SUPPLEMENTAL MATERIALS

Low-intensity pulsed ultrasound therapy promotes recovery from stroke by enhancing angio-neurogenesis in mice in vivo

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Supplemental Files

Figures S1 to S10

Tables S1 to S4

Movies S1 to S2
Figure S1. Body weight and blood pressure

Graphs showing time-course of body weight (a) and mean blood pressure (b) measured in the indicated groups (n=10 per group).
Figure S2. Cerebral blood flow during MCAO

Cerebral blood flow was determined by laser Doppler flowmetry (n=10 per group).
Figure S3. Results of behavioral tests

Results of corner test and barnes maze test.
Figure S4. Effects of the LIPUS therapy on angiogenesis after MCAO

a. Representative images of double immunostaining of Ki67 and CD31 at 500-750 μm from the SVZ on day 7. The scale bar represents 50 μm. Quantification of Ki67+/CD31+ cells at 250-500 μm (b) and 500-750 μm (c) from the SVZ on day 7 (n=8 per group).
Figure S5. Effects of the LIPUS therapy on molecular expression after MCAO

a, RT-qPCR analysis of FGF and angiopoietin-1 at day 3 (n=14 per group).  
b, Western blot analysis of ERK1/2 phosphorylation and Akt phosphorylation at day 5 (n=10 per group).

*P<0.05 (Student’s t-test).  Uncropped blots are shown in Fig. S10.
Figure S6. Expression of SDF-1 and CXCR4 by CD31 positive cells

a, Representative images of double immunostaining for SDF-1 and CD31 in the ipsilateral striatum on day 7.  
b, Representative images of double immunostaining for CXCR4 and CD31 in the ipsilateral striatum on day 7.  The scale bar represents 20 μm.
Figure S7. Expression of SDF-1 and CXCR4 by DCX positive cells

a, Representative images of double immunostaining of SDF-1 and DCX in the ipsilateral striatum on day 7. b, Representative images of double immunostaining of CXCR4 and DCX in the ipsilateral striatum on day 7. The scale bar represents 20 μm.
Figure S8. Effects of the LIPUS therapy after MCAO in eNOS−/− mice
Western blot analysis of ERK1/2 phosphorylation at day 5 (n=6 per group). Uncropped blots are shown in Fig. S10.
Figure S9. Effects of the LIPUS therapy on neurological function after MCAO in the chronic phase

a, The timeline of the experiment. b, Rotarod, tightrope, and corner tests were performed on days 28, 56, 84, and 112 following MCAO or sham surgery; n=3 (sham), n=10 (MCAO).
Figure S10. Uncropped images of Western blots

The uncropped blots are shown in the main and supplemental figures. Boxed areas indicate the cropped regions shown in the respective figure.
### Tables S1 to S4

#### Table 1. Animal number (Survival/total) in each group

| Group                               | Behavioral tests (Day 28) | Behavioral tests (Day 112) | IF | CV | RT-PCR | WB |
|-------------------------------------|---------------------------|----------------------------|----|----|--------|----|
| Sham+Non-LIPUS (WT)                | 10/10                     | 10/10                      | 3/3|
| Sham+LIPUS (WT)                    | 10/10                     | 10/10                      | 3/3|
| MCAO+Non-LIPUS (WT)                | 10/14                     | 10/14                      | 24/31 | 8/12 | 14/19 | 10/15|
| MCAO+LIPUS (WT)                    | 10/13                     | 10/13                      | 24/32 | 8/11 | 14/18 | 10/13|
| MCAO+Non-LIPUS (eNOS\(^{-/-}\))    | 6/17                      | 6/16                       | 6/15 | 6/15| 6/15 |
| MCAO+LIPUS (eNOS\(^{-/-}\))        | 6/18                      | 6/15                       | 6/15 | 6/15| 6/13 |
| **Total:**                          | **52/82**                 | **40/47**                  | **60/94** | **34/59** | **28/37** | **32/56** |

IF: immunofluorescence staining; CV: cresyl violet staining; RT-PCR: real-time PCR; WB: western blotting; WT: wild type.

*Note: The table illustrates the number of survivors (Survival) out of the total number of animals (total) in each group for behavioral tests on Day 28 and Day 112, as well as the results for IF, CV, RT-PCR, and WB.*
Table S2. Assessment of tightrope test performance

| Score | Time (s) | Platform arrival |
|-------|----------|------------------|
| 20    | 1-6      | +                |
| 19    | 7-12     | +                |
| 18    | 13-18    | +                |
| 17    | 19-24    | +                |
| 16    | 25-30    | +                |
| 15    | 31-36    | +                |
| 14    | 37-42    | +                |
| 13    | 43-48    | +                |
| 12    | 49-54    | +                |
| 11    | 55-60    | +                |
| 10    | 55-60    | -                |
| 9     | 49-54    | -                |
| 8     | 43-48    | -                |
| 7     | 37-42    | -                |
| 6     | 31-36    | -                |
| 5     | 25-30    | -                |
| 4     | 19-24    | -                |
| 3     | 13-18    | -                |
| 2     | 7-12     | -                |
| 1     | 1-6      | -                |
| 0     | 0        | -                |

Tightrope test results were assessed according to both time on the rope (in sec) and platform arrival (“+” for arrival and “-” for non-arrival). The scores ranged from 0 (minimum) to 20 (maximum).
Table S3. Antibodies used for Western blot analysis (WB) and immunofluorescence (IF)

| Antibody          | Application | Dilution | Manufacturer     | Cat. no  |
|-------------------|-------------|----------|------------------|----------|
| Doublecortin (DCX)| IF          | 1:50     | Abcam            | ab124824 |
| Doublecortin (DCX)| IF          | 1:50     | Santa Cruz       | Sc-271390|
| CXCR4             | WB          | 1:1000   | Abcam            | ab108319 |
| CXCR4             | IF          | 1:200    | Abcam            | ab124824 |
| BDNF              | WB          | 1:1000   | Abcam            | ab18723  |
| CD31              | IF          | 1:50     | MERCK            | MAB1398Z |
| CD31              | IF          | 1:50     | Dianova          | DIA-310  |
| Ki67              | IF          | 1:200    | Invitrogen       | 14-5698-82|
| SDF-1             | WB          | 1:1000   | Abcam            | ab9797   |
| SDF-1             | IF          | 1:100    | R&D Systems      | MAB350   |
| Phospho-eNOS      | WB          | 1:1000   | Cell signaling   | 9571     |
| eNOS              | WB          | 1:1000   | BD                | 610296   |
| VEGF              | WB          | 1:1000   | Santa Cruz       | Sc-7269  |
| α-tubulin         | WB          | 1:10000  | Sigma            | T5168    |
| Phospho-ERK1/2    | WB          | 1:1000   | Cell Signaling   | 9106     |
| Total-ERK1/2      | WB          | 1:1000   | Cell Signaling   | 9102     |
| Phospho-Akt       | WB          | 1:1000   | Cell Signaling   | 9271     |
| Total-Akt         | WB          | 1:1000   | Cell Signaling   | 9272     |
| Gene   | Forward primer (5’>3’) | Reverse primer (3’>5’) |
|--------|-----------------------|-----------------------|
| SDF-1  | 5’CAGAGCCAACGTCAAGCACAC | 5’TAAATTTCGGGTCAATGCAC |
| CXCR4  | 5’CCATGGAACCGATGCTGTA | 5’GCCGACTATGCCAGTCAAGAA |
| BDNF   | 5’TCAAGTTGGAAGCCTGAATGAT | 5’CTGATGCTCAGGAACCAGG |
| eNOS   | 5’ATCTTTGCTACAGCAAGACAG | 5’TCCCGGATAGATGGTCCAG |
| VEGF   | 5’ACATTGGCTCAGCAGAGCAGA | 5’TGGTTGAACCGGCATCTTTA |
| GAPDH  | 5’TGTGTCGGCTCGATCTGCAATC | 5’TGTCTTGAAGTGCCAGGAG |
| FGF2   | 5’AAGCGGCTCTACTGCAAGAA | 5’TACCGTTGCAACACACACTC |
| Angptl | 5’TGATAACGCCAGCCACAAAG | 5’CTGTAATGTCGACATAC |

Table S4. Primers used for gene expression analysis
Legends to Movies S1 to S2

Movie S1. Tightrope testing on day 28 (non-LIPUS group). This movie shows tightrope testing performed by a mouse of the non-LIPUS group on day 28 after MCAO surgery.

Movie S2. Tightrope testing on day 28 (LIPUS group). This movie shows tightrope testing performed by a mouse of the LIPUS treated group on day 28 after MCAO surgery.