Supplement. BV2 cells were treated with control media or media containing Hcrt-1 (100 nmol/L) for 1 hour before treatment with LPS (10 ng/mL). Four hours after LPS treatment, supernatant was collected and TNF-α measured using the TNFα Mouse ELISA Kit (Life Technologies). Cell number was assessed by 4′,6-diamidino-2-phenylindole staining and counting with NIH ImageJ. Fluorescence images were acquired at ×2.5 magnification. TNF-α measurements were normalized to cell number.

Statistical Analyses

Data are expressed as mean±SEM. Differences were considered statistically significant for P<0.05. Student t tests were used when 2 groups were compared. Two-way ANOVAs were used when both genotype and treatment were taken into account, followed by Bonferroni posttests using Prism 5 (GraphPad Software for Science, San Diego, CA). All assessments were by blinded observers. Power analysis was completed using the POWER procedure in SAS 9.3 (Cary, NC).

Results

Infarction Volume and Neurological Deficits Are Increased in AT Mice

Infarct volumes at 24 and 48 hours post-MCAO were significantly larger (Figure 1A and 1B), and neurological score was...
significantly worse at 24 hours in AT compared with WT mice (Figure 1C). Physiological variables were not significantly different between WT and AT mice before, during MCAO, or 10 minutes after reperfusion (Table I in the online-only Data Supplement).

### Spontaneous Locomotor Activity Is Reduced in AT Mice After MCAO

Twenty-four and 48 hours after surgery, spontaneous activity was monitored using the SmartCage system. AT mice exhibited decreased activity during the dark phase, but did not differ from WT during light phase (our unpublished data). Consistent with this, light-phase activity measurements showed no difference between sham WT and AT mice. After MCAO, AT mice exhibited more profound and significant reductions in active time (Figure 2A) and distance traveled (Figure 2B) when compared with WT mice 24 and 48 hours post-MCAO. AT mice also exhibited a significant decrease in rearing activity, indicative of reduced exploration, compared with WT mice (Figure 2C). AT and WT mice had similar average velocities before and after MCAO (Figure 2D). Together, these results are consistent with the differences in neurological scores and infarct volumes observed between the genotypes (Figure 1).

### AT Mice Exhibit Increased Macrophage/Microglia and Neutrophil Infiltration After MCAO

Infiltration of macrophages and neutrophils is prominent after MCAO. Morphometric analysis revealed that the total number of activated macrophages/microglia significantly increased in the ischemic core (IC) of AT compared with WT mice (Figure 3A and 3B). However, no significant differences were observed in the cortical penumbra (WT=36.8±4.3 versus AT=39.0±2.0; P=0.65). The increased number of activated macrophages/microglia in the IC was associated with significantly increased infiltration of leukocytes, detected as myeloperoxidase (MPO)-positive cells (Figure 3A and 3C). MPO-positive cells were restricted to the IC.

### TNF-α and IL-6 mRNA Are Increased in Sham AT Compared With WT Mice

To assess levels of inflammatory cytokines acutely after sham and MCAO surgery (4 hours), reverse transcription quantitative real-time polymerase chain reaction was used to measure Ccl2, Ccl3, IL-10, IL-1α, IL-1β, IL-6, and TNF-α. After MCAO, these cytokines all markedly increased compared with sham, but there were no significant differences between genotypes (Table II in the online-only Data Supplement). Both IL-6 and TNF-α were found to be significantly higher in sham AT compared with sham WT (Table).

### Hcrt-1 Decreases Infarct Volume and Inflammation

Hcrt-1 administered either 30 minutes before or 30 minutes after MCAO significantly reduced infarct volume in WT and AT mice 48 hours after reperfusion (Figure 4A and 4B). While Hcrt-1 pretreated AT mice showed a significantly improved neurological score, pre- or posttreatment had no effect on neurological score of WT mice (Figure 4A and 4B). Hcrt-1 administration also decreased CD68+ cells in the IC but did not change the number of CD68+ cells in the cortical penumbra (Figure 4C).

### Hcrt-1 Attenuates Microglial TNF-α Production

Immunostaining of brains 48 hours after MCAO demonstrated that the only cells expressing Hcrt-1R were CD68+ microglia in the ischemic penumbra (Figure 5A) with little to no expression in the infarct core. Glial fibrillary acidic protein-positive astrocytes (Figure 5A) and neurons (data not shown) exhibited no detectable expression. To further investigate effects of Hcrt-1 on microglial response, we measured TNF-α levels in response to LPS, an inducer of inflammation. LPS exposure significantly increased expression of Hcrt-1R on BV2 microglial cells (Figure 5B and 5C). Untreated BV2 cells express TNF-α about the detection limit of our method, 2 to 5 pg/mL, whereas LPS treatment induced a very large increase in TNF-α production, ≈300-fold. When the cells were treated with Hcrt-1 1 hour before LPS stimulation, TNF-α production was significantly reduced ≈15% (Figure 5D).

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**Figure 1.** Infarct volume and neurological score are increased in orexin/ataxin-3 (AT) mice. **A**, Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal sections showing infarcts in wild-type (WT) (left) and AT (right) mice. **B**, Quantification of infarct volume expressed as a percent of hemispheric volume at 24 and 48 hours. **C**, Neuroscore was assessed 24 and 48 hours after middle cerebral artery occlusion (MCAO). Numbers in bars represent n/group. Two-way ANOVA revealed a significant genotype effect for both infarct size and neuroscore. Post hoc tests: *P*<0.05, **P**<0.01 compared with WT.
In the present study, using the transgenic AT mice, which develop normally but exhibit degeneration of Hcrt neurons in young adulthood, we found worsened outcome after experimental stroke. Increased infarct size correlated with more severe neurobehavioral deficits in the AT mice by both standard neurological scoring and automated quantitation of spontaneous activity. Because the velocity of the mice did not differ between genotypes, it is unlikely that the deficits in

**Discussion**

In the present study, using the transgenic AT mice, which develop normally but exhibit degeneration of Hcrt neurons in young adulthood, we found worsened outcome after experimental stroke. Increased infarct size correlated with more severe neurobehavioral deficits in the AT mice by both standard neurological scoring and automated quantitation of spontaneous activity. Because the velocity of the mice did not differ between genotypes, it is unlikely that the deficits in

**Figure 2.** Orexin/ataxin-3 (AT) mice have reduced light-phase spontaneous activity after middle cerebral artery occlusion (MCAO). AT mice showed significantly greater reductions in active time (A), travel distance (B), and rear-up counts (C), but no significant difference in average velocity compared with wild-type (WT) mice (D). For all panels, Sham n=5 to 7/group, MCAO n=18 to 22/group. Two-way ANOVA revealed significant genotype and surgery (Sham versus MCAO) differences for active time (24 and 48 hours), travel distance (24 and 48 hours), and rear-ups (24 hours, 48 hours only surgery effect). Two-way ANOVA revealed a significant surgery effect in average velocity at 24 hours. Post hoc tests: *P<0.05, **P<0.01, ***P<0.001 compared with WT.

**Figure 3.** Orexin/ataxin-3 (AT) mice exhibit increased numbers of activated macrophages/microglia and MPO-positive cells after middle cerebral artery occlusion (MCAO). A, The top image shows a representative coronal brain section with cresyl violet staining on which the squares represent the area where immunostaining was taken and cells were counted. The middle panels are representative immunofluorescence images of CD68-stained, and the bottom panels of MPO-stained, sections counterstained with 4',6-diamidino-2-phenylindole 48 hours after stroke in the ischemic core. B and C, Quantification of CD68- (B) and MPO-positive (C) cells in IC. n=5/group. Bar=50 μm. *P<0.05 versus wild type (WT). CP indicates cortical penumbra; and IC, ischemic core.
active time, distance traveled, and rearing activity are because of physical impairment in the AT mice. Instead, this likely reflects reduced alertness or neuropsychological impairment in the AT mice. Administration of Hcrt-1 reduces infarct size in both AT and WT mice, consistent with previous reports.\textsuperscript{20,21}

Importantly, we report here that Hcrt-1 treatment after MCAO effectively reduces infarct volumes. Improved neurobehavioral function was only seen in pretreated AT mice, suggesting that release of endogenous Hcrt during brain injury and reperfusion might reach a level to produce maximal functional improvement in WT mice under our experimental conditions, or that sensitivity to detect differences is reduced at 48 hours. Although our results are promising, lack of neuroscore improvement after posttreatment suggests that additional work is needed to optimize posttreatment and further assess whether Hcrt-1 treatment could be a potential therapy after stroke.

A further limitation of this study is the lack of activity assessment after Hcrt-1 treatment.

Thus far, a few potential mechanisms of Hcrt-induced protection against ischemia have been proposed. Administration of Hcrt restored hepatic and skeletal insulin receptor levels close to sham and decreased postischemic glucose intolerance.

<table>
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<th>Table. TNF-α and IL-6 mRNA Values in Sham and MCAO WT and AT Mice 4 Hours After Surgery</th>
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<td>WT 1.10±0.25</td>
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<td>AT 2.19±0.35*</td>
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Values shown are means±SEM, normalized to sham WT levels. n=3 to 5/group.

AT indicates orexin/ataxin-3; IL-6, interleukin-6; MCAO, middle cerebral artery occlusion; TNF-α, tumor necrosis factor-α; and WT, wild type.

\*P<0.05 compared with WT.

Figure 4. Administration of hypocretin-1/orexin-A (Hcrt-1) before or after middle cerebral artery occlusion (MCAO) protects both wild-type (WT) and orexin/ataxin-3 (AT) mice. A, Infarct volumes in WT mice decreased with Hcrt-1 treatment 30 minutes prior or after MCAO compared with vehicle (Veh) groups, n=6 to 15/group, but there was no improvement in neuroscore at 48 hours. B, Infarct volumes in AT mice decreased with Hcrt-1 treatment 30 minutes or after MCAO compared with Veh groups, n=6 to 15/ group, but there was no improvement in neuroscore at 48 hours.

Figure 5. The hypocretin/orexin (Hcrt) system affects inflammation. A, The Hcrt-1 receptor (Hcrt-1R) is expressed on CD68⁺ microglia (top panel), but not on glial fibrillary acidic protein-positive astrocytes (bottom panel) 48 hours after stroke. Bar=25 μm. B and C, Treatment of BV2 cells with lipopolysaccharide (LPS) increased Hcrt receptor expression. B, Fluorescence micrographs representative of cells treated with vehicle (left) or LPS (right) then immunolabeled for Hcrt-1R. Bar=25 μm. C, Quantification of Hcrt-1R immunostaining shows a significant increase after LPS treatment. n=5/group. D, Hcrt-1 treatment (100 nmol/L) significantly reduced the production of tumor necrosis factor-α (TNF-α) in BV2 cells stimulated by LPS. Values normalized to LPS treatment without Hcrt-1. n=16/group. \*P<0.05, \***P<0.001.
that leads to neuronal death.\textsuperscript{21} In addition, Hcrt-1 was shown to increase levels of protective hypoxia-induced factor-1α.\textsuperscript{20} Moreover, a study of gastrointestinal ischemia/reperfusion found that exogenous Hcrt-1 resulted in decreased lipid peroxidation and MPO-positive cells,\textsuperscript{3} in agreement with our results showing decreased neutrophils in the cortical IC with Hcrt-1 treatment.

In our study, CD68\textsuperscript{+} microglia in the cortical penumbra were the predominant cells expressing the Hcrt-1R, 48 hours after MCAO. This is in contrast to a previous study showing that reactive oxygen species can directly induce proinflammatory cytokine production.\textsuperscript{3} Also, Hcrt increases insulin receptor expression.\textsuperscript{21} Insulin has been identified as an anti-inflammatory mediator,\textsuperscript{23} suggesting that increases in insulin sensitivity by Hcrt may be partially responsible for Hcrt’s anti-inflammatory actions. Although there is no previous documentation of Hcrt-regulated changes in toll-like receptors or proinflammatory signaling, these are potential future directions to explore.

In conclusion, we have shown that the endogenous Hcrt system is protective against transient MCAO in mice, in part likely attributed to Hcrt-1–mediated anti-inflammatory actions. Our findings are consistent with previous studies suggesting that Hcrt can be a potentially useful therapeutic or element of a protective cocktail to reduce stroke-induced brain damage, even if given during reperfusion, and suggest further studies are needed to understand how Hcrt attenuates inflammation after ischemia.

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Disclosures

Dr Xie is the founder and a stockholder of AfaSci, Inc. The other authors have no conflicts to report.

References


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