Supplemental Materials and Methods

Animals

Animals were group-housed under standard laboratory conditions and kept on a normal light/dark cycle. All studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees at Stanford University and AfaSci, Inc., and were conducted according to the NIH Guide for Care and Use of Laboratory Animals. AT and WT littermates were kindly provided by SRI International (Menlo Park, CA). The procedure for producing the transgenic mice was described by Hara et al.[1] Founder animals were backcrossed with C57BL/6 mice for more than 10 generations. Presence and copy numbers of the transgene in the offspring were identified by polymerase chain reaction analysis of tail DNA.

Focal cerebral ischemia

To induce focal cerebral ischemia, we introduced a silicone-coated 6-0 monofilament into the left external carotid artery and advanced it from the carotid bifurcation to occlude the MCA. Anesthesia was discontinued after suture insertion. Mice were re-anesthetized 60 min later and the filament was withdrawn. Heart rate, oxygen saturation, and respiratory rate were monitored continuously (STARR Life Sciences Corp., Allison Park, PA). Mean blood pressure was measured using the CODA computerized non-invasive blood pressure system (Kent Scientific, Torrington, CT). Sham-operated mice underwent an identical procedure, except that the suture was not inserted.

Immunofluorescence

Immunofluorescent staining was carried out on free-floating sections under moderate shaking. All washes and incubations were done in 0.1M PBS containing 0.1% triton X-100 (Sigma-Aldrich, Co. St. Louis, MO). Sections were incubated for 1 hour with blocking solution (0.1M PBS, 0.1% Triton X-100 and 5% normal goat serum), then incubated for 48 hours at 4ºC with goat anti-orexin-B antibody (diluted 1:100, #SC-8071, Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-Orexin receptor 1 (diluted 1:100, #sc-8073, Santa Cruz Biotechnology, Inc.), rat anti-CD68 antibody (reactive macrophages/microglia, diluted 1:100; MCA1957GA, AbD Serotec, Kidlington, Oxford, UK), rabbit anti-human myeloperoxidase antibody (MPO, neutrophils, diluted 1:50, #A0398, DAKO, Carpinteria, CA), or rabbit anti-GFAP (astrocytes, diluted 1:2000, #Z0334, DAKO). Sections were then rinsed and incubated for 2 hours at room temperature with Alexa Fluor 488-conjugated donkey anti-goat (orexin-B, orexin receptor-1), goat anti-rabbit (MPO), or goat anti-rat (CD68), all diluted 1:400 (Invitrogen, Carlsbad, CA). The sections were then washed and mounted on glass slides using Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Negative control experiments without primary antibodies were performed in parallel.

The number of CD68- or MPO-positive cells were counted using the optical fractionator method on epifluorescent photomicrographs (Zeiss Axiovert Inverted Fluorescent Microscope, Zeiss, Germany) covering 0.14 mm^2. For each animal, the number of CD68- and MPO-immunoreactive cells in the ischemic core or cortical penumbra (-1.70 to -2.18 relative to Bregma) was counted using ImageJ software as

described previously.[2] Representative micrographs were photographed at 40x magnification using a digital camera attached to a Zeiss LSM 510 META inverted laser scanning confocal microscope (Zeiss, Germany).

Immunocytochemistry

Cells were incubated for 1 hour in blocking solution, followed by overnight incubation at 4°C in blocking solution containing rabbit anti-Orexin receptor 1 (1:100, Santa Cruz Biotechnology Inc.). Following washes, cells were incubated for 2 hours at room temperature in blocking solution containing AlexaFluor 594 goat anti-rabbit secondary antibody (1:400, Invitrogen) and DAPI (1:500; Invitrogen). Immunofluorescence intensity was measured using the FLEXstation II-384 fluorescent plate reader, and intensity of Hcrt-1R immunofluorescence was normalized to DAPI fluorescence to account for differences in cell number.

Supplemental References

1. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron*. 2001;30:345-354.

2. Barreto GE, Sun X, Xu L, Giffard RG. Astrocyte proliferation following stroke in the mouse depends on distance from the infarct. *PLoS One*. 2011;6:e27881.

Supplemental Table 1. Physiological measurements in wildtype and transgenic orexin/ataxin-3 mice

	WT (n=7)	AT (n=7)
Before MCAO		
Heart rate (bpm)	452.3 ± 62.5	456.45 ± 44.1
Respiratory rate (per min)	81.6 ± 25.4	93.1 ± 31.5
SpO2 (%)	98.2 ± 0.4	98.5 ± 0.3
Temperature (°C)	37.4 ± 0.73	37.3 ± 0.57
MBP (mmHg)	92.3 ± 6.8	94.5 ± 5.9
During MCAO		
Heart rate (bpm)	470.5 ± 50.9	442.97 ± 43.1
Respiratory rate (per min)	59.4 ± 18.1	65.6 ± 24
SpO2 (%)	98.5 ± 0.5	97.8 ± 2.1
Temperature (°C)	37.1 ± 0.25	37.53 ± 0.64
MBP(mmHg)	88.7 ± 17.6	99.2 ± 8.1
After MCAO		
Heart rate (bpm)	472.1 ± 51.9	407.2 ± 37.8
Respiratory rate (per min)	76.4 ± 7.9	72.3 ± 19.6
SpO2 (%)	98.7 ± 0.4	98.6 ± 0.4
Temperature (°C)	37.0 ± 0.80	37.3 ± 0.40
MBP(mmHg)	94.3±9.4	97.8±9.5

Values are mean ± SEM. WT = wild-type mice; AT = transgenic orexin/ataxin mice; Before MCAO = 10 min before MCAO; During MCAO = 10 minute after MCAO; After MCAO= 10 min after reperfusion; bpm = beats per minute; SpO_2 = oxygen saturation; MBP = mean blood pressure. There were no significant differences between genotypes.

Supplemental Table 2. mRNA values in sham and MCAO WT and AT mice 4h following surgery.

	WT Sham	AT Sham	WT MCAO	AT MCAO
Ccl2	1.13 ± 0.32	1.56 ± 0.31	42.84 ± 10.82	63.98 ± 20.19
Ccl3	1.35 ± 0.59	2.66 ± 0.09	82.32 ± 43.11	41.90 ± 15.46
IL-1α	1.08 ± 0.20	1.62 ± 0.13	5.63 ± 2.40	6.40 ± 1.69
IL-1β	1.15 ± 0.27	1.53 ± 0.26	10.82 ± 2.78	13.50 ± 4.15
IL-10	1.04 ± 0.16	1.42 ± 0.36	116.5 ± 115.1	7.07 ± 2.82

Values shown are mean ± SEM, normalized to sham WT levels. n=3-5/group. Ccl2 = chemokine (C-C Motif) ligand 2; Ccl3 = chemokine (C-C Motif) ligand 3; IL-1α = interleukin 1 alpha; IL-1β = interleukin 1 beta; IL-10 = interleukin 10. No significant differences were found in these cytokines between genotypes.

Supplemental Table 3. Number of animals excluded from analyses.

Genotype/Treatment	Time	#Surgeries	#Dead	#Fail	#Included
WT/none	24h	11	2	2	7
AT/none	24h	17	3	1	13
WT/none	48h	21	1	1	19
AT/none	48h	17	2	1	14
WT/pre Hcrt-1	48h	16	8	1	7
WT/pre Vehicle	48h	15	3	2	10
AT/pre Hcrt-1	48h	16	7	3	6
AT/pre Vehicle	48h	16	2	3	11
WT/post Hcrt-1	48h	11	2	1	8
WT/post Vehicle	48h	11	2	0	9
AT/post Hcrt-1	48h	8	2	0	6
AT/post Vehicle	48h	8	1	0	7
Total		167	35	15	117

Pre/post indicates pre- or post-MCAO treatment with vehicle or Hcrt-1, and time indicates time of perfusion. #Surgeries = the number of animals that underwent MCAO surgery, #Dead = the number of animals that died before time of perfusion, #Fail = the number of animals that had a normal neurological score of 0 at the time of perfusion, #Included = the number of animals used in analyses.