Online Supplement

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

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1. Supplemental Methods

Animal Handling, Anesthesia and Tissue Collection

Animals were fed with standard rodent diet *ad libitum* while kept on a 12-h light/12-h dark cycle. All types of surgery and manipulations were performed under general anesthesia with ketamine (10mg/kg) and xylazine (4mg/kg) and body temperature maintenance. At surgery, buprenorphine (50µg/kg) was applied subcutaneously to treat possible pain. The number of animals used was n=3 per group. *Per-2*$^{-/-}$ mice were kept in constant darkness (DD, free running conditions) with free access to food and water for 10 days prior to and after SAH to achieve light-independent rhythms. Handling for experiments in DD including SAH was done under dim red light (<1 lux). Actinograms for *Per-2*$^{-/-}$ mice were obtained for the whole pre- and post-SAH period.
7 days after SAH, animals were deeply anesthetized with ketamine and xylazine and transcardially perfused with TBS. Brains were removed and postfixed in PFA 4% for 18 h. After cryoprotection in sucrose, brains were frozen and cut in 9 µm sections.
At indicated time points during the light-dark (LD) cycle, where "ZT" ("zeitgeber") denominates the time since the start of the 12 hour light cycle, animals were euthanized and heart, kidney and brain tissue was harvested. In experiments using *LyzCre-Hmox1*$^{fl/fl}$ and *Hmox1*$^{fl/fl}$ mice, tissue harvest was done at ZT12 for all animals on day 7 after SAH and 2h after the last CO treatment. The brain was dissected to obtain a 1 x 2mm punch biopsy of the SCN-region. Hippocampus (HC) and Cortex were dissected separately. Tissue samples were immediately homogenized in Trizol and further processed for RNA purification. RNA-concentration and purification was done using spin columns (RNEasy mini kit, Qiagen).

Subarachnoid Hemorrhage Model

SAH was achieved by pre-chiasmatic injection of autologous blood. In brief, after induction of anesthesia, the head was fixed in a stereotactic apparatus. With a midline incision, the skin overlying the anterior skull was opened. 4.5 mm anterior to the bregma, a burr hole was drilled into the skull with a caudal angel of 40°, using a 0.9 mm drill. 60µl of blood was withdrawn from a C57BL/6 WT blood donor and injected over 10sec with a 27-G needle advanced through the burr hole at a 40° angle until the base of the skull. The needle was left in place for 5 min to avoid backflow.

Primer Sequences for qPCR

I. Mouse

1. Per-1
   Forward: CGCATCCACTCTGGTTATGAAG
   Reverse: GAATAGGGGAATGGTCAAAGGG
2. Per-2
   Forward: AGCGGCTTAGATTCTTTCACTC
   Reverse: ATCTCATTCTCGTCCTCTTTCC
3. Cry-1
   Forward: TGAGAAATATGGCGTTCCTTCC
   Reverse: GTAAGTGCCTCAGTTTCTCCTC
4. NPAS-2
   Forward: CCCACTACTACATCACCTACCA
   Reverse: GTCTCCTTTCCACTCGAACATC
5. BMAL-1
   Forward: CCAAGAAAGTATGGACACAGACAAA
   Reverse: GCATTCTTGATCCTTCCTTGGT
6. Clock
   Forward: ACGTTCACTCAGGACAGACA
   Reverse: ACCACCTGACCCATAAGCAT
7. Rplp0
   Forward: GAGGAATCAGATGAGGATATGGGA
   Reverse: AAGCAGGCTGACTTGGTTGC

II. Human

1. Per-2
   Forward: AGCGGCTTAGATTCTTTCACTC
   Reverse: ATCTCATTCTCGTCCTCTTTCC
2. Rpl13a
   Forward: CGCTGTGAAGGCATCAACATTTC
   Reverse: GCTGTCACTGCCTGGTACTTC

Immunohistochemistry

Sections were post-fixed with 2% PFA for 10 min and permeabilized with 0.5% Triton/PBS for 10 min. Slides were then blocked in horse serum (7% in PBS) for 30 min at room temperature. Staining was performed with primary antibodies against anti-ionized calcium binding adaptor molecule 1 (Iba-1) (1:500, Wako 019-19741) at 4°C overnight. Sections were then conjugated with their corresponding secondary antibody for fluorescent (Alexa Fluor 488 donkey anti-mouse 1:600, Invitrogen). Nuclear counterstain was done with Hoechst 33258 (Sigma, 1:10,000, 3min) and slides were examined under a fluorescence microscope.

For TdT-mediated dUTP-biotin nick end labeling (TUNEL), slides were fixed in 4% PFA for 20 min and permeabilized with 0.1% Triton X for 2 min. After washing with PBS, sections were covered with enzyme and label solution (Roche, In situ cell death detection kit, TMR red)

for 1h at 37°C in a humidified atmosphere. Nuclear counterstain was done with Hoechst 33258 (Sigma, 1:10,000, 3min) and slides were examined under a fluorescence microscope. To achieve appropriate consistency in quantification of cells, from each area of interest 3 images were obtained and positive cells were quantified.

Western Blot

Brain tissue samples were lysed in RIPA buffer with one freeze/thaw cycle and sonification. Equal amounts of protein were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk in Tween20/TBS and incubated in the recommended dilution of specific antibodies (HO-1, Abcam, 1:1000) overnight at 4°C. Membranes were then incubated with the corresponding secondary antibody for infrared fluorescent detection and developed on an infrared imaging system (Licor Odyssey).

2. Supplemental Figure Legends

Supplemental Figure I: ***Kinetic of circadian rhythm gene expression after SAH***
A-B. *Per-2* mRNA expression measured by qPCR after SAH over time in the brain, heart and kidney comparing the relative change over the course of the day vs. baseline expression at ZT2. (HCN, suprachiasmatic nucleus; HC, Hippocampus) **C-D** Per-2 expression in the heart and hippocampus (HC) over time. Results show n=1-2 animals/time point. Note that few animals were used in the above experiments simply to obtain an indication of what time point to focus on to expand the studies. The data presented in Figure 3 show that ZT2 and ZT12 were chosen where 3-4 mice/time point were used.

Supplemental Figure II: ***Per-1, Per2, BMAL and NPAS2 expression in kidney over time after SAH***
A-D. Gene expression by qPCR over time in the kidney. Results show n=1-2 animals/time point. Note that few animals were used in the above experiments simply to obtain an indication of what time point to focus on to expand the studies. The data presented in Figure 3 show that ZT2 and ZT12 were chosen where 3-4 mice/time point were used.

Supplemental Figure III: ***Neuronal apoptosis after SAH with onset times ZT2 and ZT12***
A: Representative images from TUNEL staining in the cornu ammonis refion (CA) 7 days after SAH. **B:** Representative images from TUNEL staining in the cortex 7 days after SAH.

Supplemental Figure IV: ***Expression of CLOCK at ZT2/ZT12 and in microglial HO-1 deficient mice***
A: Changes in CLOCK expression in the SCN, HC and Cortex of animals subjected to SAH at ZT2 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. p= 0.0303 for cortex. **B:** Expression of *CLOCK* in the designated brain regions (SCN=suprachiasmatic nucleus, HC=hippocampus, 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=0.0078.

Figure I

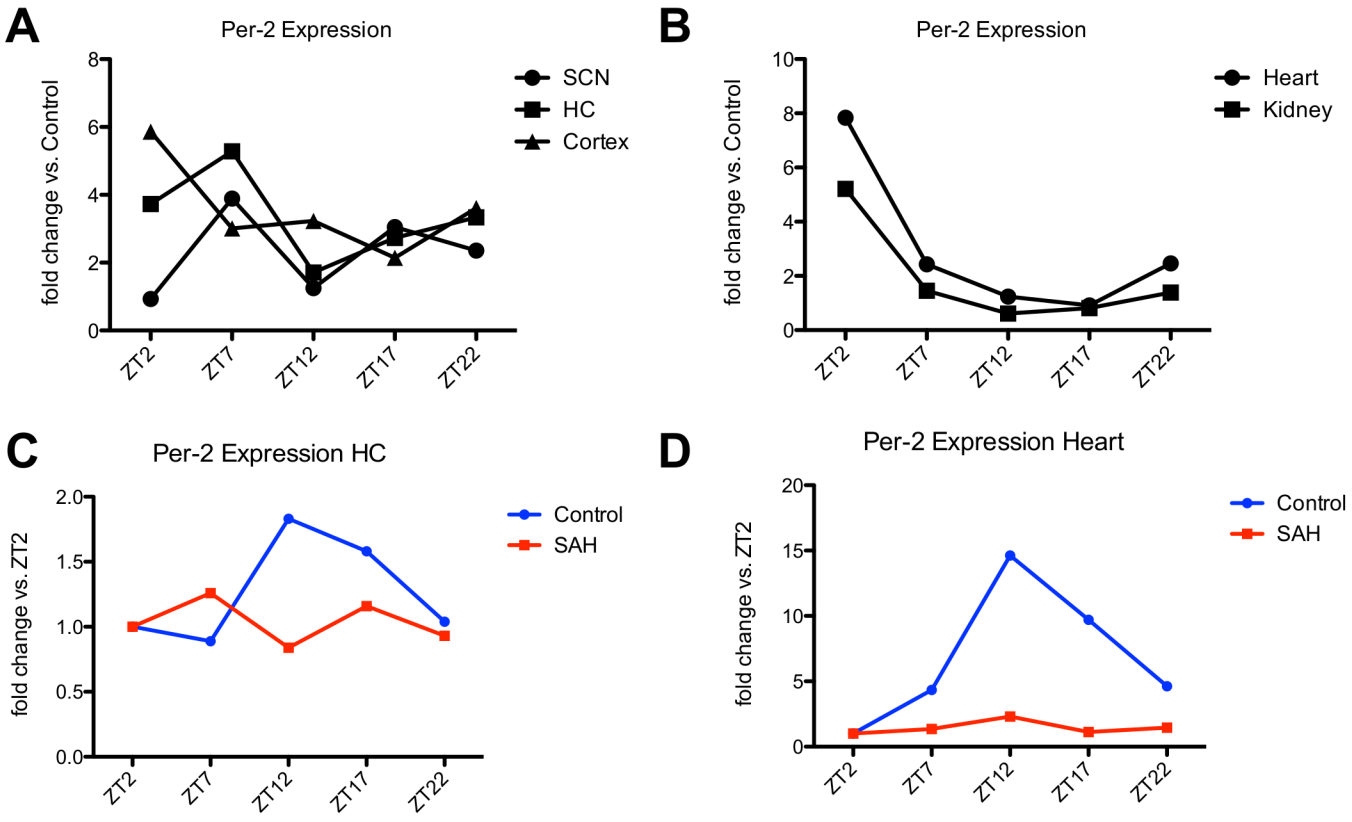
A
Per-2 Expression
fold change vs. Control
SCN
HC
Cortex
ZT2
ZT7
ZT12
ZT17
ZT22
B
Per-2 Expression
fold change vs. Control
Heart
Kidney
C
Per-2 Expression HC
fold change vs. ZT2
Control
SAH
D
Per-2 Expression Heart
fold change vs. ZT2
Control
SAH

Figure II

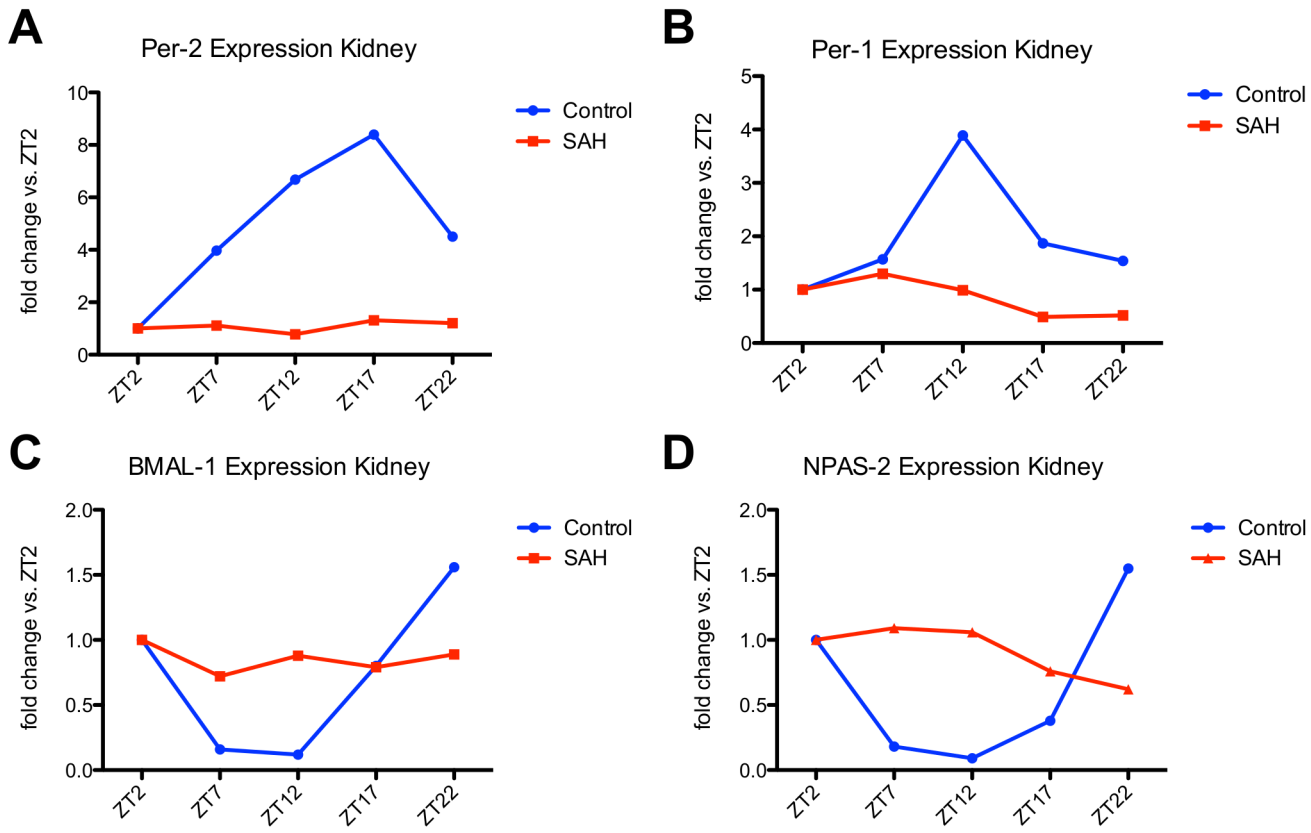
A
Per-2 Expression Kidney
fold change vs. ZT2
10
8
6
4
2
0
ZT2
ZT7
ZT12
ZT17
ZT22
Control
SAH
B
Per-1 Expression Kidney
fold change vs. ZT2
5
4
3
2
1
0
ZT2
ZT7
ZT12
ZT17
ZT22
Control
SAH
C
BMAL-1 Expression Kidney
fold change vs. ZT2
2.0
1.5
1.0
0.5
0.0
ZT2
ZT7
ZT12
ZT17
ZT22
Control
SAH
D
NPAS-2 Expression Kidney
fold change vs. ZT2
2.0
1.5
1.0
0.5
0.0
ZT2
ZT7
ZT12
ZT17
ZT22
Control
SAH

Figure III

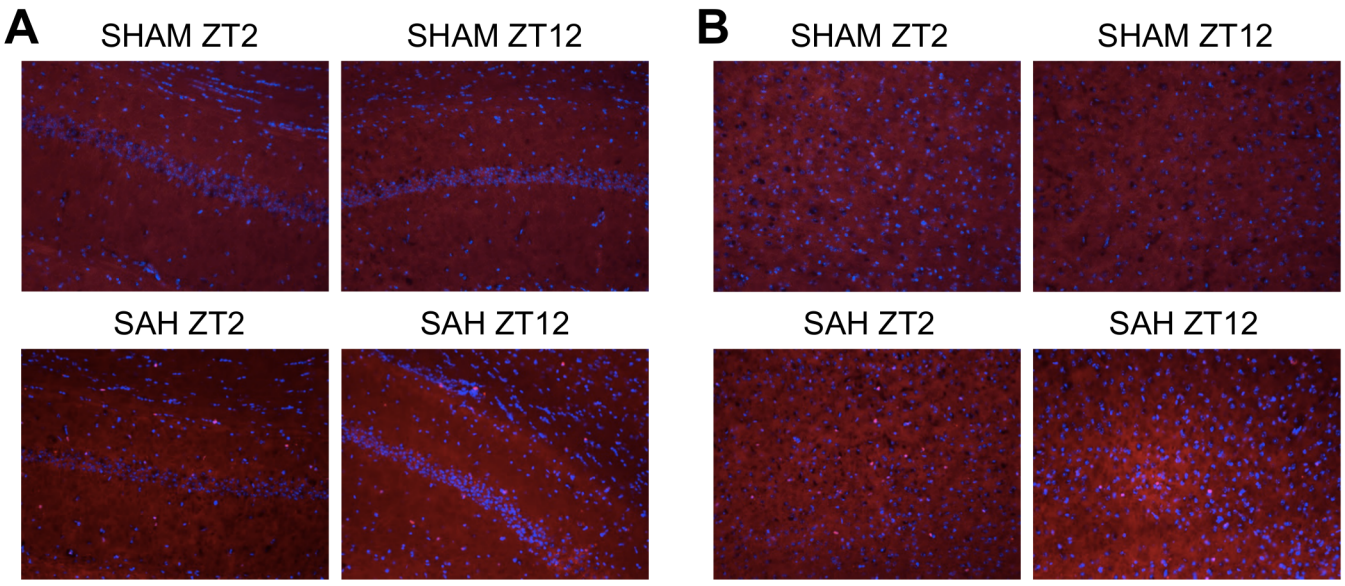

Figure IV

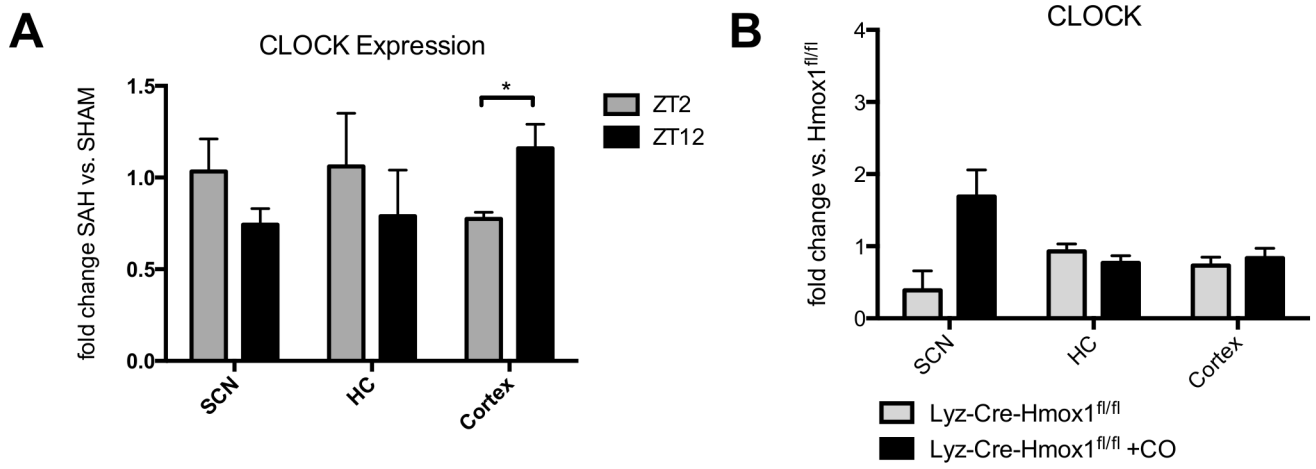

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☒ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☒ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☒ An overall study timeline is provided.
Inclusion and exclusion criteria	☐ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☒ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☒ Type and methods of randomization have been described. ☒ Methods used for allocation concealment have been reported.
Blinding	☒ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☐ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☒ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☒ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☒ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☒ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☒ Statistical methods used have been reported. ☒ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☒ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☒ Different sex animals have been used. If not, the reason/justification is provided. ☒ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☒ Statements on funding and conflicts of interests have been provided.