Supplementary Information for
Spine dynamics of PSD-95-deficient neurons in the visual cortex link silent synapses to structural cortical plasticity

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This PDF file includes:

Supplementary text: Materials and Methods
Figure S1
Legend for Figure S1
SI References
SI Materials and Methods

Experimental animals. All experiments were performed by strictly following the procedures approved by the animal care and use committees and governmental agencies of the listed institutions. Heterozygote PSD-95 female mice with a mixed 129SV/C57BL/6J background (1, 2), bred at in-house animal facility, were mated with heterozygote males of the same line to get embryonic day (E) 15.5 PSD-95 WT and KO embryos for in utero electroporation. For generating layer (L) 2/3-specific PSD-95 knockdown mice, C57BL/6J mice with timed pregnancies were obtained from the mouse colony of the central animal facility of the University Medical Center Göttingen. All mice were housed in groups of <5 in standard cages (26×20×14 cm) with a 12-h light/dark cycle with food and water ad libitum. Mice were separated after the cranial window implantations and single-housed until the end of the experiments. The sex ratio between the WT and KO (or control and sh95) groups were similar.

In utero electroporation. In utero electroporation was performed as previously described (3, 4). Pregnant mice were anesthetized with isoflurane (3% for induction and 1.5% during surgery), in O₂ (0.8 l/min) and received injections of buprenorphine (0.1 mg/kg) pre-operatively and carprofen (5 mg/kg) after the surgery. Using a sterile surgical technique, uterine horns were exposed through a midline incision made in the abdominal wall. The left ventricle of E15.5 mouse embryos was injected with ~0.5 μl Tris-EDTA solution containing 2 μg/μl exdotoxin-free plasmid DNA and a visualizing dye (0.1% Fast Green) through a pulled glass micropipette using a pedal-controlled pressure injector. All embryos of PSD-95 heterozygote females were injected a plasmid expressing CAG-driven eGFP (eGFP.CAG). For PSD-95 knockdown, another plasmid expressing shRNA against PSD-95 with a CAG-driven GFP tag (sh95.eGFP.CAG) or the control (eGFP.CAG) plasmid was injected into WT C57BL/6J embryos. Injections of lower volumes (~0.5μl), but high concentration (2 μg/μl) of DNA solution was critical to achieving sparse L2/3 cellular expression. 5 mm tweezer type round platinum electrodes (NepaGene, CUY650P5) were then positioned to target the dorsolateral wall of the left hemisphere and a series of 5 square-wave current pulses was delivered (35 V, 50-ms duration, 950-ms interval), generated by a pulse generator (Nepa21 electroporator, NepaGene). The abdominal wall was sutured shut after returning the embryos into the mother's womb, and the dam was allowed to recover in a cage kept on a heating pad.
**Chronic cranial window implantation.** For repeated *in-vivo* imaging of eGFP-labeled dendrites, a cranial window was implanted over the left visual cortex of all *in-utero*-electroporated mice (~P45) under 3-component general anesthesia (fentanyl 0.075 mg/kg, midazolam 7.5 mg/kg, medetomidine 0.75 mg/kg, i.p.) as described before (5-7). Briefly, following a scalp incision and fascia cleaning, a circular craniotomy overlying binocular visual cortex was performed using steel drills (3 mm lateral to midline, 1 mm anterior to lambda). The bone was carefully removed to not damage the dura, and the exposed surface was kept moist by applying 0.9% saline gel foam. A 4-mm round glass coverslip was then gently placed onto the brain and secured to the surrounding bone using light curing cement (Tetric EvoFlow). A custom made titanium ring (0.7g, 14mm) for head restraining was subsequently glued to the cement around the cover slip (8), and the skin was sutured back to enclose the ring. Anesthesia was terminated by an antidote (naloxon 1.20 mg/kg, flumazenil 0.50 mg/kg, atipamezol 2.50 mg/kg, s.c.), and general analgesia was provided by carprofen injection (5 mg/kg) postoperatively. Recovery of animals was closely monitored for at least five days before starting the imaging and habituation steps.

**Optical imaging of intrinsic signals.** To locate binocular V1, visual cortical responses were recorded through the implanted cranial window using intrinsic signal optical imaging (9, 10) as described before (11, 12). Mice were anesthetized with 0.6-0.8% isoflurane in oxygen, and the body temperature was maintained at 37° using a feedback-controlled heating pad. A temporally periodic visual stimulus was continuously presented to the animal, and the cortical responses at the stimulus frequency were extracted by Fourier analysis. Optical images of intrinsic cortical signals were obtained using a 135×50-mm tandem lens configuration (Nikon), and a Dalsa 1M30 CCD camera. The surface vascular pattern and intrinsic signal images were visualized with illumination wavelengths set by a green (550±10 nm) or red (610±10 nm) interference filter, respectively. After acquisition of a surface image, the camera was focused 600 μm below the superficial blood vessels. An additional red filter was interposed between the brain and the CCD camera. Frames were acquired at a rate of 30 Hz temporally binned to 7.5 Hz and stored as 512×512 pixel images after spatial binning of the camera image. A drifting horizontal bar restricted to the binocular visual field of the left primary visual cortex (20° wide, −5° to +15° azimuth) was presented to both eyes at a distance of 25 cm on a high refresh rate monitor (Dell, P170Sb). Two consecutive activity maps obtained from each five
minute imaging sessions with drifting bars moving in either 90° or 270° direction were averaged to compute the retinotopic map of binocular V1. Animals were anesthetized for less than 30 minutes in total.

Two-photon imaging. Awake 2-photon dendritic spine imaging was performed using a custom-made two-photon microscope. A mode-locked Ti:Sapphire laser (Chameleon laser, Coherent) was tuned to 920 nm, and the maximum laser power reaching the tissue was below 40 mW. Emission light was collected by a 40x water-immersion objective (NA 0.8; Olympus), filtered by emission filter (525/20; BrightLine HC) and captured by a photomultiplier tube (Hamamatsu H10770PA). Scanning and image acquisition was controlled by ScanImage software (13). One day after localizing V1, mice were briefly screened for GFP expression in binocular V1 while anesthetized (0.6% isoflurane in oxygen). Animals with sparse and bright labeling of dendrites were proceeded to habituation training for head-fixation under the two-photon microscope, as described before (6, 14), using a restrainer box. Briefly, mice were gradually trained to be restrained for up to 1 hour after about 2-3 weeks of daily training, ensuring stress-free habituation to head restraining. Blood vessel patterns and XY coordinates were used to re-localize the imaged dendrites with a few micrometers precision on each imaging session. Per animal, 15-40 μm Z-stacks (0.5 μm steps) of three to five regions within binocular V1 were acquired at 1024x1024 (0.05 μm/pixel) resolution with 2.5x digital zoom at a maximum depth of ~80μm from pia. Each z plane was scanned four to seven times in order to perform post-hoc correction of motion artefacts caused by small movements and breathing.

Monocular deprivation and eye reopening. Immediately after the last baseline imaging session on day 4 (d4), the contralateral (right) eye was deprived of vision for the next 4 days as described previously (15). Mice were anesthetized with 2% isoflurane in 1:1 O₂:N₂O (<15 minutes in total), and analgesia was provided by carprofen (sc, 5mg/kg). Lid margins were trimmed, and an antibiotic gel (Isopto-Max, Novartis) was applied. The eye was closed with two mattress sutures (Ethicon, 7-0). Mice were checked twice daily to make sure that the eyes remained closed. After 4-d of MD, the formerly deprived eye was reopened immediately after the 2-photon spine imaging session on d8, under brief (< 5 min) 2% isoflurane (1:1 O₂:N₂O) anesthesia and carprofen (sc, 5mg/kg) analgesia, and an antibiotic gel (Isopto-Max, Novartis) was applied. The reopened eye was checked daily and received antibiotic gel treatments to make sure that it remained open and clear.
Data Analysis and Statistics. Image stacks were motion corrected using a custom-written MATLAB script that performs an initial rigid x-y transformation followed by a non-rigid frame warping based on Lukas Kanade method (16). Resulting images were then deconvoluted using AutoQuant X (Media Cybernetics), and contrast adjusted with Fiji using Enhance Contrast function (17). Spine counting was performed blind to both genotype and imaging session. A custom-written MATLAB script (6) was used to align dendrites in several stacks to follow spines across imaging days. >20µm segments of dendrites lying in the horizontal plane were selected for further analyses. We defined spines as all protrusions that appeared on at least 2 consecutive z planes and were longer than 0.4 µm (18). Spine elimination and formation ratios were quantified as (number of lost or gained spines) / (number of pre-existing spines + number of resulting spines).

Due to the hierarchical and longitudinal design of our study we opted for linear and generalized linear mixed-effects models (LME and GLME, respectively) for our statistical analyses of spine elimination and formation ratios, allowing us to account for all the fixed effects, such as genotype, visual experience, and time, as well as random effects such as animal and dendrite ID. The dependent variable was either spine elimination or formation ratio. Normality of the data was checked by Shapiro-Wilk test, and only normally distributed data were presented as mean ± SEM. As the distribution of spine elimination and formation ratios were right skewed, we have used GLME to fit a gamma distribution with log link to analyze the data. The best fit for GLME was determined according to Akaike information criterion (AIC) and the Bayesian information criterion (BIC). Model was built by adding new terms one at a time until the best fit was obtained. The most parsimonious model was preferred, which included genotype, time, vision, two-way interaction between genotype and vision, three-way interaction between genotype, visual experience, and time, as well as animal ID as a random effect. To assess their significance, we reported F and P values of the fixed effects and interactions. The differences between the experimental conditions were then examined post-hoc by pairwise comparisons within the GLME with a Bonferroni correction applied to the P values. To further confirm our GLME analysis we normalized the data by calculating MD-induced changes in spine elimination or formation ratios (ΔE and ΔF, respectively) as R_{MD} – R_{NV} (see Results), and performed 2-way ANOVA or LME analysis. Statistical analyses were done using IBM SPSS Statistics (Version 26) and Prism 8.4.0.
Figure S1. 4-d monocular deprivation (MD) induced a significantly stronger decrease in spine density in PSD-95 KO mice. Mean (± SEM) change in spine density during 4-d normal vision (NV) and 4-d MD in PSD-95 WT (n=6 mice; 40 dendrites) and KO mice (n=6; 36 dendrites). *P < 0.05; ns, P > 0.05.
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