

Supplementary material for:

Shared and distinct circadian and molecular effects of ADAR2 deficiency and the *Gria2^{R/R}* rescue allele

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Immunohistochemistry

Forty mice (equally male and female) were used in this experiment. All animals were released into DD at the time of dark-to-light transition (designated as circadian time 0; CT0) and separated into four groups. Groups of *Gria2^{R/R}* mice and *Adar2^{-/-}* mice (8 mice per group) were kept in DD, and parallel experimental groups of *Gria2^{R/R}* mice and *Adar2^{-/-}* mice (12 mice per group) received 15-min LP at CT15. Four animals from each group were anesthetised with thiopental (50 mg/kg, i. p.) after 15–30 min, four animals of each experimental group were anesthetised after 50–70 min and four animals from each group were anesthetised approximately 2 h after the LP. All animals were perfused through the ascending aorta with heparinized saline, followed by PBS (0.01 M sodium phosphate/0.15 M NaCl, pH 7.4), and then with 4% paraformaldehyde in PBS. Brains were sectioned into series of 30 µm-thick free-floating coronal slices.

Phospho-p44/42 MAPK (pERK1/2) (Thr202/Tyr204) was detected using a rabbit polyclonal antibody (Cell Signaling Technology, Cat# 9101S, RRID: AB_331646, dilution: 1:1500), and c-FOS levels were assessed using polyclonal antibodies (Merck Group, Darmstadt, Germany; Cat# PC38, RRID: AB_2106755, dilution: 1:15000). Staining was visualized using the avidin/biotin method, with

diaminobenzidine as the chromogen (Vectastain ABC Kit, Vector, Burlingame, California, United States), as described by Bendová et al. [1]. All brain sections were processed simultaneously and under identical conditions. Immunopositive cells in the SCN of the mid-caudal region were manually tagged and counted using an image analysis system (NIH ImageJ software). The mean value of immunopositive cells from the left and right SCN constituted $n = 1$. To verify the extent of pERK1/2 immunoreactive staining, images were converted to greyscale, and the relative optical density (OD) of the specific immunohistochemical signal in the central part of the SCN was determined.

Mass spectrometry-based proteomic analysis

Frontal cortex samples collected at CT15 from the corresponding experimental cohort were processed for mass spectrometry-based proteomic analysis ($n = 4$ per genotype). Tissue samples were cryopulverized (CryoMill, Retsch) and subsequently lysed by boiling at 95°C for 5 min in 100 mM TEAB (Triethylammonium bicarbonate) containing 2% SDC (sodium deoxycholate), 40 mM chloroacetamide, 10 mM TCEP (Tris(2-carboxyethyl)phosphine) and further sonicated (Bandelin Sonoplus Mini 20, MS 1.5). Protein concentration was determined using BCA protein assay kit (Thermo) and 20 µg of protein per sample was used for MS sample preparation. The sample volume was then adjusted to 65 µl in total by adding 100mM TEAB containing 2% SDC.

Samples were further processed using SP3 beads on the Thermo KingFisher Flex automated Extraction & Purification System in a 96-well plate. Briefly, 65 µl of sample was added to 65 µl of 100 % ethanol and mixed with the SP3 beads. After binding, the beads were washed three times with 80% ethanol. After washing, samples were digested in 50 mM TEAB at 40 °C with 0.5 µg of trypsin for two hours, then another 0.5 µg of trypsin was added and digested overnight. After digestion samples were acidified with TFA to 1% final concentration and peptides were desalted using in-house made stage tips packed with C18 disks (AttractSPE, Affinisep, France) according to Rappsilber et al. [2]

Nano Reversed phase columns (Ion Opticks, Aurora Ultimate TS 25×75 C18 UHPLC column) were used for LC/MS analysis. LC analysis was performed on Vanquish NEO (Thermo Scientific). Mobile phase buffer A was composed of water and 0.1% formic acid. Mobile phase B was composed of acetonitrile and 0.1% formic acid. Samples were loaded onto the trap column (C18 PepMap100, 5 µm particle size, 300 µm x 5 mm, Thermo Scientific) in loading buffer composed of water and 0.1% formic acid. Peptides were eluted with Mobile phase B gradient from 2% to 35% B in 31.7 min. Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analyzed on a Thermo Orbitrap Astral (Thermo Scientific) by data-independent approach. Survey scans of peptide precursors from 380 to 980 m/z were performed in Orbitrap at 120K resolution (at 200 m/z) with a 5×10^6 ion count target. DIA scans were performed in Astral analyzer. AGC target was set to 5×10^4 and maximum injection time to 3 ms. Precursor mass range 380 – 980 m/z was covered by 4 m/z windows. Activation type was set to HCD with 25 % collision energy.

Proteomic data analysis

All data were analyzed and quantified with the Spectronaut 20.4 software [3] using directDIA analysis. Data were searched against *Mus musculus* database (downloaded from UniProt in November 2025, containing 21 859 entries). Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds and a maximum of two missed cleavages. Carbamidomethylation of cysteines was set as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. FDR was set to 1 % for PSM, peptide and protein. Quantification was performed on MS2 level. Precursor PEP cutoff and precursor and protein cutoff was set to 0.01, protein PEP was set to 0.05. Data were exported and data analysis was performed using Perseus 1.6.15.0 [4]

Contaminants were removed, intensity values were log₂-transformed, and proteins with insufficient quantification, defined as fewer than three valid values in at least one experimental group, were excluded. Differential protein abundance between genotypes was assessed using Student's t-tests with permutation-based FDR correction. Proteins passing a 5% FDR threshold were considered significantly altered.

1. Bendova, Z., Sladek, M. & Svobodova, I. The expression of NR2B subunit of NMDA receptor in the suprachiasmatic nucleus of Wistar rats and its role in glutamate-induced CREB and ERK1/2 phosphorylation. *Neurochem. Int.* **61**, 43–47 (2012).
2. Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**, 1896–1906 (2007).
3. Bruderer, R. *et al.* Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics* **14**, 1400–1410 (2015).
4. Tyanova, S. *et al.* The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* **13**, 731–740 (2016).

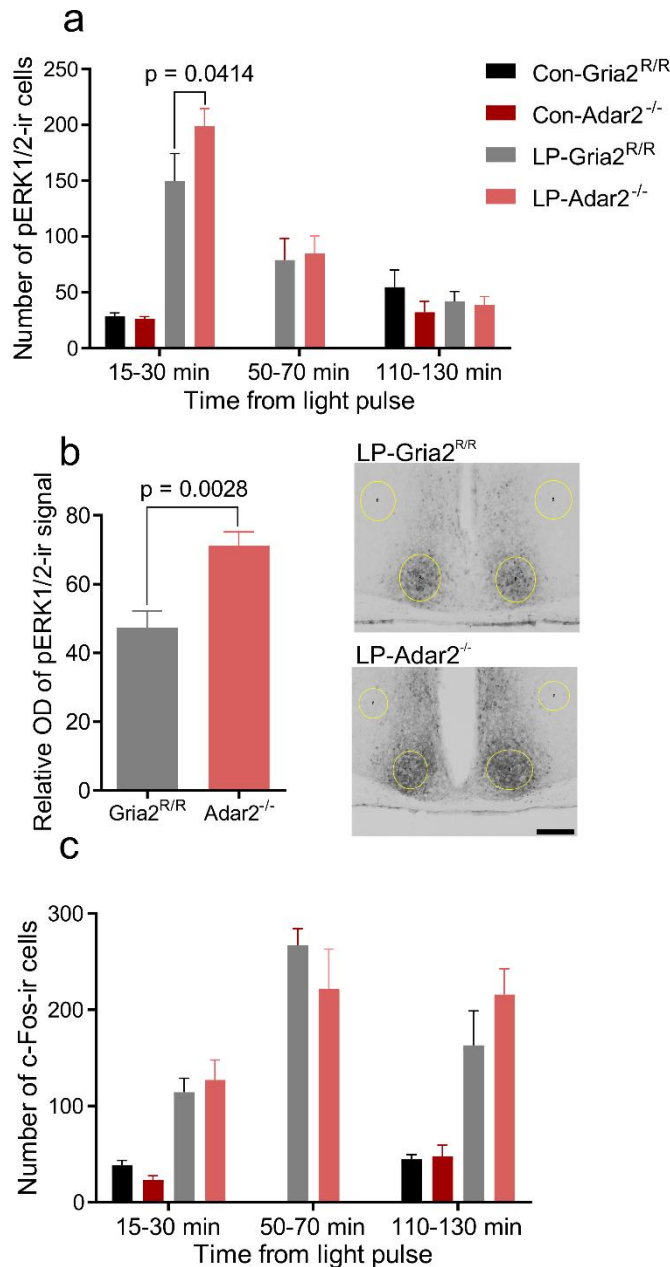


Fig. S1. The difference in light-induced changes in the levels of pERK1/2, and c-FOS in the SCN.

Gria2^{R/R} and *Adar2^{-/-}* mice were either maintained in DD as non-pulsed controls or exposed to a 15-min light pulse at CT15. Light-pulsed animals were sampled 15–30, 50–70 and 110–130 min after the pulse, whereas control animals were sampled at the corresponding early and late intervals. The sampling interval was established considering the time of intracardiac perfusion. Levels of immunopositivity were assessed for the pERK1/2 (a) and c-FOS (c). OD measurement of pERK1/2 signal in SCN collected 15–30 min after LP (b) confirmed the difference determined by cell counts. The *p*-value (*p*) indicates the difference assessed by an unpaired *t*-test. The data are plotted as mean $\pm$ SEM, *n* = 4. Representative photomicrographs of coronal sections of the SCN demonstrate the intensity and distribution of pERK1/2 in both experimental groups. The yellow circles depict the central parts of the SCNs where a specific immunohistochemical signal was determined and the area depicted for background subtraction. Scale bar = 200 μ m.

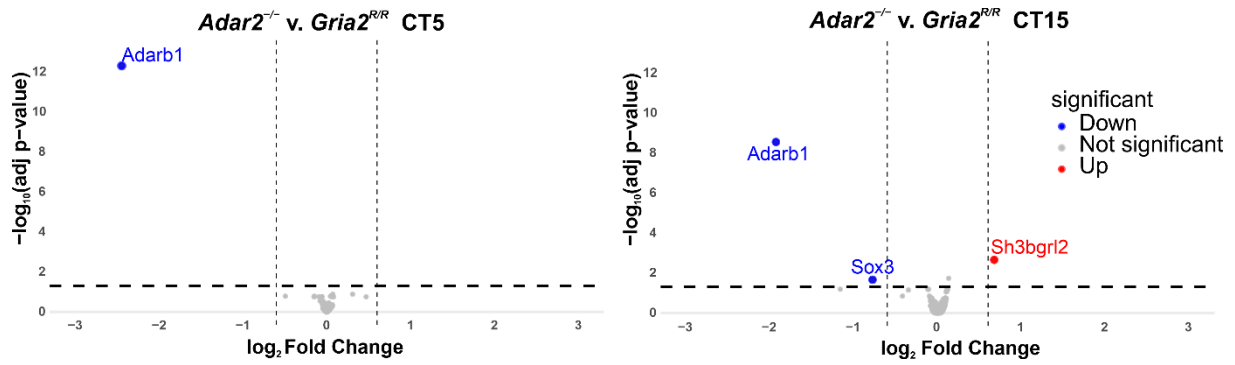


Fig. S2. Differential gene expression between *Adar2*^{-/-} and *Gria2*^{R/R} mice in the SCN.

Volcano plots show differential gene expression in *Adar2*^{-/-} relative to *Gria2*^{R/R} mice at CT5 (left) and CT15 (right). The x-axis represents log₂ fold change and the y-axis -log₁₀ adjusted p-value. Genes meeting both significance criteria (absolute LFC ≥ 0.58, corresponding to an approximately 1.5-fold difference, and adjusted p ≤ 0.05) are shown in blue when expressed at lower levels and in red when expressed at higher levels in *Adar2*^{-/-} mice. Genes not meeting both criteria are shown in grey. Vertical and horizontal dashed lines indicate the fold-change and adjusted p-value thresholds, respectively.

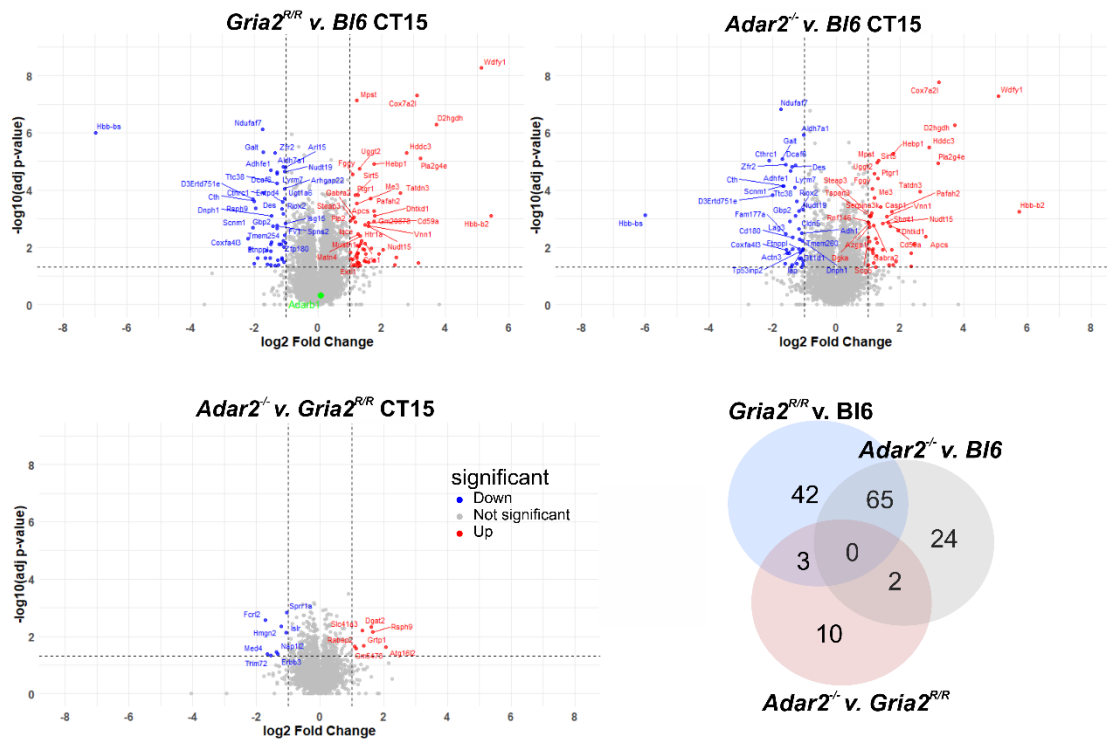


Fig. S3. Differentially abundant proteins in frontal cortex at CT15.

Volcano plots show mass spectrometry-based proteomic comparisons of frontal cortex samples collected at CT15 from *Gria2*^{R/R}, *Adar2*^{-/-} and Bl6 mice. Differential protein abundance is shown for *Gria2*^{R/R} versus Bl6, *Adar2*^{-/-} versus Bl6 and *Adar2*^{-/-} versus *Gria2*^{R/R} comparisons. The x-axis indicates log₂ fold change and the y-axis indicates -log₁₀ p-value. Proteins with significantly higher abundance in the first genotype of each comparison are shown in red, proteins with significantly lower abundance are shown in blue, and non-significant proteins are shown in grey. Dashed lines indicate the statistical and fold-change thresholds used to define differentially abundant proteins. The Venn diagram summarizes the overlap of differentially abundant proteins among the three CT15 cortical proteomic contrasts. The two modified lines showed extensive proteomic differences relative to Bl6, whereas the direct *Adar2*^{-/-} versus *Gria2*^{R/R} comparison identified a much smaller set of differentially abundant proteins.

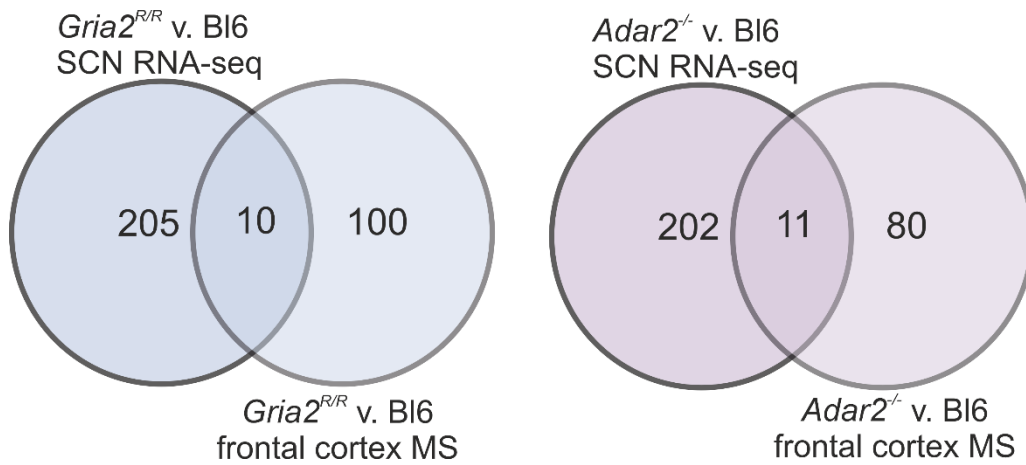


Fig. S4. Overlap between SCN transcriptomic and frontal-cortex proteomic differences at CT15.

Venn diagrams show the overlap between differentially expressed genes identified by RNA sequencing in the SCN and differentially abundant proteins identified by mass spectrometry (MS) in the frontal cortex for *Gria2*^{R/R} versus Bl6 (left) and *Adar2*^{-/-} versus Bl6 (right). Numbers indicate genes/proteins identified exclusively by RNA-seq, exclusively by MS, or in both datasets. The 10 and 11 overlapping gene–protein pairs, respectively, showed concordant directions of change relative to Bl6 at the transcript and protein levels. Differential-expression criteria were $LFC \geq 1$ and adjusted $p \leq 0.05$ for RNA-seq and $LFC \geq 1$ and Student's t -test $p \leq 0.05$ for proteomic data.