Supplemental Information

The clock modulator Nobiletin mitigates astrogliosis-associated neuroinflammation and disease hallmarks in an Alzheimer’s disease model

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This SI file contains 6 supplemental figures (Figure S1-S6) with figure legends and 3 supplemental table (Table S1-S3).
Figure S1. Characterization of circadian free-running behavior, sleep and systemic metabolism in control or NOB-treated WT and APP/PS1 mice. (A) Circadian period measurements. (B) Piezo sleep measurements. Left to right panels: percent sleep, sleep bout duration, and number of bouts. Data are presented as mean ± SEM. *, p<0.05: two-way ANOVA with Tukey’s multiple comparisons indicating significant difference between WT.Cntl and APP/PS1.Cntl. #, p<0.05: t-test showing significant difference between WT.Cntl and WT.NOB. (C) Metabolic chamber measurements. Left to right: average of oxygen consumption, carbon dioxide production, respiratory exchange ratio (RER) and heat production. Data are presented as mean ± SEM. *, p<0.05: t-test showing significant statistical difference between WT.Cntl and WT.NOB.
Figure S2. Cortical mRNA expressions of core clock genes and AD-related genes in WT and APP/PS1 mice. RT-qPCR analysis of (A) core clock genes and (B) AD-related genes in cortex tissues collected at ZT6 and ZT18 (n ≥ 3/each group). Data are presented as mean ± SEM in bar graph. *, p<0.05; **, p<0.01; ***, p<0.001: three-way ANOVA with Tukey’s multiple comparisons. Statistical significance and F distribution of interaction are shown in Table S2.
Figure S3. NOB alters proteomic landscape in the cortex. (A) List of AD-related proteins upregulated and downregulated in APP/PS1 and rescued by NOB treatment. (B) Venn Diagram of differentially expressed proteins in WT.Cntl (Top), APP/PS1.Cntl (Right) and APP/PS1 NOB (Left) groups. “WT.Cntl ZT6 vs ZT18 (199)”: Yellow circle indicates differentially expressed proteins in WT.Cntl at two circadian time points (ZT6 and ZT18). “APP/PS1 NOB ZT6 vs 18 (224)”: Purple circle indicates differentially expressed proteins in APP/PS1.NOBS at two circadian time points (ZT6 and ZT18). “APP/PS1 ZT6 vs 18 (335)”: Red circle indicates differentially expressed proteins in APP/PS1.Cntl at two circadian time points (ZT6 and ZT18). (C) Heat map showing the top enrichment clusters by Metascape analysis in WT.Cntl, APP/PS1.Cntl, APP/PS1.NOBS cortex at two circadian time points (ZT6 and ZT18).

Proteins upregulated in APP/PS1 (103+27)
- CAMKK1, CSMD2, ENTPD2, LETM1, NDFIP1, OGG1, PCBP3, RhoA, RPL12, RWDD2a, UBA3, USF1, WFS1, ZNRF2, CENPJ, KIT

Proteins downregulated in APP/PS1 (31+83)
- ABCG2, ABLIM2, AGAP2, AGBL4, ARFGAP3, BAG6, CKAP4, CPLX2, DAAM1, DNAJB4, ERC2, EXOG, FBXL16, GGA3, LRRC7, MAP3K5, MAPK8IP3, NECT1N1, PLCB1, RABE7F1, SUMO1

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Figure S4. Immunofluorescence staining revealed diminished Aβ pathology by NOB. (A) Double immunofluorescence of brain regions containing both the cortex and the hippocampus. Scale bar: 500 µm. Green: GFAP (astrocyte); red: 4G8 (Aβ); DAPI (blue). (B) Quantification of 4G8 immunofluorescence in the cortex and the hippocampus. Right panels: Quantifications based on plaque size. T-test shows significant statistical difference between APP/PS1.Cntl and APP/PS1.NOB (**, p<0.01; ***, p<0.001; ****, p<0.0001).
Figure S5. NOB significantly affects astrocyte cell morphology and density. (A) Process thickness of GFAP+ astrocytes in the cortex (top) and hippocampus (bottom) at ZT6. Data are presented as mean ± SEM. *, p<0.05; ****, p<0.0001: two-way ANOVA with Tukey’s multiple comparisons. (B) Quantification of GFAP+ astrocyte cell size. Data are presented as mean ± SEM. ***, p<0.001; ****, p<0.0001: two-way ANOVA with Tukey’s multiple comparisons. This analysis revealed a significant effect for interaction (treatment × genotype): cell size in the cortex, F(1,21)=13.54, p<0.01; cell size in the hippocampus, F(1,21)=11.34, p<0.01. (C-D) S100β immunostaining of the cortex and the hippocampus regions. (C) Quantification data are presented as mean ± SEM. *, p<0.05; ****, p<0.0001: two-way ANOVA with Tukey’s multiple comparisons. #, p<0.05: t-test showing significant difference between APP/PS1.Cntl and APP/PS1.NOB. (D) Representative images. Scale bar: 100 µm. Green: S100β (astrocyte); red: 4G8 (Aβ); blue: DAPI.
Figure S6. NOB did not alter immunoreactivity of the microgliosis marker IBA1 in APP/PS1 mice. Double immunofluorescence staining in the cortex (upper two rows) and the hippocampus (lower two rows) at two different time points (ZT6 and ZT18). Scale bar: 100 µm. Green: IBA1 (microglia); red: 4G8 (Aβ). Right panels: Quantification of IBA1 immunoreactivity. Data are presented as mean ± SEM. ****, p<0.0001: two-way ANOVA with Tukey’s multiple comparisons.
Table S1. Primer sequences for RT-qPCR.

| Gene     | Forward (5'-3')             | Reverse (5'-3')          |
|----------|-----------------------------|--------------------------|
| Clock    | CCTTCAGGAGTCAGTCCATAAAC     | AGACATCGCTGGCTGTGTTAA    |
| Bmal1    | CCACCTCAGAGCCATTTGATACA     | GAGCAGGTTCATTTCCACTTTGCT |
| Per1     | TTCGTGGAAGTCACACCTCTT       | GGGAAAGTGTGGCTTTTAGAT    |
| Per2     | ATGCTCGCCATCCACAAGA         | GCGGAATCGAATGGGAGAAT     |
| Per3     | AAAAGCACCAGGGATAGATGG       | GGGAGGCTGTAGCTGTTCA      |
| Cry1     | CTGCGCTGGAAGTCATCTGTG      | CGTGCGCCATTTGAGTTTATG    |
| Cry2     | TGTCCCTTCAGTGTGGGAAGA       | GCTTCCAGGCTTGCTGTTGA     |
| Npas2    | CAACAGAGCAAGCACATCTCT       | TTCTGATCCATCCGATCCG     |
| Rora     | GCACCTGACCAGAGACGCAA       | GAGCGATCCACGTACATCA      |
| Nr1d1    | CATGGTGACTGTGTAAGGTGTG      | CACAGGCGTGCACTCCATAG     |
| Dbp      | CTGCCCCAGTTTCTTCTGTG       | CCAGGTCCAGTATCCACG       |
| App      | AGCACCCAGAGAGAATGTCG       | GCCAGTTCTTGCGTACGC       |
| Bace1    | ACATGCTGCGCTGACTGAA        | GCCTGCGAAATCTCAGCATAG    |
| Bace2    | TGAGGAGCTGTACCCACATCCAAA   | TGGCCAAAGCAAGCATACGCAAGTC |
| Apoe     | ATTGCGAAGTAGGGCTCTGCTG     | CCAGTCGAGTAGCTGTCTCA     |
| Scna     | TGACAGCAGTCGTCGCTGA        | CATGTCTTCCGAGATTCTTC     |
| Scnb     | GAGAGGAGCTGTGCTGCTG        | TCCCTGCGTTCAGGACTGT      |
| Lrp1     | ATTGAGGCAAGAGATGACAG       | CCAGTCTGCTCCAGAATCCAC    |
| Adam10   | ACAGACTTGCTCTGCTGTAATCTT   | GGTATGTCATTGGGCAAGTGATG |
| Atxn3    | TGTCGTTTACGAAAGATCAG       | GTCAAAGAAGACAGGCTGACT    |
| Atxn10   | TCAGAGTGCCCGTTCTGTGAT      | ATCCCTTGTGCTAGTCTCT     |
| Tnfa     | CTGTAGCCAGCTGCTGACTG       | TTAGAGATCCATCCGGTCTG     |
| IL1b     | TGTTGGCAGTACTCTGCTGTCT     | TCGTCCGAGCCTGATG        |
| IL6      | TACCATCTGCAGTGCTGAGGAG     | CTGAAGTGCGATCTGTCTGT     |
| IL4      | ACAAGGAGAAGGACGCCAT        | GAAGCCCTTACAGAGCCCTCA    |
| IL17     | GGTCCAGCCAGGGCCCTACAAG     | AGGCTTCCCTCCGACTGA       |
| IL18     | CAGGACGTGACACTCTTGCAAG     | TCGTACATGGGACGCATTGT     |
| Ifngr    | TCAAGTGGCATAGTGAGGAAGA     | TGGCTCTGCGAGTCTTTCATG    |
| Gapdh    | CAAGGTCATCCATGACACCTTG     | GGGCCATCCAGCTTTGTG       |
Table S2. Statistical significance and F distribution of interaction by three-way ANOVA for Figures 2 and S2. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. The F distribution shows two parameters including degrees of freedom numerator (dfn) and degrees of freedom denominator (dfd).

| Gene Name | P value | Treatment | Genotype | Time point | Treatment X Genotype | Treatment X Timepoint | Timepoint X Genotype |
|-----------|---------|-----------|----------|------------|----------------------|----------------------|----------------------|
| Clock     | F(dfn,dfd)=3.744 | ns       | ns       | ns         | *                    | ns                   | ns                   |
| Brmal1    | F(dfn,dfd)=20.27 | ns       | *        | ***        | ns                   | ns                   | ns                   |
| Rora      | F(dfn,dfd)=97.14 | ns       | ns       | ***        | ns                   | *                    | ns                   |
| Nr1d1     | F(dfn,dfd)=8.073 | ns       | ns       | ns         | **                   | ns                   | ns                   |
| Per1      | F(dfn,dfd)=10.83 | ns       | *        | ns         | ns                   | ns                   | ns                   |
| Per2      | F(dfn,dfd)=6.877 | ns       | ***      | ****       | ns                   | ns                   | ns                   |
| Per3      | F(dfn,dfd)=1.662 | ns       | ns       | ns         | ***                  | ns                   | ns                   |
| Npas2     | F(dfn,dfd)=29.93 | ns       | ns       | ns         | ***                  | ns                   | ns                   |
| Cry1      | F(dfn,dfd)=13.74 | ns       | ***      | ns         | ns                   | ns                   | ns                   |
| Cry2      | F(dfn,dfd)=0.588 | ns       | ns       | ns         | *                    | ns                   | ns                   |
| Dbp       | F(dfn,dfd)=12.40 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| App       | F(dfn,dfd)=25.98 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Bace1     | F(dfn,dfd)=18.44 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Bace2     | F(dfn,dfd)=1.230 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Apoe      | F(dfn,dfd)=12.51 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Scna      | F(dfn,dfd)=11.05 | ns       | ns       | ***        | ns                   | ns                   | ns                   |
| Scnb      | F(dfn,dfd)=1.606 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Lrp1      | F(dfn,dfd)=27.32 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Adam10    | F(dfn,dfd)=23.57 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Axtn3     | F(dfn,dfd)=1.377 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
| Axtn10    | F(dfn,dfd)=0.028 | ns       | ns       | ns         | ns                   | ns                   | ns                   |
Table S3. Statistical significance and F distribution of interaction by three-way ANOVA for Figure 4. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. The F distribution shows two parameters including degrees of freedom numerator (dfn) and degrees of freedom denominator (dfd).

| Gene and Protein Name | P and F values | Treatment | Genotype | Time point | Treatment | Timepoint | Timepoint | Timepoint |
|-----------------------|---------------|-----------|----------|------------|-----------|-----------|-----------|-----------|
|                       |               |           |           |            |           |           |           | Genotype  |
| Tnfa                  | F(df,df)      | F(1,26)=32.77 | F(1,26)=8.358 | F(1,26)=1.562 | F(1,26)=7.359 | F(1,26)=0.87 | F(1,26)=10.13 | F(1,26)=4.137 |
| IL1b                  | P value       | ****       | **        | ns         | *          | ns         | **        | ns         |
| IL6                   | P value       | ****       | **        | ns         | ns         | ns         | ns         | ns         |
| IL4                   | P value       | **         | ***       | ns         | ***        | ns         | ns         | ns         |
| IL17                  | P value       | **         | **        | ns         | **         | ns         | ns         | *          |
| IL18                  | P value       | ****       | ***       | ns         | ns         | ns         | ns         | ns         |
| Ifngr                 | P value       | **         | ****      | ns         | ns         | ns         | ns         | ns         |
| NLRP3                 | P value       | *          | ****      | ns         | ns         | ns         | ns         | ****       |