Supplemental Material

MicroRNA-targeting of neurotropic flavivirus: effective control of virus escape and reversion to neurovirulent phenotype.

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2x mir-124aT(1,2):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
AGGCTATTGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAATAATGGGAGGCGTAATAATCCCC
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CAGGTTCT-3′

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GACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTGGTGTTGTTGATCCAA
CAGGTTCT-3′

3x mir-124aT(1,2,3):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
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mir-9T-124aT-124aT(1,2,3):

5′–**TAA**TTCGAAGGTAACC***TCATACAGCTAGATAACCAAAGA***TTACCAACAACA***TGGCATTCACCGCGTCCTTAA***CTCGAGAACACCAA
AGGCTAT***TGGCATTCACCGCGTGCCTTAA***
*ATCGATT*GAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAATAATGGGAGGCGTAATAATCCCCA
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ACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTGGTGTTGTTGATCCAAC
AGGTTCT-3′

3x mir-124aT(1,2,4):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
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AGGGAGGCCATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTCCCATCACTGACAA***TGGCATTCA***
CCGCGTGCCTTAATCGATAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTCCTGGTGGAAGGACTAGAGGTTAGAGGAG
ACCCCCCCAACACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCG
CAAGATGGATTGGTGTTGTTGATCCAACAGGTTCT-3′

3x mir-124aT(1,2,5):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
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CAAGATGGATTGGTGTTGTTGATCCAACAGGTTCT-3′

4x mir-124aT(1,2,3,4):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
AGGCTAT***TGGCATTCACCGCGTGCCTTAA***ATCGATTGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCC
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CCCCTCCCATCACTGACAA***TGGCATTCACCGCGTGCCTTAA***TCGATAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTC
CTGGTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCT
GCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTGGTGTTGTTGATCCAACAGGTTCT-3′

4x mir-124aT(1,2,3,5):

5′–**TAA**TTCGAAGGTAACC***TGGCATTCACCGCGTGCCTTAA***TTACCAACAACA***TGGCATTCACCGCGTGCCTTAA***CTCGAGAACACCAA
AGGCTAT***TGGCATTCACCGCGTGCCTTAA***ATCGATTGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCC
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CCCCTCCCATCACTGACAAAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTCCTGGTGGAAGGACTAGAGGTTAGAGGA
GACCCCCCCAACACA***TGGCATTCACCGCGTGCCTTAA***TCGATAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCT
GCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTGGTGTTGTTGATCCAACAGGTTCT-3′

Figure S1. Sequence of the 3'NCRs of the TBEV/DEN4 genomes carrying target sequences for mir-124a and mir-9 microRNAs. For each virus construct, the 3'NCR sequence from a TAA-stop codon of the polyprotein ORF to end of TBEV/DEN4 genome is shown (nts 10278 - 10664). The inserted sequences of miRNA targets and their flanked restriction sites are underlined; the sequences of miRNA targets are shown in red italics.

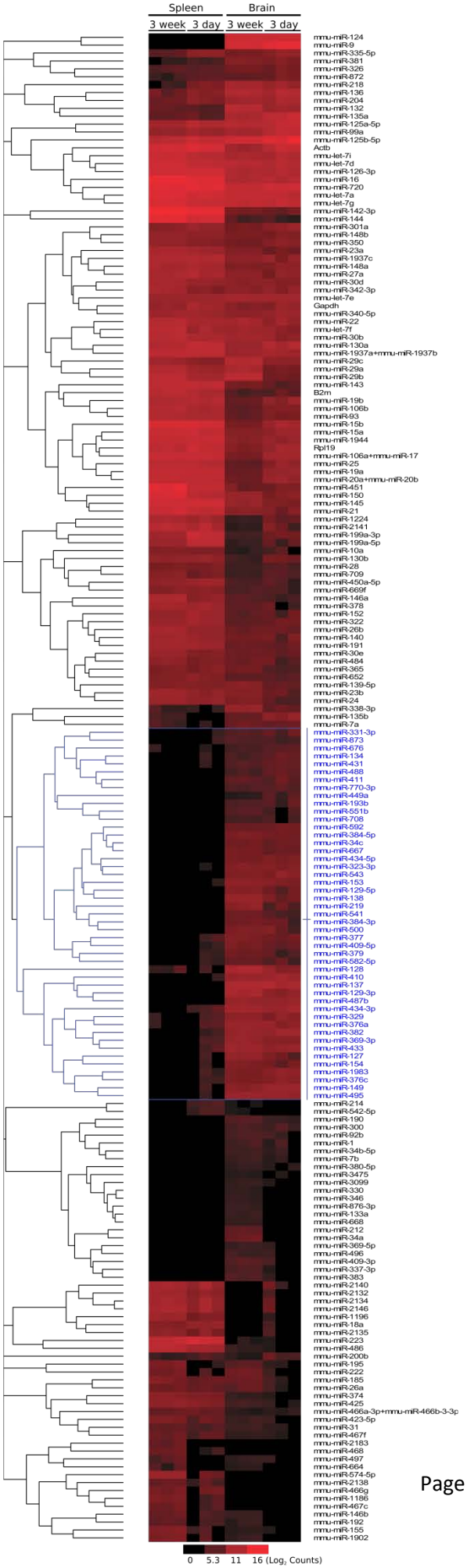

Figure S2. Hierarchical cluster analysis of microRNAs expressed in brains and spleens of suckling and adult mice. Color scale bar at the heatmap bottom represents $\log_2$ expression levels. To determine miRNA profiles, total RNA from spleens (SP; n=3) or brains (LB, left-hemisphere of brain; n=3) of 3-day or 3-week-old Swiss Webster (SW) mice were isolated using the Qiagen miRNeasy Mini Kit and QIAcube robot (Qiagen). Each RNA sample (100 ng) was analyzed for more than 600 murine miRNAs (miRBase v15 database), using the nCounter Mouse miRNA Expression Assay Kit (NanoString Technologies) in accordance with the manufacturer's protocols. After NanoString nCounter digital reading, counts for each microRNA were extracted and analyzed. Two types of controls were used: 1) negative controls, eight non-specific probes for background calculation and 2) positive controls, probes that bind six spiked-in targets at known concentrations to assess the sample scaling. The miRNA quantities values were $\log_2$ transformed and used for t-test (P-values), heatmap clustering, and ANOVA. From 600 mouse miRNAs, 250 were not detected in any sample, and 159 probes were excluded by ANOVA with P-value > 0.01. The remaining 191 probes were clustered using Euclidian distance and Average linkage and shown in the heatmap. With the exception of the mir-9 and mir-124, miRNAs enriched in the brain and spleen are shown in the top portion of the heatmap and whose expression was not higher in the brain are represented in the bottom section. A cluster of 47 miRNAs are shown in blue, in the middle portion and represented miRNAs mainly expressed in the brain.

Figure S3. Deletion mutations that accumulated in the 3'NCR of miRNA target viruses isolated from the mouse brains. Brains of suckling mice (MB) were harvested on the indicated day p.i., and virus RNA was isolated from the 10% brain homogenate for the sequence analysis. The sequence of miRNA targets (shown in red italics) and flanked restriction enzyme sites (underlined) were inserted between indicated nucleotides of the TBEV/DEN4 genome. The ORF stop codon is shown in bold. GR: a group of mice as shown in Table 2. Deletions identified in the brain-derived escape mutants are shown as dashes and their sizes are provided. The prototype virus is shown in the first line of each sequence comparison.

```
                                .10280
2x mir-124aT(1,2)          5'-TAATTCGAAGGTAACCTGGCATTCACCGCGTGCC
GR 3, MB 1 day 8           5'-TAATTCGAAGGTAAC------------------
GR 3, MB 2 day 8           5'-TAATTCGAAGGTAAC------------------
GR 1, MB 3 day 16          5'-TAATT----------------------------
GR 1, MB 4 day 19          5'-TAATTCGAAGGTAACCTGGCATTC----------

    .10281     .10292                          .10293
TTAATTACCAACAACATGGCATTCACCGCGTGCCTTAACTCGAGAACACCAAAGGCTAT
-----------------------------------TCGAGAACACCAAAGGCTAT
-----------------------------------TCGAGAACACCAAAGGCTAT
------------------------------------------------------------
-----------------------------------------------------GGCTAT


TGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAA
TGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAA
TGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAA
------------------------------------------------------------
TGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAA

    .10371
TAATGGGAGGCGTAATAA-3'     No. of nts deleted:
TAATGGGAGGCGTAATAA-3'          58
TAATGGGAGGCGTAATAA-3'          58
----GGGAGGCGTAATAA-3'          151
TAATGGGAGGCGTAATAA-3'          63
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                                    .10280
mir-9T-124aT(1,2)          5′-TAATTCGAAGGTAACCTCATACAGCTAGATAACC
GR 6, MB 1 day 9           5′-TAATTCGAAGGTA--------------------
GR 6, MB 2 day 11          5′-TAATTCGAAGGTAACCTCATACAGCTAGATAACC
GR 6, MB 3 day 19          5′-TAATTCGAAGGTAACCTCATACAGCTAGATAACC

     .10281     .10292                        .10293
AAAGATTACCAACAACATGGCATTCACCGCGTCCTTAACTCGAGAACACCAAAGGCTAT
-------------------------------------------------------------
-----------------------------------------------------GGCTAT
-----------------------------------------------------GGCTAT


TGAAGTCAGGCCACTTGTGCCACGGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAA
-------------------------------------------------------------
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               .10382
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TAATGGGAGGCGTAATAATCCCCAGGGAG-3′         53
TAATGGGAGGCGTAATAATCCCCAGGGAG-3′         53
```

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                                  .10280
3x mir-124aT(1,2,3)    5'-TAATTCGAAGGTAACCTGGCATTCACCGCGTGCC
GR 3-3, MB 1 day 9     5'-TAATTCGAAGGTA---------------------
GR 3-3, MB 2 day 11    5'-TAATTCGAAGGTAA--------------------
GR 3-3, MB 3 day 11    5'-TAATTCGAAGGTAA--------------------
GR 3-1, MB 4 day 17    5'-TAATTCGAAGGT----------------------
GR 3-2, MB 5 day 17    5'-TAATTCGA--------------------------
GR 3-2, MB 6 day 17    5'-TAATTCGAAGGTAAC-------------------
GR 3-3, MB 7 day 17    5'-TAATTCGAAGGTAAC-------------------

   .10281      .10292                       .10293         .
TTAATTACCAACAACATGGCATTCACCGCGTGCCTTAACTCGAGAACACCAAAGGCTAT
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------

10307                        .10308
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-------------------------GATTGAAGTCAGGCCACTTGTGCCACGGTTTGAG
----------------------ATCGATTGAAGTCAGGCCACTTGTGCCACGGTTTGAG
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                                    .10375
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CATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTC
CATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTC
CATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTC
CATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTC
CATGCGCCACGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTC
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                .10473
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CCATCACTGACAAAACGCAGCAAAAGGGGGCCCGAA-3'      110
CCATCACTGACAAAACGCAGCAAAAGGGGGCCCGAA-3'      185
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----------------CGCAGCAAAAGGGGGCCCGAA-3'      271
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                                        .10280
mir-9T-124aT-124aT(1,2,3)          5′-TAATTCGAAGGTAACCTCATACAGCT
GR 12, MB 1 day 14                 5′-TAATTCGAAGGTAACCTCATACAGCT
GR 12, MB 2 day 16                 5′-TAATTCGAAGGTAACCTCATACAGCT
GR 12, MB 3 day 16                 5′-TAATTCGAA----------------
GR 12, MB 4 day 19                 5′-TAAT----------------------

              .10281     .10292                          .10293
AGATAACCAAAGATTACCAACAACATGGCATTCACCGCGTCCTTAACTCGAGAACACCA
AGATA-----------------------------------------------------
AGAAAA----------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------

       .10307                          .10308
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------------------------------------------------------------
------------------------------------------------------------
----------------------------CGATTGAAGTCAGGCCACTTGTGCCAC
------------------------------------------------------------

                                                      .10383
GGTTTGAGCAAACCGTGCTGCCTGTAGCTCCGCCAATAATGGGAGGCGTAATAATCCCC
------------------------------------------------------------
-----------------------------------------------------AATCCCC
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                                                  .10435
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-------------------------------------------CTAGCGGTTAGAGGA


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------------------------------------------------------------
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                        .10530
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TACTCCTGGTGGAAGGACTAGAGGTTAGAGGAGACC-3′      108
TACTCCTGGTGGAAGGACTAGAGGTTAGAGGAGACC-3′      243
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                                        .10280
3x mir-124aT(1,2,4)            5′-TAATTCGAAGGTAACCTGGCATTCACCG
GR 4-3, MB 1 day 11            5′-TAATTCGAAGGTAACC------------
GR 4-3, MB 2 day 16            5′-TAATTCGAAGGTAACCTGGCATT-----
GR 4-3, MB 3 day 20            5′-TAATTCGAAGGTAACCTGGCATT-----

          .12081     .10292                          .10293
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GGCTATT
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-------------------------------------------------------------
-------------------------------------------------------------

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-------------------------------------------------------------
-------------------------------------------------------------

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-------------------------------------------------------------
-------------------------------------------------------------
                                                    .10469
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-------------------------------------------------------------
-------------------------------------------------------------
             .10470
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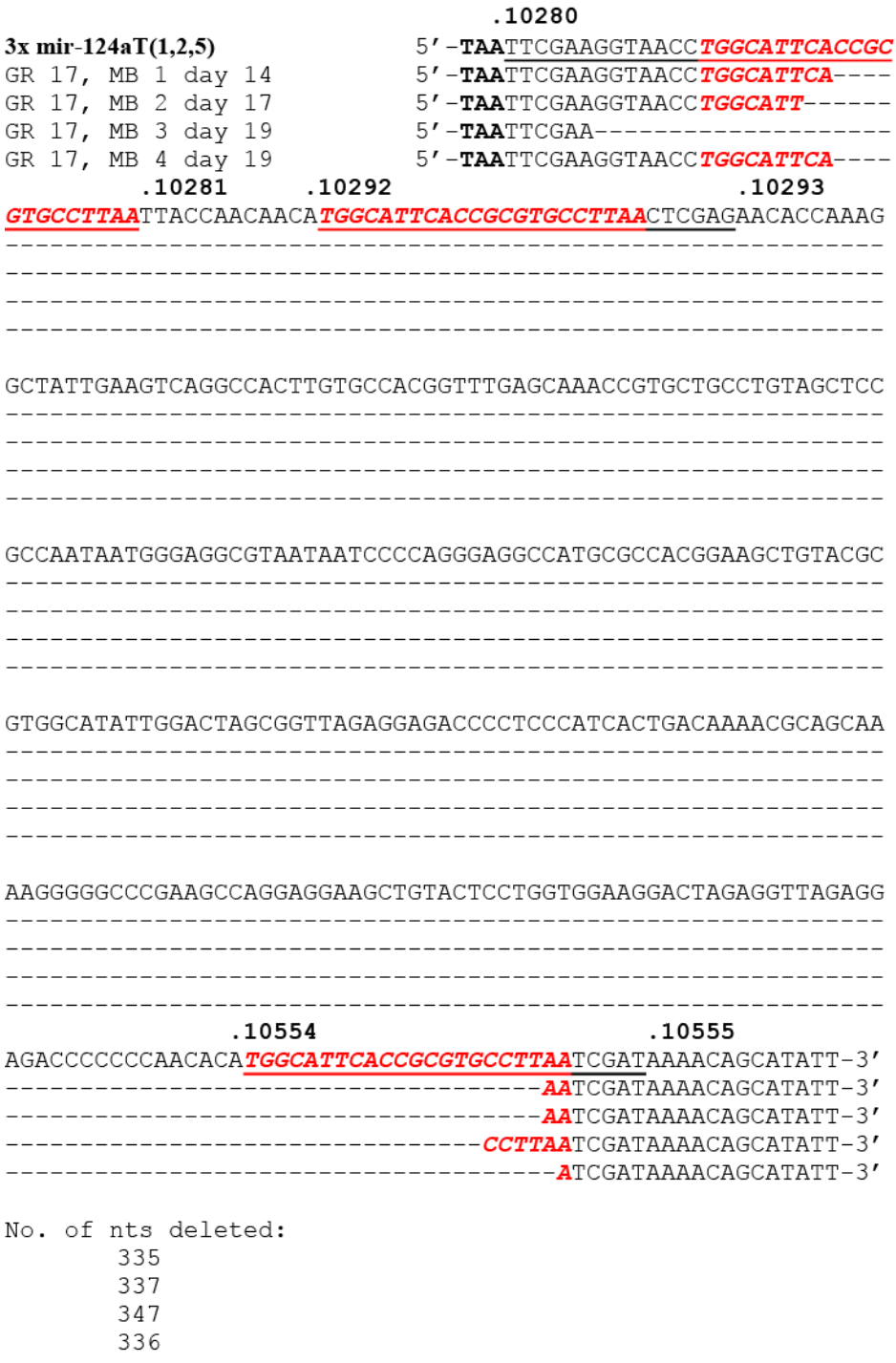

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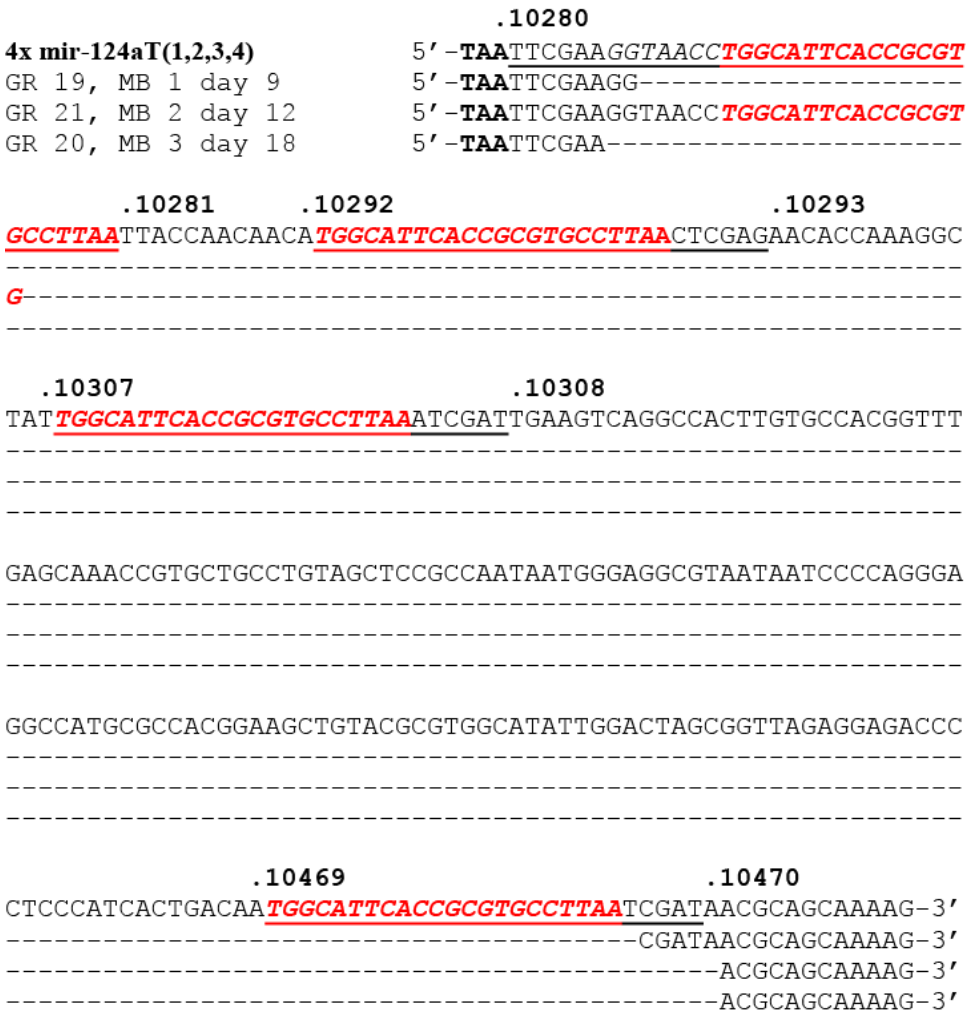
.10280
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GR 19, MB 1 day 9 5′-TAATTCGAAGG----------
GR 21, MB 2 day 12 5′-TAATTCGAAGGTAACCTGGCATTCACCGCGT
GR 20, MB 3 day 18 5′-TAATTCGAA----------
.10281
.10292
.10293
GCCTTAATTACCAACAACATGGCATTCACCGCGTGCCTTAACTCGAGAACACCAAAGGC
G
.10307
.10308
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.10469
.10470
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CGATAACGCAGCAAAAG-3′
ACGCAGCAAAAG-3′
ACGCAGCAAAAG-3′
No. of nts deleted:
295
279
302


.10280
4x miR-124aT(1,2,3,5)
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GR 23, MB 1 day 20
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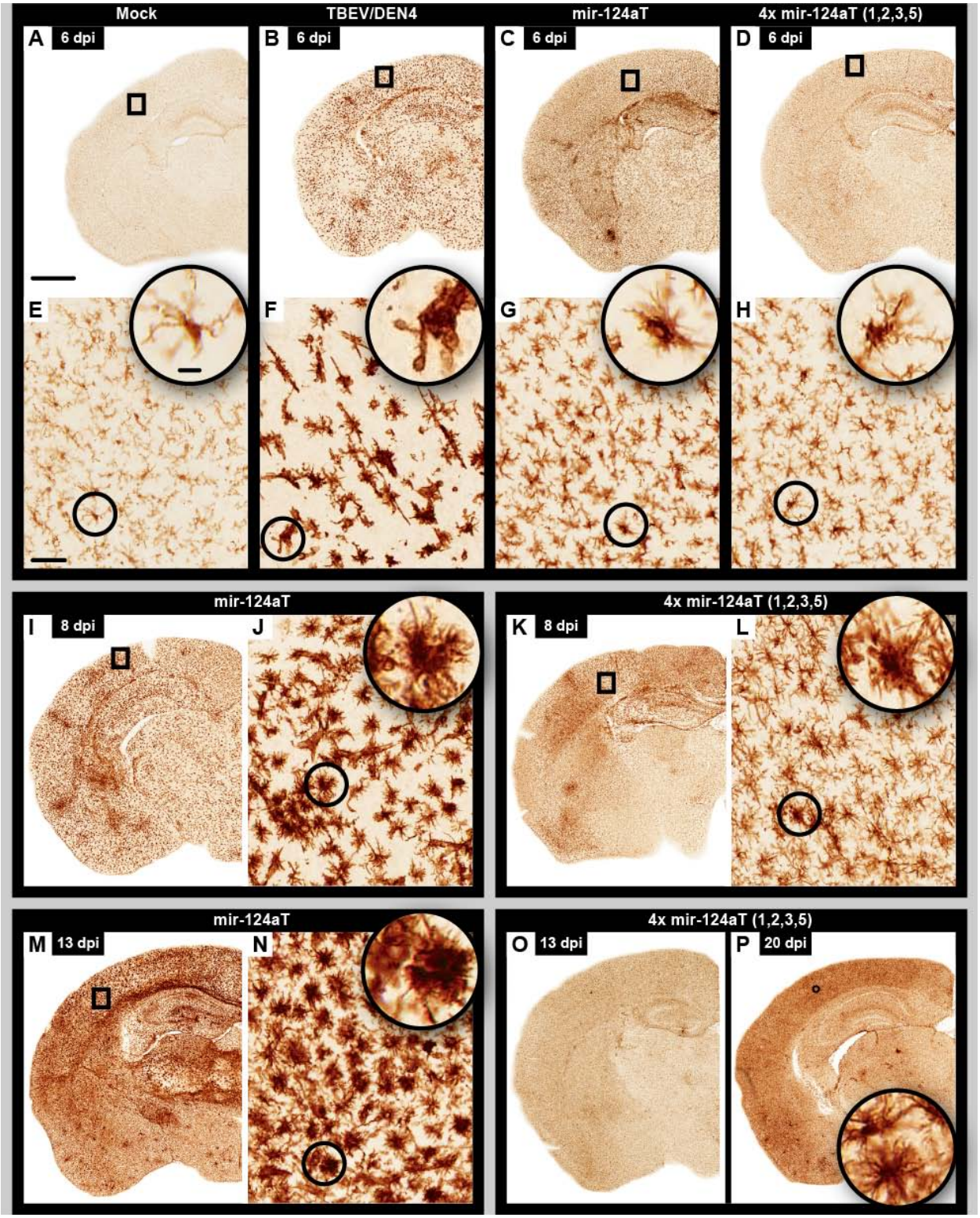

Figure S4. Microglial activation. Microglial activation was revealed by specific immunoreactivity (IR) for the ionized calcium binding adapter molecule 1 (Iba1-IR) in the CNS of mice infected with the TBEV/DEN4 (B and F), mir-124aT (C, G, I, J, M, and N), or 4x mir-124aT(1,2,3,5) (D, H, K, L, O, and P) virus on indicated dpi. Mock control is shown in A and E. Boxed areas of the cortex in (A – D, I, K, and M) are shown at higher magnification in each panels (E – H, J, L, and N). Round insets show circled microglial cells at higher magnification. Bar (1000 µm) for the brain hemisphere in (A) also applies to (B –D, I, K, M, O, and P). Bar 50 µm in (E) also applies to (F - H, J, L, and N). Bar 10 µm for the inset in (E) applies to all round insets.

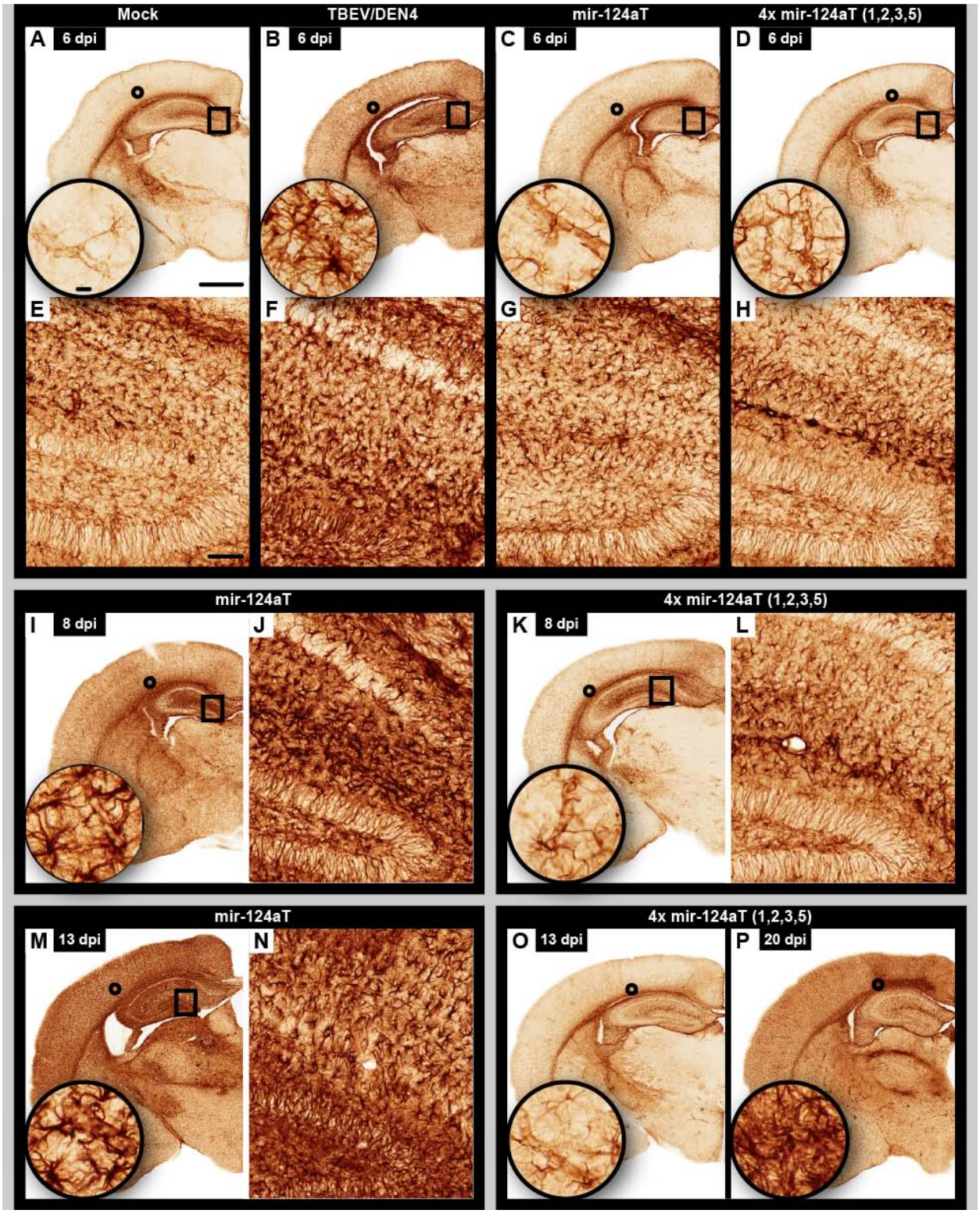

Figure S5. Reactive astrocytosis. Reactive astrocytosis was revealed by specific IR for the glial fibrillary acidic protein (GFAP-IR) in the CNS of mice infected with the TBEV/DEN4 (B and F), mir-124aT (C, G, I, J, M, and N), or 4x mir-124aT(1,2,3,5) (D, H, K, L, O, and P) virus on indicated day p.i. Mock control is shown in A and E. Boxed areas of the hippocampus in (A – D, I, K, and M) are shown at higher magnification in corresponding panels (E – H, J, L, and N). Round insets show circled cortical areas at higher magnification. Bar (1000 µm) for the brain hemisphere in (A) also applies to (B –D, I, K, M, O, and P). Bar 50 µm in (E) also applies to (F - H, J, L, and N). Bar 10 µm for the inset in (E) applies to all round insets.

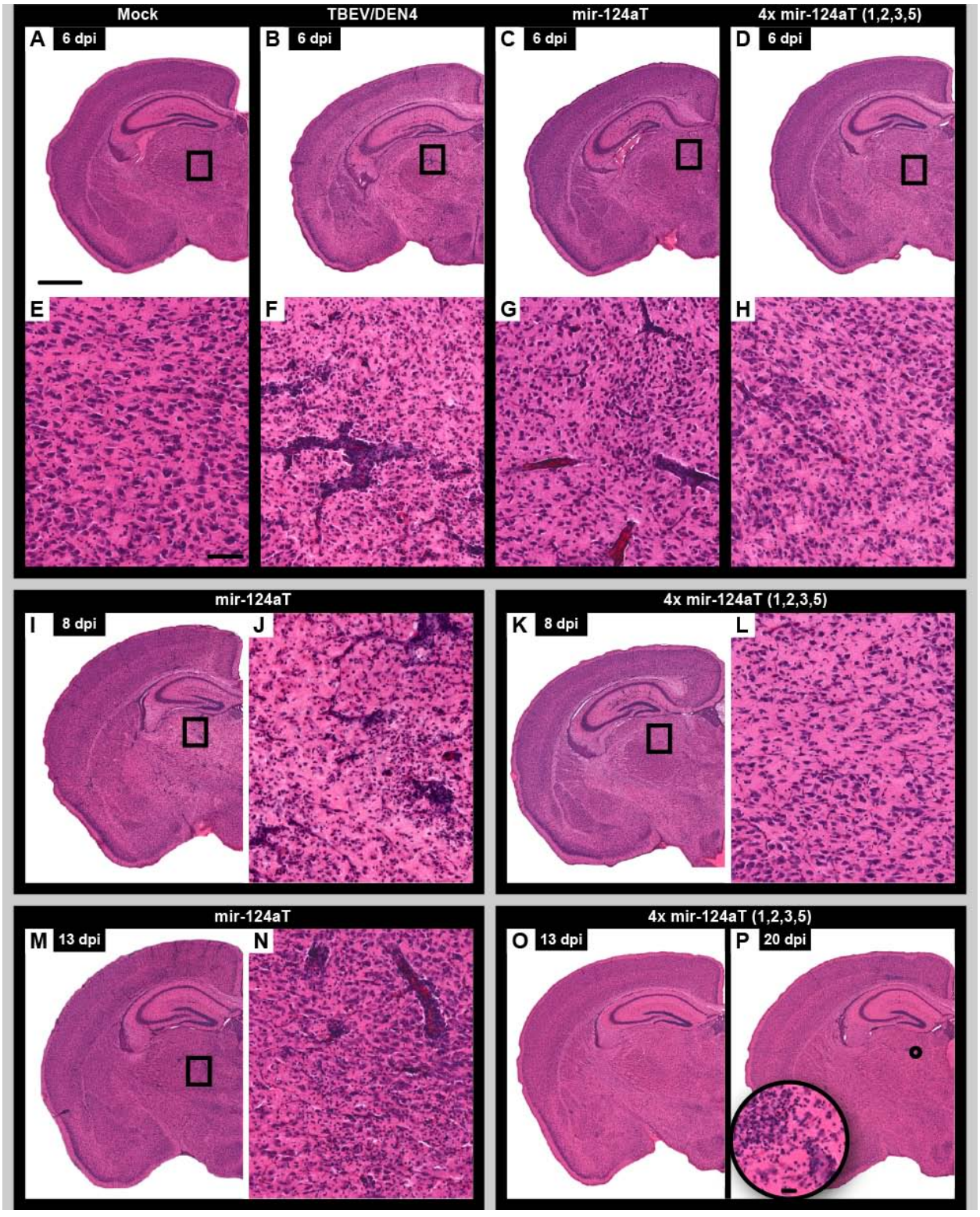

Figure S6. Histopathological changes (H&E) with inflammatory cell infiltration. Brain sections of mice infected with the TBEV/DEN4 (B and F), mir-124aT (C, G, I, J, M, and N), or 4x mir-124aT(1,2,3,5) (D, H, K, L, O, and P) virus are shown on indicated day p.i. Mock control is shown in A and E. Boxed areas of the cortex in (A – D, I, K, and M) are shown at higher magnification in corresponding panels (E – H, J, L, and N). Round inset in (P) show circled area of the thalamus at higher magnification. Bar (1000 µm) for the brain hemisphere in (A) also applies to (B –D, I, K, M, O, and P). Bar 50 µm in (E) also applies to (F - H, J, L, and N). Bar for the inset in (P) is 10 µm.

Table S1. Differences in the expression profiles of selected miRNAs in the brain and spleen of suckling and adult mice.

miRNA[a]	Brain[b]	Spleen[b]
let-7a	1.28	2.00*
let-7b	-1.25	2.64*
let-7c	-1.10	2.94*
let-7d	1.22	2.04*
let-7e	1.18*	-1.30*
let-7f	2.13	3.54*
let-7g	1.00	2.33*
let-7i	-1.38	2.44*
mir-124a	1.21*	1.00
mir-128a	4.55*	4.11
mir-218	2.03	-30.39*
mir-9	-2.86*	1.00

[a] Only data for miRNAs that are complementary to the corresponding target sequences introduced into the TBEV/DEN4 virus genome are shown. The members of the let-7 miRNA family (from let-7a to let-7i) are included since they are closely related and share the seed sequence.

[b] Fold changes in the expression of miRNAs between 3-week-old versus 3-day-old mice.

* Indicates statistically significant difference in miRNA expression between 3-week-old versus 3-day-old mice (two-tailed t-test, $P < 0.05$).