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Carbon Monoxide Preserves Circadian Rhythm to Reduce the Severity of Subarachnoid Hemorrhage in Mice

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Abstract

Background and Purpose—Subarachnoid hemorrhage (SAH) is associated with a temporal pattern of stroke incidence. We hypothesized that natural oscillations in gene expression controlling circadian rhythm impact the severity of neuronal injury. We moreover predict that heme oxygenase-1 (HO-1/*Hmox1*) and its product carbon monoxide (CO) contribute to restoration of rhythm and neuroprotection.

Methods—Murine SAH model where blood was injected at various time points of the circadian cycle. Readouts included circadian clock gene expression, locomotor activity, vasospasm, neuroinflammatory markers, and apoptosis. Additionally, cerebrospinal fluid and peripheral blood leukocytes from SAH patients and controls were analyzed for 'clock gene' expression.

Results—Significant elevations in the clock genes *Per-1*, *Per-2* and *NPAS-2* were observed in the hippocampus, cortex and suprachiasmatic nucleus (SCN) in mice subjected to SAH at zeitgeber time (ZT) 12 when compared to ZT2. Clock gene expression amplitude correlated with basal expression of HO-1, which was also significantly greater at ZT12. SAH animals showed a significant reduction in cerebral vasospasm, neuronal apoptosis, and microglia activation at ZT12 compared to ZT2. In animals with myeloid-specific HO-1 deletion (*Lyz-Cre-Hmox1*$^{fl/fl}$) *Per-1, Per-2,* and *NPAS-2* expression was reduced in the SCN, which correlated with increased injury. Treatment with low dose CO rescued *Lyz-Cre-Hmox1*$^{fl/fl}$ mice, restored *Per-1, Per-2 and NPAS-2* expression and reduced neuronal apoptosis.

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Disclosures

LEO is a scientific advisor for Hillhurst Biopharmaceuticals.

Conclusions—Clock gene expression regulates, in part, the severity of SAH and requires myeloid HO-1 activity to clear the erythrocyte burden and inhibit neuronal apoptosis. Exposure to CO rescues the loss of HO-1 and thus merits further investigation in patients with SAH.

Keywords

Circadian Rhythm; Subarachnoid Hemorrhage; Carbon Monoxide; Brain Injury

Subject Codes

[130] Animal models of human disease; [50] Cerebral Aneurysm, AVM, & Subarachnoid hemorrhage

Introduction

There is a striking rhythm in hemorrhagic stroke onset across the 24-hour day.[1, 2] We questioned whether the response to SAH would also vary as a function of time of day. Whether this temporal pattern of occurrence has implications for stroke severity and outcome remains unclear. This daily rhythm of stroke occurrence cannot be attributed to external factors like physical activity, which suggests that the central circadian clock may be implicated in the pathophysiology of stroke.[3] The central circadian clock is located in the hypothalamic suprachiasmatic nuclei (SCN) and provides temporal organization for virtually all biochemical, neurobiologic and physiologic processes.[4] By tracking the solar day, the SCN is able to synchronize rhythmicity throughout the body with the external environment.[5,6]

This robustness of circadian rhythms can be explained, in part, by the oscillating expression of genes that form transcriptional, translational, and post-translational negative feedback loops that govern rhythmicity.[7] This family of clock genes consists of several transcription factors including *CLOCK, BMAL-1, RevErb-α,* and *NPAS-2* as well as regulatory proteins including *Period (Per) 1–3* and *Cryptochrome (Cry) 1–2.* Disturbance or deficiency in the activity of these transcription factors leads to pathophysiology in the cardiovascular system[8] and the brain,[9] whereas their stabilization is protective.[8] Since there are distinct gene expression cycles that occur throughout the course of a day, it is tempting to speculate that these differences in expression levels represent a key determinant of the likelihood for a stroke occurrence as well as the severity of tissue injury.

A remarkable link has been shown to exist between the circadian auto-regulatory feedback loop and the endogenous bioactive gas carbon monoxide (CO), which is produced ubiquitously by the body, including the brain, by the heme oxygenase (HO). Indeed, *NPAS-2* and *RevErb-α* contain heme, thus their turnover involves the degradation of heme but also results in the generation of CO and the bile pigments, biliverdin and bilirubin as breakdown products. We have shown that the transcription factor *RevErb-α* plays an important role in regulation of endothelial cell migration and is modulated in part by CO.[10] Transcriptional activity of *NPAS-2*[11] and *CLOCK*[12], two of the transcription factors that control expression of *Per* and *Cry*, have been shown to be CO-dependent by a heme-based gas sensor mechanism.

We therefore hypothesized that SAH would lead to disturbances of both central and peripheral rhythmicity in mice. We further predicted that the time of day of SAH occurrence would impact overall sensitivity to neuronal injury. Finally, we asked whether the inducible isoform of HO HO-1, and CO are critical determinants of the severity of neuronal injury after SAH, and if so, does this link to an influence on clock gene expression.

Materials and Methods

Animals

Wild-type male C57BL/6, *Lyz-Cre*, and *Per-2*$^{-/-}$ mice were obtained from the Jackson Laboratory. Myeloid specific HO-1 knockout (*Lyz-Cre-Hmox1*$^{fl/fl}$) was achieved by crossing *Hmox1*$^{fl/fl}$ mice (Riken) with mice expressing Cre recombinase under the lysozyme (Lyz) promoter.

Subarachnoid Hemorrhage (SAH) Stroke Model in Mice

SAH was modeled as described previously[13] and in the online supplement. Sham treatment consisted of the same surgical procedure, without injection of blood. SAH and Sham surgery were performed at zeitgeber time (ZT)0 (the beginning of the 12h light cycle), ZT2, or ZT12.

CO Gas Treatment

After SAH, *Lyz-Cre-Hmox1*$^{fl/fl}$ and *Hmox1*$^{fl/fl}$ animals were randomly assigned to receive treatment with CO (250ppm) or air for 1h as described.[14] Treatment was started immediately after the SAH procedure and repeated every 24h for 7 days.

Hematoxylin/Eosin Staining and Evaluation of Cerebral Vasospasm

Frozen brains were cut in 9μm serial coronal sections and stained with hematoxylin and eosin. Representative digital images of three consecutive middle-cerebral artery (MCA) cross-sections from each animal were obtained and the lumen radius/wall thickness ratio was quantified to assess vasospasm using ImageJ.

Immunohistochemistry, TUNEL Staining and Western blot

9μm serial coronal sections were stained for ionized calcium binding adaptor molecule 1 (Iba-1) (Wako) and conjugated with the corresponding secondary antibody for fluorescent imaging (Alexa Fluor 488 donkey anti-mouse; Invitrogen) as specified in the online supplement. Nuclear counterstain was done with Hoechst 33258 (Sigma). TdT-mediated dUTP-biotin nick end labeling (TUNEL; Roche) was done as specified in the online supplement and previously reported.[15] Western Blot from mouse brains was performed as described previously[15] and as described in the online supplement.

Gene expression analysis

RNA was obtained and processed as specified in the online supplement. cDNA libraries were acquired by reverse transcription (iScript cDNA synthesis kit, BioRad). Gene expression was analyzed by real-time (rt)-PCR (SYBR green master mix, Agilent

Technologies) with *Rplp0* as a reference gene. Primer sequences are detailed in the online supplement.

Human sample collection and analysis

After acquiring patient consent, CSF and blood samples were obtained from SAH patients admitted to the intensive care unit (ICU) on day 1 and day 7. Patients with unruptured cerebral aneurysms served as controls, and therefore CSF samples in controls were only acquired once, during surgery. RNA from cerebrospinal fluid (CSF) cells was isolated with Trizol, concentrated by spin-column purification (RNEasy Micro Kit, Qiagen) and cDNA libraries were acquired by reverse transcription (iScript cDNA synthesis kit, BioRad). Gene expression of *Per-2* was analyzed by rt-PCR with *Rpl13a* as a reference gene. Primer sequences are detailed in the online supplement.

Statistics

Data were analyzed with a computerized statistical program (GrapPad Prism Version 5). Results are presented as means (±SD). Two groups were compared with Student's t-test, while multiple groups were compared with one-way ANOVA with post hoc Bonferroni multiple comparison. A p-value of <0.05 was considered to be statistically significant.

Study Approval

All procedures involving the animals were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center, Boston, MA. Patients were studied under a protocol for acquiring CSF and blood samples that was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center. Patients or their legally authorized representative provided written informed consent.

Results

Cerebrospinal fluid (CSF) from subarachnoid hemorrhage (SAH) patients shows increased and sustained Per-2 expression

We first looked for gene expression patterns indicating disruption of normal rhythmicity in patients with SAH. On day 1 after SAH onset, *Per-2* expression in cells isolated from the CSF was significantly higher as compared to control patients with unruptured aneurysm (Figure 1A). Though there was a trend for the same effect on day 7, higher variability in gene expression levels likely prevented these results from achieving statistical significance in part due to restoration of circadian rhythms in some patients (Figure 1B, p=0.09). *Per-2* expression in peripheral blood leukocytes was unchanged in all groups (Figures 1C–D).

SAH disturbs circadian-dependent activity patterns

To study the effect of SAH on circadian-dependent motor activity, we chose *Per-2*$^{-/-}$ mice. These mice were housed and treated in continuous darkness (DD, free running conditions) because under these conditions, mice exhibit a shortened circadian rhythm that is easy to manipulate. DD was also used to eliminate the possibility of light entrainment that would override SAH-related changes. *Per-2*$^{-/-}$ mice housed in DD before SAH showed a shortened

circadian cycle of 22.3h (Figure 2A). After SAH, the circadian period of activity pattern was approximately 30 minutes longer as compared to pre-SAH (Figure 2B–C).

SAH increases central and peripheral circadian rhythm gene expression

We next asked whether SAH in mice leads to disruption of clock gene expression in the brain and peripheral organs. SAH was initiated at ZT 0 and gene expression was analyzed over time. Compared to control samples taken at the same ZT times, the expression of *Per-2* was induced early after SAH (Suppl. Figures IA–B). Similar effects were observed for *Per-1* and *NPAS-2* (data not shown). This increase in expression led to disruption of central and peripheral organ rhythmicity, as the normal cyclic change in expression of *Per-2*, *Per-1*, *BMAL-1*, and *NPAS-2* was disrupted in the brain and kidney after SAH (Suppl. Figures IC–D, Suppl. Figure II).

Time of the day influences circadian gene and heme oxygenase (HO)-1 expression

The naturally occurring oscillation of circadian rhythm gene expression as well as the fact that this rhythm is disrupted after SAH, prompted us to next study the influence of different SAH onset times on circadian gene expression. For this purpose, SAH and Sham operations were performed at either ZT2 (nadir of *Per*- and *Cry*-gene expression) or ZT12 (peak), and circadian rhythm gene expression was evaluated after 7 days. Animals with SAH occurring at ZT12 showed higher expression levels of *Per-1*, *Per-2*, *NPAS-2*, and *CLOCK* in the hippocampus (HC) and cortex as compared to animals with SAH at ZT2 (Figures 3A–C; Suppl. Figure IVA). Two transcription factors involved in the circadian rhythm feedback loop (*NPAS-2, CLOCK*) are modulated by the bioactive gas carbon monoxide that is generated by HO-1. Therefore, we investigated whether HO-1 expression in the brain also follows a circadian pattern. Compared to cerebral HO-1 expression at ZT2, expression at ZT12 was markedly induced (Figure 3D).

Time of day influences susceptibility to hemorrhage-induced vasospasm and neuronal injury

We next investigated whether the differences in circadian rhythm gene expression would alter the severity of vasospasm and neuronal injury after SAH. We measured vasospasm of the middle cerebral artery (MCA) on SAH post-operative day (POD) 7 via H&E staining (Figure 4A). SAH performed at either ZT2 or ZT12 induced vasospasm as compared to sham, but vasospasm was most severe in animals with SAH at ZT2 (Figure 4B). We also analyzed whether changes in circadian rhythm genes and the greater extent of vasospasm at ZT2 would result in increased neuronal cell death. SAH at both zeitgeber times showed increased neuronal apoptosis in the dentate gyrus (DG) and *Cornu Ammonis* (CA) regions of the HC as well as the cortex, when compared to sham-treated mice (Figures 4C–D and Suppl. Figures IIIA–B). . Importantly, neuronal apoptosis was markedly higher when SAH was initiated at ZT2, as compared to ZT12 (No. TUNEL-positive cells; DG 21 ±3.5 vs. 15 ±2.6; CA 18±2.0 vs. 10 ±1.0; Cortex 42 ±3.1 vs. 28 ±4.6).

The extent of neuroinflammation induced by SAH depends on the time of day

Microglia, the tissue resident macrophage of the brain, respond to injurious stimuli with changes in morphology, surface molecule expression, and migration to the site of injury. Microglial-induced neuroinflammation has been found critical to the pathology of many neurological diseases, including hemorrhagic stroke. We therefore analyzed morphological changes to microglia by immunostaining for Iba-1 surface expression in different brain regions at ZT2 and ZT12 ± SAH, which at these time points we believe are consistent for microglia. At ZT2, microglia showed a more activated morphology as compared to ZT12 with elongated cell bodies and thicker processes (Figures 5A–D). Furthermore, in the peri-hematoma region at the base of the brain, Iba-1 positive microglia were much more abundant at ZT2 compared to ZT12, indicating a higher degree of microglia migration to the site of injury. We conclude that the time of day when the injury occurs directly impacts the severity of the tissue injury.

Microglial HO-1 and CO modulate circadian rhythm gene expression and neuronal injury

As shown above, increased expression of *Per-1*, *Per-2, NPAS-2* and HO-1 were associated with less neuronal injury and neuroinflammation after SAH. We therefore investigated whether a causative link exists between CO signaling in the brain, circadian rhythm gene expression, and SAH-induced neuronal injury. Since HO-1 is predominantly upregulated in microglia following brain injury[16], we utilized myeloid-specific HO-1-deficient mice (*Lyz-Cre-Hmox1*$^{fl/fl}$) to study the role of endogenously generated CO by HO-1. Compared to *Hmox1*$^{fl/fl}$ control mice, the expression of *Per-1*, *Per-2*, *NPAS-2*, and *CLOCK* was markedly suppressed in the SCN in *Lyz-Cre-Hmox1*$^{fl/fl}$ mice (Figures 6A–C, Suppl. Figure IVB). Rescue experiments performed by exposure to CO strongly increased expression of all four circadian rhythm genes in the SCN (Figures 6A–C, Suppl. Figure IVB). In different brain regions, including the cortex, neuronal apoptosis was increased in *Lyz-Cre-Hmox1*$^{fl/fl}$ mice compared to *Hmox1*$^{fl/fl}$ controls, which was reversed when *Lyz-Cre-Hmox1*$^{fl/fl}$ mice were treated with CO (Figure 6D).

Discussion

We show for the first time that mice subjected to SAH have disturbed molecular and behavioral circadian rhythms. Similar dysregulated clock gene expression was also observed in the CSF of SAH patients. It is clear from these studies that changes in expression levels of *NPAS-2* and *Period* genes depend on what time of day SAH occurs. We posit that this, in turn, influences the severity of neuronal injury as measured by changes in vasoreactivity, neuronal apoptosis, and myeloid-driven neuroinflammation. Lastly, we show that the heme degradation pathway that involves HO-1 and the generation of CO, influences clock gene expression in the SCN and ultimately neuronal injury. We find that HO-1 expression is highest during the early active period for mice or ZT12 (early subjective night). Mice subjected to SAH at night showed significantly less injury and cell death when compared to ZT2 or daytime controls; this corresponded with lack of HO-1 expression. Moreover, administration of exogenous CO rescued daytime SAH mice, thus recapitulating the protection observed with increased HO-1 expression.

SAH, like other stroke subtypes, presents with a distinct temporal pattern of occurrence with the greatest frequency occurring in the early morning hours.[1, 17, 18] Numerous explanations have been proposed to explain this phenomenon including changes in blood coagulation[19], autonomic nervous system activation[20], and blood pressure.[21] However, in human stroke, disruption of circadian rhythmicity *per se* has not been studied as a potential etiology. This is somewhat surprising given the abundance of animal data. Reduced expression of *BMAL-1*[22], *CLOCK*, and particularly *Per-2* increases susceptibility to multiple organ dysfunction including the brain and cardiovascular system.[8, 23, 24]

Only a few reports have analyzed the influence of varying clock gene expression on neuronal injury. The connection between the expression of these genes and susceptibility to injury might not be as straightforward as observed in peripheral organs due to the fact that circadian gene expression is differentially regulated depending on the specific brain region.[25] Nonetheless, a connection between the quantity of *Period* gene expression and severity of neuronal injury after ischemic insult has been reported.[9, 26] Moreover, others have demonstrated injury-dependent upregulation of *Per-2* after traumatic brain injury.[27] The expression pattern of circadian rhythm genes and their role in neuronal injury after SAH has not been studied. In both humans and mice, SAH leads to upregulation of clock genes in what appears to be a stress response in an effort to protect the brain from further injury. This is best supported by knockout studies, where absence of *Per-2* results in enhanced injury.[8]

Although the auto-regulatory feedback loop controlling clock gene expression has been well described[7], explicit downstream targets in specific organs remains poorly described. It is known that up to 10% of the genome participates in rhythmic patterns of expression and in a highly tissue-specific manner.[28, 29] How this pattern influences susceptibility to injury and what signaling pathways and genes are expressed remains an ongoing area of study. There is likely a host of stress response genes that are involved. HO-1 is one of the more potent cytoprotective genes. When activated prior to an insult, HO-1 is highly protective in the brain and other organs; absence of HO-1 results in heightened injury. Importantly, CO, a product of HO-1 breakdown of heme, when administered exogenously, is now accepted as a protective molecule and has been shown to ameliorate hemorrhagic and ischemic stroke.[15,30] CO has been shown to function as a neurotransmitter akin to nitric oxide[31] and there is evidence that CO participates in long-term potentiation.[32] Thus, CO is clearly involved and important in a variety of neurologic functions including inflammation and tissue stress. The mechanism by which CO exerts its neuroprotective effects in the brain is intriguing and clearly requires further study.

The role of microglia in neuronal injury is two-sided in that they can induce neuronal apoptosis via neuroinflammation[13], but they can also impart neuroprotection.[33] The neuronal response to activated microglia most likely depends on the etiology and degree of neuronal injury. Here, we demonstrate increased neuronal cell death, potentially due to increased microglia activation. Our data in regards to myeloid-specific HO-1 deficient mice suggests a link between severity of injury, clock gene expression, and HO-1 expression. Why HO-1 is elevated in animals at night is likely linked to their activity where we know that increases in heme-containing proteins such as those involved in metabolism, vasomotor tone, neurotransmission, and oxidative processes, all require heme turnover, and thus

increased HO-1 expression. We would therefore propose that CO contributes to maintenance of proper circadian rhythms. Conversely, during the daytime when the animal sleeps, many of these same hemoproteins are not as prevalent. Collectively, we put forth a novel mechanism as to how changes in circadian rhythm might be transmitted under pathological conditions in the brain. The sudden change in clock gene expression observed after SAH may serve as a rapid signal that leads to increased HO-1 expression either due to the increased heme burden or via regulation by one or more of the clock gene transcriptional regulators such as *NPAS2*. It is known that HO-1 and HO-2 are involved in the activity of several transcription factors responsible for the regulation of circadian rhythm. While HO-2 is present in the brain, its expression does not change in response to external factors or stimuli. These clock gene transcription factors have been shown to be responsive to CO through their heme moieties that serve as gas-sensors.[11, 12, 34] Our data show that exogenous CO modulates circadian gene expression and reduces injury. These new insights might explain the temporal pattern observed in SAH and may also help in understanding the mechanisms underlying the beneficial effects of HO-1 and CO in the brain after SAH. In the future, CO might prove to be a beneficial adjunct to stroke therapy. Understanding how the central and peripheral clocks influence the injury response may offer critical clues as to how, and perhaps more importantly, when, to intervene with therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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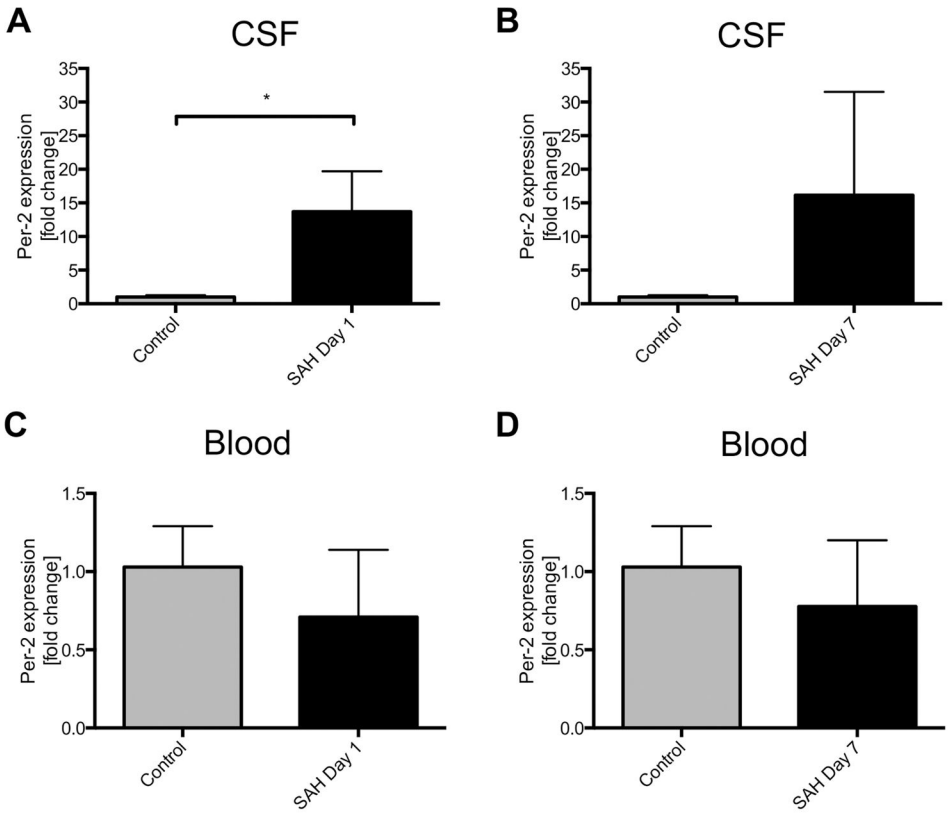

Figure 1. Per-2 expression in human subarachnoid hemorrhage (SAH) patients

A–B: *Per-2* expression in cells isolated from the cerebrospinal fluid (CSF) of SAH patients compared to control patients with unruptured cerebral aneurysms on day 1 (**A**) and day 7 (**B**) after SAH onset. [fold change vs. control]; *p=0.0072 for day 1, p=0.09 for day 7. **C–D** *Per-2* expression in peripheral leukocytes isolated from the whole blood of SAH and control patients. Results represent mean±SD of 13 patients.

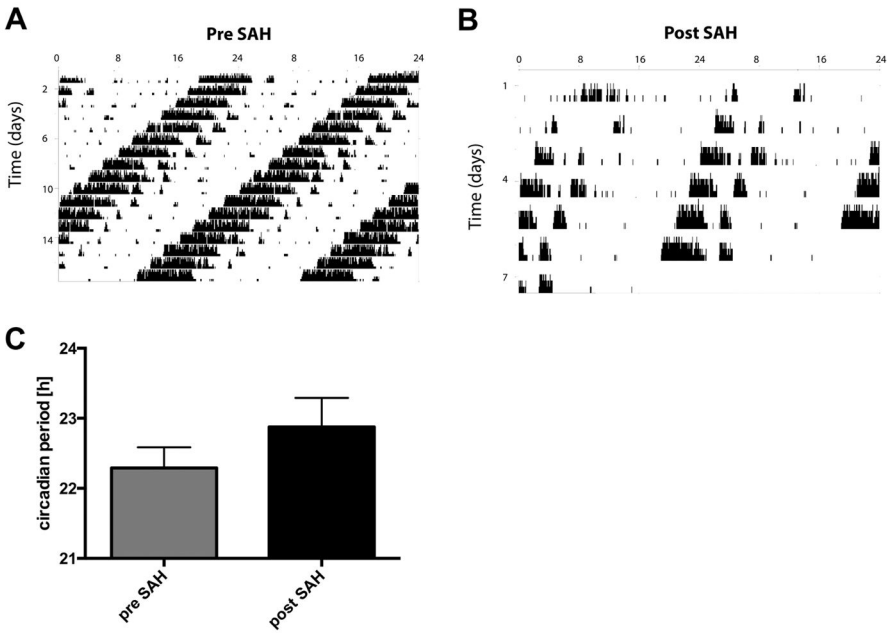

Figure 2. Effects of SAH on Activity Patterns in Per2$^{-/-}$ mice

A–B: Representative wheel-running actograms of *Per-2*$^{-/-}$ mice under free running conditions (constant darkness, DD) pre-SAH (**A**) and post-SAH (**B**). Results are representative of 3 independent experiments with n=3–4mice/group. **C:** Mean circadian period in minutes before and after SAH (pre-SAH vs. post-SAH, p=0.06).

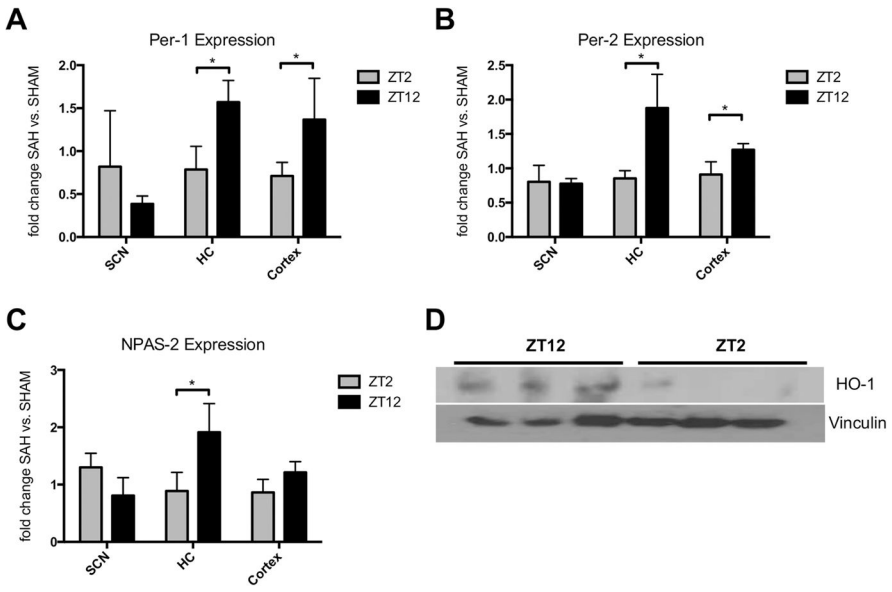

Figure 3. SAH onset time determines changes in circadian rhythm gene expression

A–C: Changes in *Per-1* (**A**), *Per-2* (**B**) and *NPAS-2* (**C**) expression in the suprachiasmatic nucleus (SCN), hippocampus (HC), and cortex of animals subjected to SAH at zeitgeber time (ZT) 2 and ZT12 compared to animals with SHAM procedures at the same time points. Samples were collected exactly 7 days after procedures to analyze the effect purely related to SAH and onset time. **A:** ZT2 vs. ZT12 *p=0.0212 for HC, *p=0.044 for cortex; **B:** *p=0.0242 for HC, *p=0.0386 for cortex; **C:** *p=0.0412 for HC; **D:** Cerebral heme oxygenase (HO)-1 protein expression analyzed by Western Blot and normalized to vinculin in animals at ZT2 and ZT12. Results represent mean ± SD with n=3–4 mice/group.

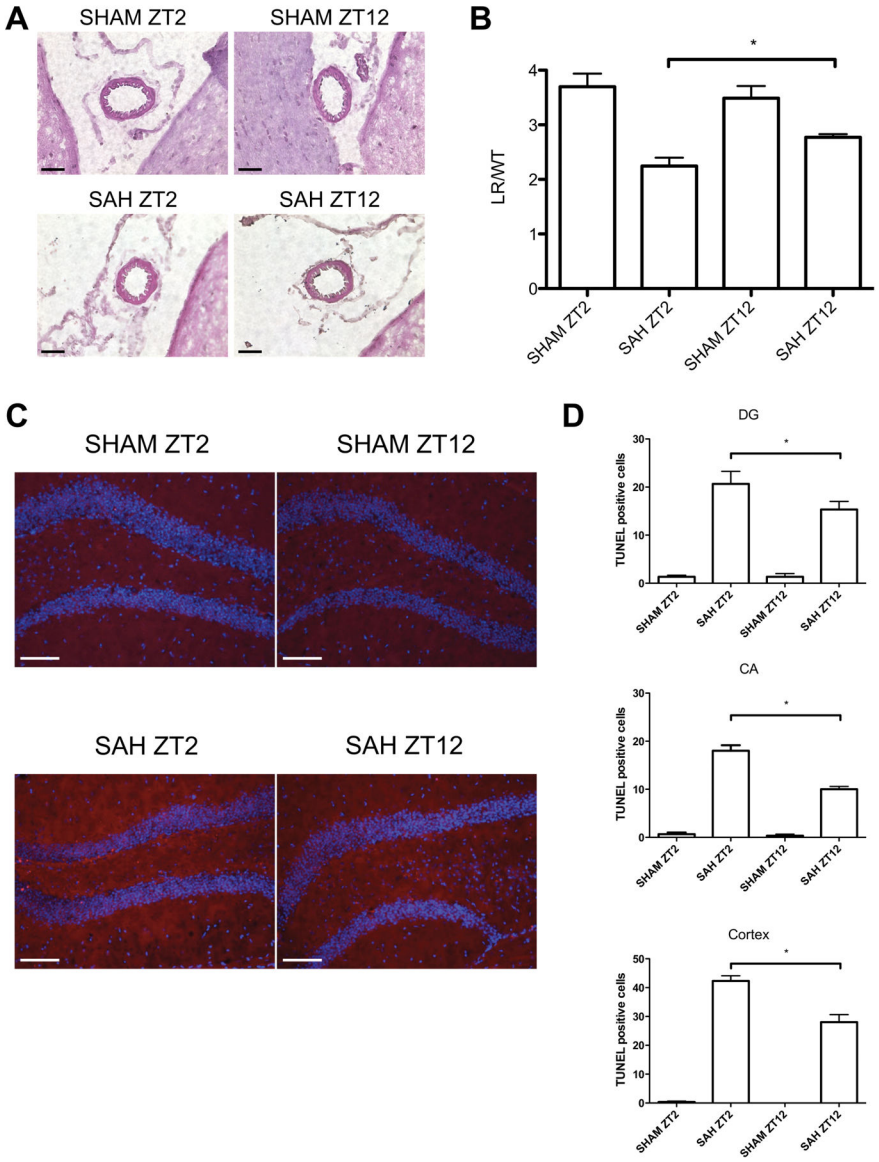

Figure 4. Influence of SAH onset time on vasospasm and neuronal apoptosis

A. Representative cross sections of the middle cerebral artery (MCA) in animals with either SHAM (upper panel) or SAH (lower panel) at ZT2 (left panel) and ZT12 (right panel), respectively, 7 days after surgery. Images are representative of 3–4 mice/group with 3 sections/brain. 40× magnification **B.** Quantification of MCA vasospasm was defined as the quotient of lumen radius (LR) and wall thickness (WL); results represent mean ± SD of 3 images. *p=0.0313 SAH ZT2 vs. SAH ZT12. **C.** TUNEL staining in the dentate gyrus (DG) 7 days after SHAM (upper panel) or SAH (lower panel) at ZT2 (left panel) and ZT12 (right panel). Representative images from 3–4 mice/group with 3 sections/brain. 20× magnification **D.** Quantification of TUNEL-positive cells per microscopic field (DG: top, *p=0.0387 SAH ZT2 vs. SAH ZT12; *cornu ammonis* (CA): middle, *p=0.0002; cortex: bottom, *p=0.0013). Results represent mean ± SD of 3 images. Scale bar = 50μM

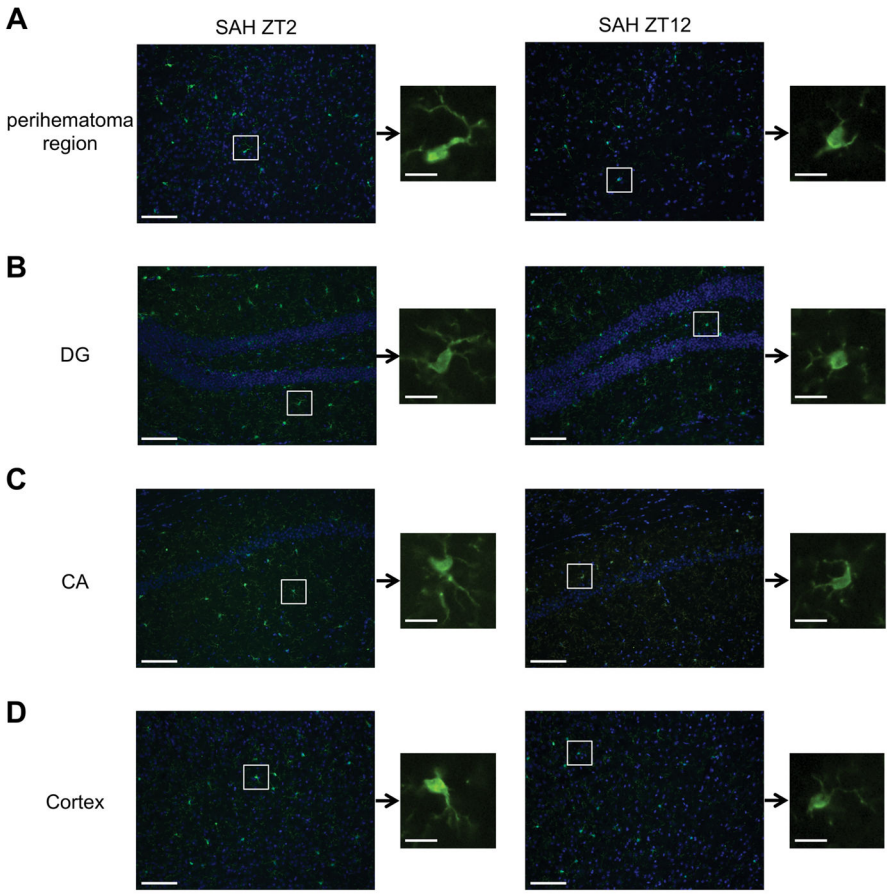

Figure 5. The extent of neuroinflammation depends on SAH onset time

A–D: Immunohistochemical staining for Iba-1 (green) showing the morphology and quantity of microglia cells after SAH at ZT2 and ZT12 in the perihematona region at the base of the brain (**A**), dentate gyrus (DG, **B**), *cornu ammonis* (CA, **C**) and cortex (**D**). Nuclei were counterstained with Hoechst 33258 (blue). Higher magnification images (20x) show the representative changes in microglia morphology. White boxes show the location of the magnified microglia. Microglia at ZT2 (left panel) show elongated cell bodies and thickened processes indicative of activation. All images are representative of 3–4 mice/group with 3 sections/brain. Scale bar = 50μM for the low magnification images and 10μM for the single cell images.

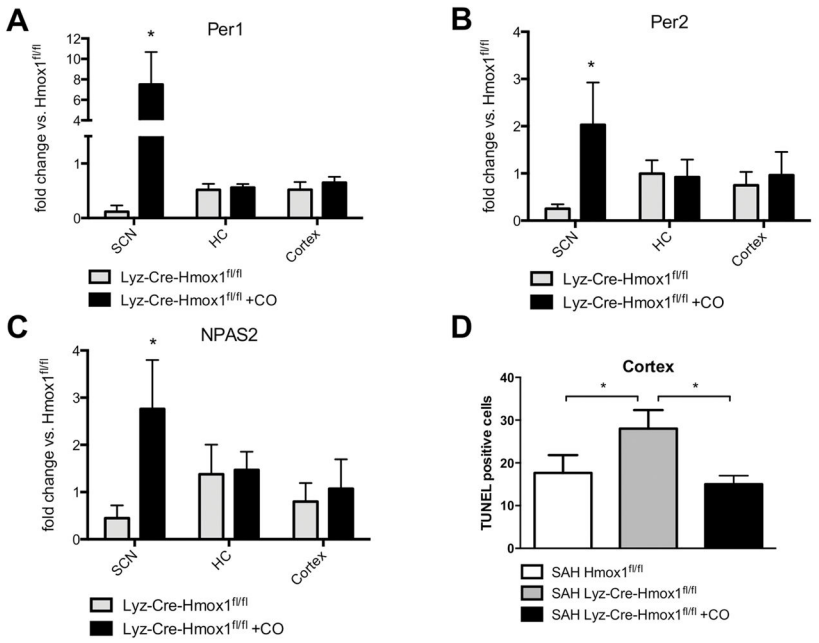

Figure 6. Myeloid HO-1 and carbon monoxide (CO) modulate circadian rhythm gene expression and susceptibility to neuronal injury

A–D. Expression of *Per-1* (**A**), *Per-2* (**B**) and *NPAS-2* (**C**) in the indicated brain regions (SCN, HC, cortex) in *Lyz-Cre-Hmox1*$^{fl/fl}$ mice ± CO treatment for 7 days after SAH compared to *Hmox1*$^{fl/fl}$ control animals. *Lyz-Cre-Hmox1*$^{fl/fl}$ vs. *Lyz-Cre-Hmox1*$^{fl/fl}$ + CO, change in the SCN: *p=0.0156 for *Per-1* (**A**); *p=0.0267 for *Per-2* (**B**); *p=0.0201 for *NPAS-2* (**C**). Results represent mean ± SD from 3–4 mice/treatment group. **D.** Quantification of TUNEL-positive cells per microscopic field in the cortex of *Hmox1*$^{fl/fl}$ and *Lyz-Cre-Hmox1*$^{fl/fl}$ mice ±CO: *Hmox1*$^{fl/fl}$ vs. *Lyz-Cre-Hmox1*$^{fl/fl}$ *p=0.0408; *Lyz-Cre-Hmox1*$^{fl/fl}$ vs. *Lyz-Cre-Hmox1*$^{fl/fl}$ +CO *p=0.0146. Results represent mean ± SD of 3–4 mice/group.