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## Mitigation of murine focal cerebral ischemia by the hypocretin/orexin system is associated with reduced inflammation

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### Abstract

**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/ataxin-3 (AT) mice, a transgenic strain in which Hcrt-producing neurons degenerate in early adulthood, and wild type (WT) 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 to WT mice.

Macrophage/microglial infiltration and myeloperoxidase-positive cells were higher in AT compared to WT 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 WT and AT mice, but had no effect on neurological score in either genotype. Microglia express the Hcrt-1 receptor following MCAO. TNF $\alpha$  production by LPS-stimulated microglial BV2 cells was significantly reduced by Hcrt-1 pretreatment. Sham AT mice exhibit increased brain TNF $\alpha$  and IL-6 mRNA, suggesting chronic inflammation.

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**Conflict of interest:** X. Xie is the founder and a stockholder of AfaSci, Inc. All other coauthors declare no conflict of interest.

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**Conclusion**—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 following ischemic insult.

## Keywords

Hypocretin; orexin; brain ischemia; inflammation; neurobehavior

## Introduction

The hypocretin/orexin (Hcrt) neurons which produce Hcrt neuropeptides (Hcrt-1 and 2, i.e., orexin A and B) are localized in the hypothalamus.<sup>1,2</sup> They project broadly throughout the brain and mediate many physiological functions, including wakefulness and sleep, energy homeostasis, glucose metabolism, autonomic function,<sup>2-11</sup> and stress-adaptive responses such as stress-induced analgesia.<sup>12, 13</sup> Loss of Hcrt neurons or dysfunction in the Hcrt system has been observed in several disorders, including narcolepsy<sup>14, 15</sup> and subarachnoid hemorrhage.<sup>16</sup> Recently, a few studies have indicated that the Hcrt system may be involved in cerebral ischemic injury. Increased expression of the Hcrt-1 receptor on neurons, astrocytes, and oligodendrocytes was observed 48 hours following mouse global ischemia,<sup>17</sup> and in neurons 4-24 hours following permanent middle cerebral artery occlusion (MCAO) in rat.<sup>18</sup> Moreover, intracerebroventricular administration of Hcrt-1 before MCAO in rat<sup>19, 20</sup> and mouse<sup>21</sup> decreased the infarct size. Separately, recent work has found that lipopolysaccharide-induced lethargy may be due in part to damage of Hcrt-positive neurons,<sup>22</sup> suggesting that the Hcrt system may play a role in inflammatory processes.

Brain ischemia causes both immediate and delayed cell death and is accompanied by a robust inflammatory response that can exacerbate injury during reperfusion. In the present study we tested whether endogenous Hcrt-producing neurons promote neuroprotection following 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 to WT mice. mRNA analysis revealed higher levels of both TNF $\alpha$  and 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 (wildtype C57/BC6 and orexin/ataxin-3 mice, 3-5 months old, 25-35g) were used. While 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.<sup>23</sup> Details of strain production and animal care can be found in Supplemental Materials and Methods.

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 Supplemental Table 3.

### **Focal cerebral ischemia**

Anesthesia was induced with 4% isoflurane and maintained by 1.5-2% isoflurane in 70% air and balanced oxygen by a facemask. Rectal temperature was maintained at  $37 \pm 0.5^\circ \text{C}$  with a heating pad (Harvard Apparatus, Hollister, MA). Transient focal ischemia was induced by middle cerebral artery occlusion (MCAO) for 60 min, which generates infarction in both cortex and striatum, as previously described.<sup>24-26</sup> Details of surgery protocol can be found in Supplemental Materials and Methods. 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,<sup>25, 26</sup> 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.<sup>26, 27</sup> The homepage 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 min during the light phase, 24 and 48 hours following reperfusion.

### **Measurement of cerebral infarction area**

Twenty-four or 48 hours after MCAO and immediately following neuroscore 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-triphenyletrazolium chloride (TTC, #T8877, Sigma-Aldrich, St. Louis, MO) and infarction core volume as defined by an absence of TTC staining (percent of hemispheric 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.<sup>25, 26</sup>

### **Immunofluorescence**

Ischemic or sham-operated mice were euthanized with an overdose of isoflurane and perfused with ice-cold phosphate-buffered saline (PBS; pH 7.4) 48 hours after MCAO, followed by 4% paraformaldehyde in PBS as previously described.<sup>28</sup> Brains were removed and post-fixed for 72 hours in 4% paraformaldehyde in PBS and cut into 50  $\mu\text{m}$  coronal sections. Details of the immunofluorescence protocol, including antibodies used and cell counting protocol, can be found in Supplemental Materials and Methods.

### **Reverse Transcription Quantitative real-time Polymerase Chain Reaction (RT-qPCR) for mRNA quantitation**

Total RNA was isolated with TRIzol® (Invitrogen) from the ischemic hemisphere (from +0.8 to -1.2mm relative to bregma) of WT or AT mice 4 hours following 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 ( $\Delta\text{Ct}$ ) and the comparison calculated as the inverse log of  $\Delta\Delta\text{Ct}$  to give relative fold change value.

### Treatment with recombinant Hcrt-1 in vivo

Hcrt-1 was injected intracerebroventricularly (icv) as previously described.<sup>26</sup> Two  $\mu$ l of either vehicle (0.1% bovine serum albumin in 0.9% PBS) or containing 2nmol of Hcrt-1 dissolved in the vehicle was infused over 10 min into the left lateral ventricle 30 min before or after MCAO. After 48 hours of reperfusion neurological score was determined, animals were sacrificed, and brains removed for TTC staining, as described above.

### Measurement of TNF $\alpha$ production by BV2 cells

BV2 microglial cells were treated with 10 ng/ml lipopolysaccharide (LPS, Sigma) for 24 hours and fixed with 4% paraformaldehyde. Details of immunocytochemistry protocol, including quantification, can be found in Supplemental Materials and Methods.

BV2 cells were treated with control media or media containing Hcrt-1 (100 nM) for 1 hour prior to treatment with LPS (10 ng/ml). Four hours following LPS treatment, supernatant was collected and TNF $\alpha$  measured using the TNF $\alpha$  Mouse ELISA Kit (Life Technologies). Cell number was assessed by DAPI staining and counting with NIH ImageJ. Fluorescence images were acquired at 2.5 $\times$  magnification. TNF $\alpha$  measurements were normalized to cell number.

### Statistical Analyses

Data is expressed as mean  $\pm$  SEM. Differences were considered statistically significant for  $p < 0.05$ . Student's  $t$ -tests were used when two groups were compared. Two-way ANOVAs were used when both genotype and treatment were taken into account, followed by Bonferroni post-tests 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,B) and neurological score was significantly worse at 24 hours in AT compared to WT mice (Figure 1C). Physiological variables were not significantly different between WT and AT mice before, during MCAO, or 10 min after reperfusion (Supplemental Table 1).

### Spontaneous locomotor activity is reduced in AT mice after MCAO

Twenty-four and 48 hours after surgery, spontaneous activity was monitored using the SmartCage<sup>TM</sup> 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. Following MCAO AT mice exhibited more profound and significant reductions in active time (Figure 2A) and distance traveled (Figure 2B) when compared to WT mice 24 and 48 hours post MCAO. AT mice also exhibited a significant decrease in rearing activity, indicative of reduced exploration, compared to 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 following MCAO.<sup>29</sup> Morphometric analysis revealed that the total number of activated macrophages/microglia significantly

increased in the ischemic core (IC) of AT compared to WT mice (Figure 3A,B). However, no significant differences were observed in the cortical penumbra (WT =  $36.8 \pm 4.3$  vs AT =  $39.0 \pm 2.0$ ,  $P = 0.65$ ). The increased number of activated macrophages/microglia in the IC was associated with significantly increased infiltration of leukocytes, detected as MPO-positive cells (Figure 3A,C). MPO positive cells were restricted to the IC.

### **TNF $\alpha$ and IL-6 mRNA are increased in sham AT compared to WT mice**

To assess levels of inflammatory cytokines acutely after sham and MCAO surgery (4 hours),<sup>30</sup> RT-qPCR was used to measure Ccl2, Ccl3, IL-10, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and TNF $\alpha$ . Following MCAO, these cytokines all markedly increased compared to sham, but there were no significant differences between genotypes (Supplemental Table 2). Both IL-6 and TNF $\alpha$  were found to be significantly higher in sham AT compared to sham WT (Table 1).

### **Hcrt-1 decreases infarct volume and inflammation**

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

### **Hcrt-1 attenuates microglial TNF $\alpha$ 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. GFAP+ 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 $\alpha$  levels in response to LPS, an inducer of inflammation. LPS exposure significantly increased expression of Hcrt-1R on BV2 microglial cells (Figure 5B,C). Untreated BV2 cells express TNF $\alpha$  about the detection limit of our method, 2-5 pg/ml, while LPS treatment induced a very large increase in TNF $\alpha$  production, approximately 300-fold. When the cells were treated with Hcrt-1 1 hour prior to LPS stimulation TNF $\alpha$  production was significantly reduced approximately 15% (Figure 5D).

## **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 following experimental stroke. Increased infarct size correlated with more severe neurobehavioral deficits in the AT mice, by both standard neurological scoring<sup>26</sup> and automated quantitation of spontaneous activity. Because the velocity of the mice did not differ between genotypes, it is unlikely that the deficits in active time, distance traveled and rearing activity are due to 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.<sup>21,20</sup> 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 post-treatment suggests that additional work is needed to optimize post-treatment and further assess whether Hcrt-1 treatment could be a potential

therapy following 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 post-ischemic glucose intolerance that leads to neuronal death.<sup>21</sup> In addition, Hcrt-1 was shown to increase levels of protective hypoxia-induced factor-1 $\alpha$ .<sup>20</sup> Moreover, a study of gastrointestinal ischemia/reperfusion found that exogenous Hcrt-1 resulted in decreased lipid peroxidation and MPO+ cells,<sup>31</sup> in agreement with our results showing decreased neutrophils in the cortical ischemic core with Hcrt-1 treatment.

In our study CD68+ 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 neurons and some glial cells expressed the Hcrt-1R 24 hours after permanent MCAO in rat.<sup>18</sup> These discrepancies may be due to differences in time course of expression, species, or antibodies used.

In light of our immunohistochemical findings, including a marked increase in activated microglia in the ischemic core in the AT mice, we hypothesized that endogenous Hcrt-1 may regulate acute inflammation, thereby contributing to its neuronal protective properties. Indeed, microglial BV2 cells pretreated with Hcrt-1 exhibited decreased LPS-induced TNF $\alpha$  production. These data, along with the reduction of MPO- and CD68-cell counts with Hcrt-1 treatment *in vivo*, suggest that Hcrt-1 can be anti-inflammatory, which may complement other postulated neuroprotective mechanisms mentioned above.<sup>20, 21</sup>

Currently, the mechanism by which Hcrt-1 affects inflammation is unknown. Although previous studies have shown that inflammatory agents, such as LPS, decrease the activity of Hcrt-positive neurons in the hypothalamus,<sup>22</sup> TNF $\alpha$ -R-deficient mice have increased expression of Hcrt mRNA,<sup>32</sup> and treatment of B35 neuroblastoma cells with TNF $\alpha$  decreases Hcrt precursor half-life.<sup>32</sup> The present study is the first to directly assess the effects of Hcrt-1 on inflammatory responses following cerebral ischemia. One possible mechanism underlying Hcrt modulation of inflammation is via Hcrt's antioxidant effects.<sup>31</sup> Recent work has suggested that reactive oxygen species can directly induce pro-inflammatory cytokine production.<sup>33</sup> Also, Hcrt increases insulin receptor expression.<sup>21</sup> Insulin has been identified as an anti-inflammatory mediator,<sup>34</sup> 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 pro-inflammatory 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 following ischemia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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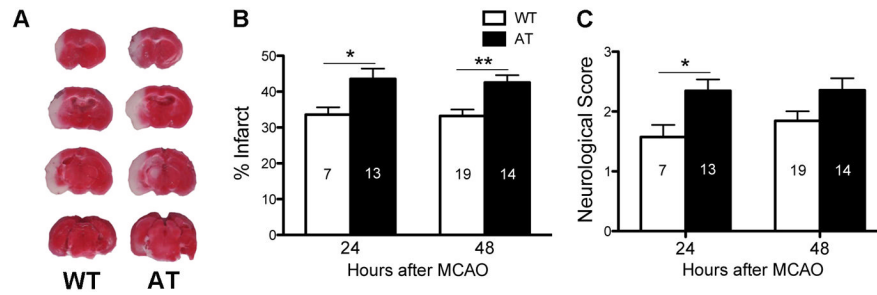
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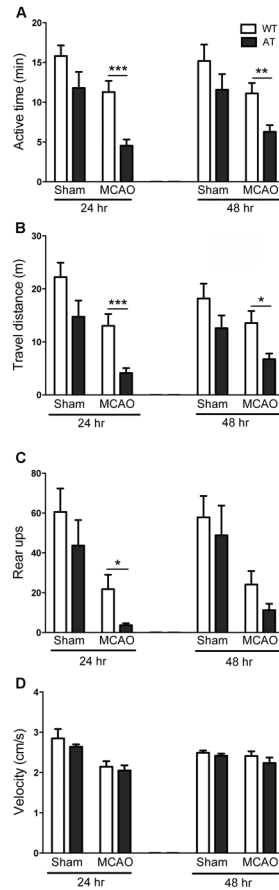
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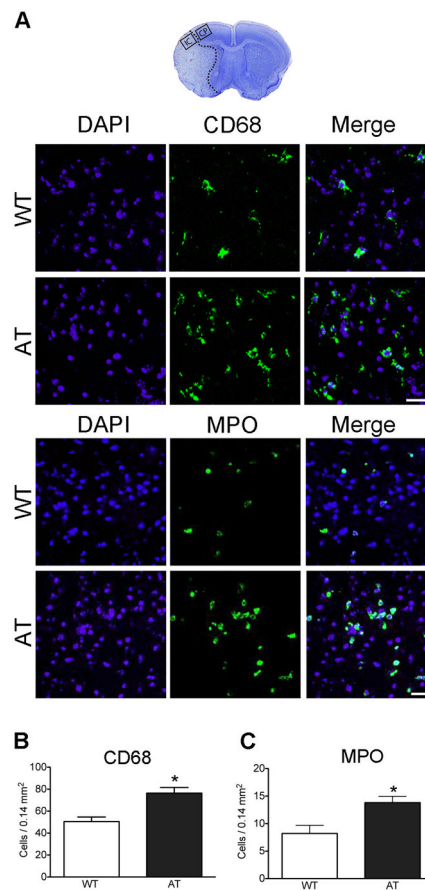


**Fig. 1.** Infarct volume and neurological score are increased in AT mice. (A) Representative TTC-stained coronal sections showing infarcts in WT (left) and AT (right) mice. (B) Quantification of infarct volume expressed as a percent of hemispheric volume at 24 and 48 hours. (C) Neuroscore was assessed 24 and 48 hours after 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 to WT.

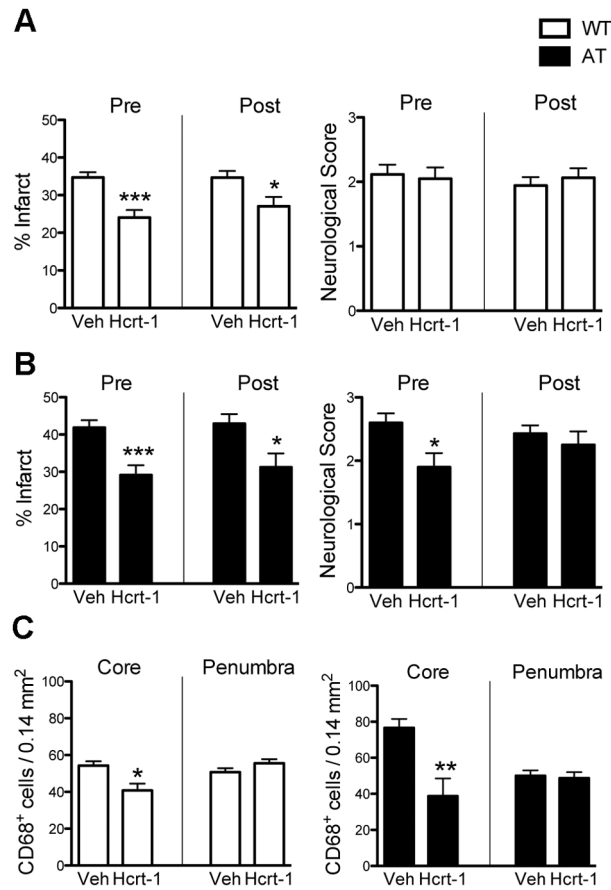


**Fig. 2.**

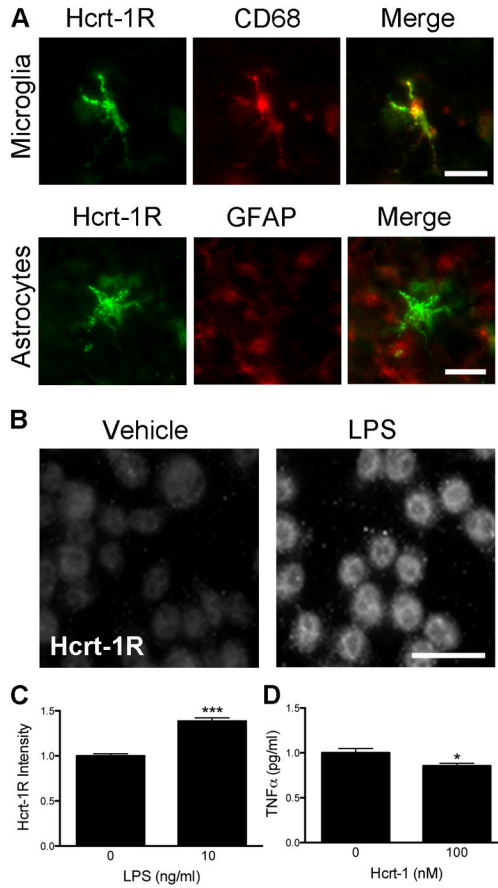
AT mice have reduced light-phase spontaneous activity after MCAO. AT mice showed significantly greater reductions in active time (A), travel distance (B), and rear up counts (C), but no significant difference in average velocity compared to WT (D). For all panels, Sham n=5-7/group, MCAO n=18-22/group. Two-way ANOVA revealed significant genotype and surgery (Sham vs. 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 24h. Post hoc tests: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to WT.



**Fig. 3.** AT mice exhibit increased numbers of activated macrophages/microglia and MPO-positive cells after MCAO. (A) The top image shows a representative coronal brain section with cresyl violet staining on which the squares represent the area where pictures of immunostaining were taken and cells were counted (IC = ischemic core, CP = cortical penumbra). The middle panels are representative immunofluorescence images of CD68-stained, and the bottom panels of MPO-stained, sections counterstained with DAPI 48 hours after stroke in the ischemic core. (B-D) Quantification of CD68- (B) and MPO- (C) positive cells in IC.  $n=5$ /group. Bar=50 $\mu$ m. \*  $P<0.05$  vs. WT.



**Fig. 4.** Administration of Hcrt-1 before or after MCAO protects both WT and AT mice. (A) Infarct volumes in WT mice decreased with Hcrt-1 treatment 30 min prior or after MCAO compared to vehicle groups,  $n=6-15/\text{group}$ , but there was no improvement in neuroscore at 48 hr. (B) Infarct volumes in AT mice decreased with Hcrt-1 treatment 30 min or after MCAO compared to vehicle groups, neuroscore was only decreased by Hcrt-1 pretreatment compared to vehicle in AT mice. (C) Hcrt-1 decreased CD68+ cells in the ischemic core in WT and AT mice compared to vehicle, but did not change counts in the penumbra in either genotype,  $n=3-6/\text{group}$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to Vehicle (Veh).



**Fig. 5.** The Hcrt system affects inflammation. (A) The Hcrt-1R receptor is expressed on CD68-positive microglia (top panel), but not on GFAP-positive astrocytes (bottom panel) 48 hours after stroke. Bar=25 $\mu$ m. (B-C) Treatment of BV2 cells with 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 $\mu$ m. (C) Quantification of Hcrt-1R immunostaining shows a significant increase following LPS treatment. n=5/group. (D) Hcrt-1 treatment (100 nM) significantly reduced the production of TNF $\alpha$  in BV2 cells stimulated by LPS. Values normalized to LPS treatment without Hcrt-1. n=16/group. \* P < 0.05, \*\*\* P < 0.001.

**Table 1**TNF $\alpha$  and IL-6 mRNA values in sham and MCAO WT and AT mice 4 hours following surgery.

	<b>Sham TNF<math>\alpha</math></b>	<b>MCAO TNF<math>\alpha</math></b>	<b>Sham IL-6</b>	<b>MCAO IL-6</b>
WT	1.10 $\pm$ 0.25	42.97 $\pm$ 13.99	1.04 $\pm$ 0.14	10.04 $\pm$ 2.36
AT	2.19 $\pm$ 0.35 *	34.81 $\pm$ 11.90	1.58 $\pm$ 0.13 *	18.67 $\pm$ 6.58

Values shown are mean  $\pm$  SEM, normalized to sham WT levels. n=3-5/group.

\* P &lt; 0.05 compared to WT.